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Alterations in bovine skeletal muscle desmin and the cytoskeleton induced by temperature and electrical stimulation

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ABSTRACT

Alterations in the myofibrillar integrating cytoskeleton and its constituent protein, desmin, were assessed in bovine skeletal muscle at 1, 24 and 168 hours of postmortem storage. Changes assessed over postmortem storage in muscle subjected to usual (control) storage conditions (2°C) were compared to changes in tissue subjected to either of two conditions known to increase meat tenderness, i.e. high temperature (25°C) storage or electrical stimulation. Quantities of desmin extractable at the selected intervals were determined in part by densitometric scanning of the desmin band in polyacrylamide tube gels of desmin-enriched fractions. Alterations in the protein were assessed with immunoautoradiographic slab gel labelling of selected protein fractions generated during the desmin-enriching protocol with monospecific anti-desmin antibodies. Results indicate that accelerated solubilization from the cytoskeleton and subsequent breakdown of desmin occur in both storage regimes associated with accelerated tenderization of bovine skeletal muscle. High temperature promotes tenderization by favoring conditions more acceptable to proteolysis, while electrical stimulation accelerates tenderization by combined physical disruption of muscle ultrastructure and activation of muscle proteases. A highly insoluble 22,000 dalton polypeptide, not related to any myofibrillar proteins with known Z line location, appeared in desmin-enriched extracts concurrent to the breakdown of desmin in muscle stored under conditions of all three experimental regimes.

Desmin was shown by immunofluorescent labelling of bovine skeletal myofibrils to be a part of a filamentous cytoskeletal network inter-

connecting myofibrils at the level of the Z line. The location of this cytoskeleton was not altered over 168 hours of storage in any of the experimental regimes.

The arrangement of desmin into cytoskeletal filaments of 100 Å diameter was unequivocally demonstrated in bovine skeletal muscle for the first time with immunoelectron microscope labelling of skeletal muscle myofibrils. These filaments had diameters that were similar to those formed from purified bovine desmin *in vitro* under suitable conditions of pH and ionic strength.

LIST OF ABBREVIATIONS

Å = angstrom = 10^{-10} meters

ATP = adenosine 5'-triphosphate

BSA = bovine serum albumin

^{o}C = degrees centigrade

CAF = calcium-activated factor

cm = centimeter

DDSA = dodecenylsuccinic anhydride

DEAE = diethylaminoethyl

DER = diglycidyl ether of polypropylene glycol

DMP-30 = 2, 4, 6-tri (dimethylaminomethyl)-phenol

DTE = dithioerythritol

EDTA = ethylenediaminetetraacetic acid

FITC = fluorescein isothiocyanate

g_{max} = relative centrifugal field at maximum tube radius

gm = gram

µg = microgram

HAMES = high ionic strength actomyosin extracting solution

hr = hour

Hz = hertz

I.D. = inner diameter

kg = kilogram

kV = kilovolt

M = molar

mM = millimolar

mm = millimeter

μm = micrometer

mA = milliampere

MCE = mercaptoethanol

mg = milligram

min = minute

ml = milliliter

nm = nanometer

O.D. = outer diameter

PBS = phosphate-buffered saline

PMSF = phenylmethylsulfonyl fluoride

ppm = parts per million

rpm = revolutions per minute

SDS = sodium dodecylsulfate

sec = second

Tris = Tris-(hydroxymethyl)-aminomethane

v = volume

w = weight

INTRODUCTION

Many eukaryotic cells contain networks of filaments which are distinct in diameter from actin filaments, microtubules and myosin filaments. These filaments, visualized with the electron microscope, were at first widely regarded as disaggregation products of either microtubules or myosin filaments, and were therefore generally neglected despite numerous observations of their existence in a variety of cell types (Lazarides, 1982). It is now apparent that these filaments, which have a diameter (100 Å) intermediate to actin filaments (70 Å) and myosin filaments (140 Å) or microtubules (250 Å), are present in many eukaryotic cells in an ordered cytoskeletal array. This cytoskeleton is thought to serve a structural function in most vertebrate cells.

Isolation of subunit proteins and subsequent immunological labelling within many cell types (Lazarides, 1982) has indicated the existence of five distinct classes of 100 Å or intermediate filaments. The five classes, and the cells from which they have been isolated, are as follows: a) keratin (tono-) filaments, present in cells of epithelial origin; b) glial filaments, present in cells of glial origin; c) neurofilaments, detected only in neurons; d) vimentin (decamin) filaments, found in mesenchymally-drived non-muscle cells such as fibroblasts; e) desmin (skeletin) filaments, found in smooth, cardiac and skeletal muscle cells. These filaments may be comprised of one or more proteins and may be polymers with similar subunits, or copolymerize with subunits from different classes. Some cells may contain two (Franke et al., 1979;

Gard et al., 1979) or even three (Wang et al., 1980) co-existing classes.

Intermediate filaments comprised of desmin form a cytoskeletal network which surrounds myofibrils at the level of the Z line in adult vertebrate skeletal muscle (Lazarides, 1978, 1980, 1982; Lazarides and Balzer, 1978; Granger and Lazarides, 1979; Gard and Lazarides, 1980; Robson et al., 1981; Lazarides et al., 1982). This lateral integration may, in part, be responsible for imposing a side by side registration of the contractile proteins in myofibrils which gives rise to the familiar banding pattern detectable at the light microscope level of resolution in skeletal muscle. It is likely that desmin also performs an important role in adjoining myofibrils to the cell membrane (Lazarides, 1978), and perhaps even in maintaining overall muscle cell size and shape (Robson et al., 1981).

Desmin, when isolated and purified from skeletal muscle, is highly susceptible to an endogenous Ca^{2+}-activated muscle protease (CAF) and other proteases of non-muscle origin (O'Shea et al., 1979). In a pilot study, Robson et al. (1980) have shown, by one-dimensional SDS-polyacrylamide gel electrophoresis, a decrease over postmortem time in the percentage of desmin in desmin-enriched muscle fractions isolated from bovine skeletal muscle. It seems plausible that the degradation of desmin, and of the integrating cytoskeletal network of 100 Å filaments which it comprises, could be important in helping to explain many of the postmortem physical changes in muscle and the beneficial increase in tenderness that occurs in fresh meat during postmortem storage (Robson et al., 1981).

The objective of this study was to determine the quantitative and qualitative effects that postmortem aging (conditioning) has on the

cytoskeleton and its constituent protein, desmin, in bovine skeletal muscle. Observations made on muscle subjected to one of two postmortem conditions known to hasten muscle breakdown and improve tenderization (high temperature (25°C) storage, and electrical stimulation with 2°C storage) were compared with observations made on tissue from the contralateral carcass side stored under usual (2°C, no electrical stimulation) aging conditions. The following approaches were used to follow changes in desmin and the cytoskeleton in bovine skeletal muscle subjected to these conditions at 1, 24 and 168 hours of postmortem storage:

1) Muscle fractions enriched in desmin were isolated from bovine longissimus dorsi muscle. The amounts of desmin in each isolated fraction were then determined in part by densitometrically scanning SDS gels.

2) An antibody elicited against highly purified desmin was used to analyze the cytoskeleton at the fluorescence and electron microscope levels of resolution. The antibody was also radioisotopically labelled and used to identify breakdown products of desmin in one-dimensional slab gels.

3) Ultrastructural changes induced by electrical stimulation were assessed by analyzing thin sections of fixed longissimus dorsi muscle in the electron microscope.

LITERATURE REVIEW

Skeletal Muscle Structure

Skeletal muscle is the most abundant tissue in the vertebrate body, comprising approximately 40% of the total tissue mass. It is the effector of a wide range of voluntary body movements, each made for the purpose of adjusting to the external environment. Movement is accomplished by the coordinated work of many muscle fibers or cells which, together with connective and adipose tissue, comprise a given muscle.

Although muscles throughout the body vary greatly in size and shape, all are characterized by an external covering of connective tissue called the epimysium. The epimysium is fairly thick and tough and is the site of intermuscular fat deposition in animals. The epimysium further subdivides into smaller sheets of connective tissue which pass into the muscle at irregular intervals to form groups of muscle fibers called fasciculi. These fasciculi, or bundles, contain from 50 - 300 muscle cells which are long, cylindrical and tapered at both ends. Each cell is surrounded by a further subdivision of connective tissue called the endomysium, which lies immediately adjacent to the outer cell membrane (sarcolemma). Cells are the smallest unit of structure in muscle capable of giving a physiological response (Huxley, 1972).

A cross-striated pattern of alternating dark and light bands is noted when viewing muscle cells at a low magnification with the phase contrast microscope. Higher magnification reveals that each muscle fiber is composed of the organelles usually present in other eukaryotic cells, plus unique, elongated, thread-like structures called myofibrils

arranged in the cytoplasm (sarcoplasm). Myofibrils are not surrounded by a continuous membrane, are 1 - 3 μm in width, are themselves cross-striated and are aligned with their dark and light bands in register. It is this register that imparts the characteristic cross-striated appearance to the entire muscle cell.

In polarized light, the dark bands appear anisotropic and the light bands isotropic. The first letter of each term was used to name these dark (A) and light (I) bands within the myofibril. Other banded regions in the myofibril were named by their appearance in the phase contrast microscope. A dense dark line seen to intersect the light I band was called the Z line, from the German "zwischenscheibe", meaning intervening disc. In the middle of the darker A band, two other regions were recognized; a wider, less dense area centrally located called the H zone (from the German "heller", meaning clearer or lighter), and, centrally located within the H zone, a dark, narrow line called the M-band (from the German "mittelscheibe", meaning median membrane) which can be seen only in the electron microscope.

Myofibrils contain 50 - 55% of the total protein in muscle and are the intracellular contractile organelles of the skeletal muscle cell. The distance in a single myofibril bounded by two adjacent Z lines is called a sarcomere and is regarded as a structural contractile unit. Myofibrils may be viewed as a series of adjacent sarcomeres (several hundred or a thousand or more, depending upon the length of the cell) in end-to-end alignment parallel to the long axis of the cell. The length of a sarcomere ranges from 1.8 - 2.8 μm, depending upon the extent

of cellular contraction.

The organization of proteins insoluble at intracellular ionic strength, not only into a three-dimensional array of interdigitating thick (140 Å diameter) and thin (70 Å diameter) filaments but also into M and Z lines, gives rise to the banding patterns of myofibrils seen in the phase contrast or electron microscope. The darker A bands contain the thick filaments, each of which is composed mainly of 300 - 400 myosin molecules. The 1.0 μm long thin filaments, comprised primarily of actin, are anchored at one end to the Z line in an ordered three-dimensional array and interdigitate between thick filaments at the other free end. The light I band is the area in the myofibril where no thick filaments are present. The Z line bisects the I band and is comprised primarily of the structural protein α-actinin. The H zone is a less dense area in the center of the A band. The width of the H zone is determined by the distance between the free ends of thin filaments. The dark M-line in the center of the A band has been identified as the location of creatine kinase (Walliman et al., 1978) and myomesin (M-protein) (Trinick and Lowey, 1977; Eppenberger et al., 1981).

Active muscle length changes occur by a sliding motion of thick and thin filaments relative to each other as was proposed independently by A. F. Huxley and R. Niedergerke and H. E. Huxley and J. Hanson in 1954. This sliding filament model of contraction was partially derived from observations that I band and H zone widths vary with the degree of contraction, while thick and thin filaments and the A band, Z line and M-line remain unaffected. Subsequent work has supported this sliding

filament model and has increased the understanding of the molecular events which occur during the contraction and relaxation of muscle.

Within the past six years many structural proteins comprising very small percentages of the total myofibrillar protein have been discovered. Many of these proteins have not been completely characterized and some are the subject of as few as one or two publications (Greaser et al., 1981). However, the characterization of two of these newly discovered proteins, connectin (titin) and desmin, has been complete enough to warrant discussion of their structural significance within the muscle cell.

Connectin was initially isolated by Maruyama et al. (1977) from the residue remaining after actomyosin extraction from rabbit skeletal myofibrils by using either 6M urea - 1% SDS or 0.1N NaOH. The highly insoluble remnant after these extractions was believed to be an elastic component of thin fibers aligned with the longitudinal axis of the myofibril and the sarcolemma (Maruyama et al., 1977; Maruyama and Natori, 1978; dos Remedios and Gilmour, 1978).

Wang et al. (1979) used agarose gel filtration to isolate a protein doublet named titin (approximate molecular weight of 1 X 10^6, as determined by electrophoretic mobility) from SDS-solubilized chicken breast myofibrils. Immunofluorescent localization of this protein doublet in glycerinated chicken breast myofibrils indicated the presence of titin in the M-line, Z line, the junction of the A and I bands, and perhaps throughout the entire A band. Maruyama et al. (1981) isolated connectin, with a method modified from their original protocol (Maruyama

et al., 1977) to include column chromatography, and showed it to be identical to titin on the basis of electrophoretic mobility, amino acid composition and immunofluorescent localization. Based on immunofluorescence and electron microscope studies, it seems that connectin may be a (the) structural subunit of filaments functioning to give longitudinal support and elasticity to the myofibril (Locker and Leet, 1976; dos Remedios and Gilmour, 1978; Young et al., 1981).

Desmin is another highly insoluble myofibrillar protein which also has an important structural role within the muscle cell. This protein, which has a molecular weight of 55,000 daltons, has been isolated from actomyosin-extracted chicken (Price and Sanger, 1979) and porcine (O'Shea et al., 1979, 1981; Rathbun, 1982) skeletal myofibrils with urea and/or acetic acid, and is estimated to comprise 0.35% of skeletal muscle myofibrillar protein (O'Shea et al., 1981). Desmin has also been shown to be present in skeletal muscle by two-dimensional peptide analysis of isolated myofibrils or myotubes (Izant and Lazarides, 1977; Lazarides and Balzer, 1978; Gard et al., 1979; Granger and Lazarides, 1979; Granger et al., 1979; Gard and Lazarides, 1980) or by immunofluorescent antibody localization in avian skeletal muscle (Lazarides and Hubbard, 1976; Lazarides and Balzer, 1978; Lazarides and Granger, 1978; Granger and Lazarides, 1978, 1979; Bennett et al., 1979; Campbell et al., 1979; Gard and Lazarides, 1980; Richardson et al., 1981). The immunofluorescence studies show desmin to predominantly surround the Z line in a distinctly filamentous pattern. This fluorescent filamentous pattern has been directly linked to electron microscope observations of 100 Å

diameter filaments connecting myofibrils at the Z line (Bennett et al., 1979; Nunzi and Franzini-Armstrong, 1980; Edstrom et al., 1980; Pierobon-Bormioli, 1981) by the work of Richardson et al. (1981), who used a method of indirect antibody labelling suitable for viewing in an electron microscope to ascertain the presence of desmin in these 100 Å filaments. Based on present knowledge, it seems that desmin is an important, and possibly the only, component of a transverse structural (cytoskeletal) network that links adjacent myofibrils at the level of the Z line. Such an intracellular matrix may provide tensile strength to muscle and ensure that all of the component myofibrils are mechanically integrated during contraction and relaxation (Lazarides, 1978).

It has been shown that unaged bovine sternomandibularis muscle fibers maintain their well-ordered structure of laterally aligned myofibrils during homogenization, but that this precise ordering was readily disrupted when aged muscle was homogenized (Davey and Gilbert, 1969). Young et al. (1981) have shown, in polyacrylamide gels of crude, desmin-containing fractions, that the percentage of desmin in these fractions from ox sternomandibularis muscle declines during six days of postmortem storage and postulate this, when considered with the observations of Davey and Gilbert (1969), to be indicative of disintegration of the colligative cytoskeletal network during postmortem aging. It, therefore, seems plausible that desmin and the cytoskeletal network of 100 Å filaments comprised by it may be responsible for some, or perhaps many, of the postmortem physical changes in muscle and for the beneficial increase in tenderness that occurs in fresh meat during postmortem storage (Young et al., 1981; Robson et al., 1981).

Desmin and the Intermediate Filament Network in Skeletal Muscle Cells

Discovery of intermediate filaments and extraction of desmin

Ishikawa et al. (1968) were the first to use the name "intermediate-sized" filaments for the approximately 100 Å diameter filaments that were observed between striated myofibrils in developing myogenic cells in culture. Although intermediate filaments are common in embryonic striated muscle, they become progressively less visible as myofibrils become laterally registered in a tightly packed, highly organized mature skeletal muscle cell. Therefore, early research on the intermediate filament network in skeletal muscle utilized techniques gleaned from work done on smooth muscle cells, where intermediate filaments are much more abundant.

Cooke and Chase (1971) were the first to detect an intracellular network of intermediate (100 Å) filaments which linked cytoplasmic dense bodies {(analogues of skeletal muscle Z lines (Somlyo et al., 1973; Schollmeyer et al., 1976; Bond and Somlyo, 1982)} to membrane-bound attachment plaques. Solubilization of intermediate filaments permitted the isolation and characterization of the component subunit, which has a molecular weight of 55,000 daltons and has been named desmin (Lazarides and Hubbard, 1976) or skeletin (Small and Sobieszek, 1977). Because the name, desmin, has been more widely used, the 55,000 dalton protein from intermediate filaments in muscle will hereafter be referred to in this dissertation as desmin.

The precedures for extracting desmin from skeletal muscle are based on those pioneered in the extraction of desmin from smooth muscle

(Cooke and Chase, 1971; Cooke, 1976; Schollmeyer et al., 1976; Huiatt et al., 1980). Well-washed myofibrils are extracted with high ionic strength solutions to remove actomyosin. Desmin is then solubilized from the remaining residue with 1M acetic acid, isoelectrically precipitated at pH 5.5, and resolubilized in 6M urea. This urea-solubilized, desmin-enriched extract can be purified by successive chromatography on hydroxyapatite and DEAE-Sepharose CL-6B columns (O'Shea et al., 1981; Lazarides et al., 1982) to 97% homogeneity. This purification procedure leads to yields of desmin from porcine skeletal muscle of 0.35% of the original weight of well-washed myofibrils, as compared to 8.0% of the original weight of washed avian smooth muscle myofibrils (Huiatt et al., 1980; O'Shea et al., 1981).

Desmin purified by these procedures remains soluble after urea removal by extensive dialysis against 10mM Tris-acetate, pH 8.5, and clarification at 183,000 X g_{max} for one hour. Porcine skeletal desmin (in 10mM Tris-acetate, pH 8.5) has an ultraviolet absorption spectrum with a maximum at 278 nm, an extinction coefficient, $E^{1\%}_{278}$, of 5.67 and a ratio of absorbance at 278 nm to that at 260 nm of 1.63 (Rathbun, 1982). These figures agree well with results reported by Huiatt et al. (1980) on purified avian smooth muscle desmin.

Purified desmin isolated from porcine skeletal muscle (O'Shea et al., 1981) or from avian smooth muscle (Huiatt et al., 1980) has been shown to form intermediate filaments with an average diameter of 100 Å when dialyzed against 150mM NaCl, 10mM imidazole, 1mM $MgCl_2$, 1mM DTE, pH 7.0. No significant interactions occur between purified porcine skeletal

desmin and purified porcine skeletal tropomyosin, α-actinin and actin or purified porcine smooth muscle filamin (Rathbun, 1982), all of which may be present in the Z line of skeletal muscle (Yamaguchi et al., 1983).

Desmin isolated from avian and mammaliam smooth (Lazarides and Balzer, 1978; Huiatt et al., 1980; Steinert et al., 1982) and avian skeletal and cardiac (Lazarides and Balzer, 1978; Steinert et al., 1982) muscle are resolved as two major (α and β) isoelectric variants by two-dimensional gel electrophoresis (O'Farrell, 1975). Further work has shown the existence of c-AMP dependent protein kinases capable of *in vivo* and *in vitro* phosphorylation of the more acidic (α) but not the more basic (β) isoelectric variant of desmin (O'Connor et al., 1979, 1981). While the catalytic subunit of the c-AMP dependent protein kinase has been isolated from rabbit skeletal muscle (O'Connor et al., 1981), only one major isoelectric variant of desmin (β) and the presence of a very minor, more acidic variant have been detected in mature mammalian skeletal muscle (Lazarides and Balzer, 1978; O'Shea et al., 1981; Rathbun, 1982).

Localization of desmin in skeletal muscle cells

Desmin has been shown to be located at the Z line in chicken skeletal myofibrils by indirect immunofluorescence labelling with specific antibodies against chicken gizzard desmin (Lazarides and Hubbard, 1976). The authors also observed that labelling was associated with filamentous structures along the length of a muscle fiber both in close association with the plasma membrane and between myofibrils. Subsequent observations on isolated Z-disc sheets (Granger and Lazarides, 1978), derived from KI-extracted chicken skeletal muscle, showed desmin to be

present at the periphery of each Z-disc where it was a component of an interconnecting filament network spanning the breadth of the entire muscle cell. This has also been substantiated by other work on intermediate filaments in skeletal muscle (Lazarides, 1978; Schmid et al., 1979; Bennett et al., 1979; Campbell et al., 1979). It is now known that desmin is also a component of cytoskeletal intermediate filaments in mammalian smooth, skeletal and cardiac cells (Lazarides et al., 1982).

While the presence of transversely oriented 100 Å filaments at the level of the Z line had been detected with the electron microscope in vertebrate and non-vertebrate skeletal muscle by many researchers (Garamvolgyi, 1967; Bennett et al., 1979; Nunzi and Franzini-Armstrong, 1980; Edstrom et al., 1980; Pierobon-Bormioli, 1981; Schreiner, 1982), it was not until recently that these 100 Å filaments were unequivocally demonstrated to be comprised, at least in part, of desmin. Richardson et al.(1981) have localized 100 Å filaments in mature avian skeletal muscle by the indirect method with horseradish peroxidase and antibodies directed against avian gizzard desmin. Their results support and provide the definitive linkage between conclusions reached in prior biochemical, morphological and fluorescence studies on the existence and location of desmin and the existence and (partial) composition of 100 Å (intermediate) filaments in skeletal muscle; that is, that desmin unequivocally exists at the level of the Z line as a component of an intermediate filament network peripheral to the Z lines in skeletal muscle.

Other proteins associated with 100 Å filaments in skeletal muscle

While desmin is the predominant intermediate filament subunit in skeletal muscle cells, there is a lack of agreement on whether it is the only intermediate filament class or subunit in skeletal muscle cells. Vimentin (Franke et al., 1978), also referred to in the literature as fibroblastic intermediate filament protein or decamin (Zackroff and Goldman, 1979), is unequivocally present in cultured skeletal muscle cells (Berner et al., 1981). The presence of vimentin in mature skeletal muscle cells is, however, ambiguous. Some researchers claim, based on two-dimensional gel electrophoresis and/or immunofluorescence, that it is not ambiguous (Bennett et al., 1979; O'Shea et al., 1981; Holtzer et al., 1981) while others state that vimentin persists in mature avian skeletal muscle (Gard et al., 1979; Gard and Lazarides, 1980; Lazarides, 1980) or chicken skeletal myofibrils (Granger and Lazarides, 1979) with a distribution seen by immunofluorescence to be similar to that of desmin. It is not certain whether vimentin exists as individual filaments or as a copolymer with desmin, because the molecules can form either homopolymer or heteropolymer intermediate filaments in vitro (Steinert et al., 1981).

The Lazarides[1] group has claimed to have isolated other intermediate filament-associated proteins from avian skeletal muscle. None of these proteins have the capability, as do desmin and vimentin, to form synthetic filaments in vitro.

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[1]All remaining references in this section which contain information on the presence of these proteins are from the research group headed by E. Lazarides at the California Institute of Technology, Pasadena, CA.

A 68,000 dalton neurofilament-associated polypeptide, usually isolated from rat spinal cord, was shown by immunoradiographically labelled two-dimensional gel electrophoresis to be present in whole cell extracts of seven-day-old chicken skeletal myotubes and in Triton X-100/KI extracted chicken pectoralis myofibrils. Antibodies to this 68,000 dalton protein bound to the Z line in myofibrils prepared from adult chicken pectoralis muscle (Wang et al., 1980). This protein has also been referred to as the heat shock protein, thermin (Wang et al., 1981). However, O'Shea et al. (1981) and Rathbun (1982) were unable to detect the presence of any 68,000 dalton polypeptide during routine preparations of desmin from adult porcine skeletal muscle, nor has its presence in skeletal muscle been noted in any other publications.

Synemin (Granger and Lazarides, 1980) and paranemin (Breckler and Lazarides, 1982) are two other intermediate filament-associated proteins which this group has identified in avian skeletal muscle. Paranemin (280,000 daltons) and synemin (230,000 daltons) were both shown by immunoradiographic labelling of two-dimensional gels to be present in cultured avian myogenic cells, and were shown by immunofluorescence to be associated with cytoplasmic intermediate filaments during early myogenesis and with the Z line during late myogenesis. However, only very small quantities of synemin were present (approximately 1 - 2% of the amount of desmin) in adult skeletal muscle (Lazarides, 1982). No other reports of these proteins are in the literature. Though desmin is generally regarded as the major subunit of intermediate filaments in mature skeletal muscle, it may indeed be one of several proteins

which copolymerize to form individual 100 Å filaments.

The proposed role of the cytoskeleton in postmortem skeletal muscle

Connecting adjacent myofibrils at the Z line has been the only function ascribed to the cytoskeletal filament network in living skeletal muscle to date (Granger and Lazarides, 1978; Lazarides, 1980; Robson et al., 1981; Lazarides et al., 1982). The breakdown of this myofibril integrating network and its component protein, desmin, during postmortem aging has recently been implicated to be partially responsible for tenderization occurring during postmortem storage of bovine skeletal muscle (George et al., 1980; Young et al., 1981; Robson et al., 1981). The mechanisms of postmortem tenderization of bovine skeletal muscle have been covered in some recent excellent reviews on chemical changes occurring during postmortem aging (Asghar and Yeates, 1978; Dutson et al., 1980b; Asghar and Pearson, 1980). Rather than discuss the mechanisms thought to be involved in postmortem aging, the focus of the remainder of this literature review will be on published research dealing with two conditions (high temperature storage and electrical stimulation) shown to accelerate tenderness during postmortem conditioning. Particular attention will be given to the possible effects of each condition on desmin and the cytoskeleton.

High Temperature Conditioning and its Effects on Tenderization of Postmortem Muscle

The tenderizing effect that early postmortem high temperature storage has on beef muscle has been realized for more than thirty years. Roschen et al. (1950) patented a process in which the freshly killed and

dressed beef carcass was "held at a temperature of approximately 96°F... for approximately 4 to 5 hours" before commencement of normal chilling. Since the rate of biophysical and biochemical reactions depends upon temperature, and since temperature also affects the kinetic energy and metabolic activities of cellular systems in muscle, it is not surprising that aging periods can be reduced by holding carcasses at higher than normal temperatures (Davey and Gilbert, 1976).

Numerous studies have shown that more rapid tenderization of carcasses occurs by aging at elevated temperatures (Busch et al., 1967; Henderson et al., 1970; Newbold and Harris, 1972; Parrish at al., 1973; Dutson et al., 1975; 1976; Locker and Leet, 1976; Davey and Gilbert, 1976; Olson et al., 1976; Moeller et al., 1976, 1977; Dutson, 1977; Cheng and Parrish, 1978; Dransfield et al., 1980; Lochner et al., 1980). While this point is well substantiated, differences in theories exist as to which biochemical mechanisms are relevant to increases in tenderness noted during high temperature conditioning.

Proposed mechanisms relevant to tenderness in high temperature aged beef

Early muscle researchers widely held the belief that the degree of muscle contraction, which is indicated by sarcomere length (Herring et al., 1967), was directly related to the degree of tenderness in muscle (Locker, 1960; Locker and Hagyard, 1963). The theory that tenderness decreased with the degree of contraction arose out of three general observations: 1) meat cooked pre-rigor, before tension development, was more tender than meat cooked soon after the development of rigor (Moran and Smith, 1929; Weidemann et al., 1963); 2) meat from pre-rigor, cold

shortened carcasses is much tougher than meat not allowed to cold shorten (Locker, 1960; Marsh and Leet, 1966); 3) muscle cut and chilled soon after slaughter (similar to cold shortening on the carcass (Busch et al., 1967)) was tougher after rigor mortis had developed than muscle which had gone into rigor on the bone (Ramsbottom and Strandine, 1949; Locker, 1960; McCrae et al., 1971). Based on these observations, and in particular the effects of cold shortening (the mechanisms of which will be discussed in the following section) on muscle contraction and toughness, studies were done to determine the effects that high temperature storage would have on reducing pre-rigor contraction in ovine and bovine muscle. It was well-documented that sarcomere lengths were greater in carcasses where substantial carcass cooling was delayed until the onset of rigor mortis (Smith et al., 1971; Parrish et al., 1973; Bouton et al., 1973, 1974), and these observations led some investigators to attribute the improvements in tenderness to the decrease in muscle fiber shortening in high temperature-aged carcasses. It was, however, realized by most investigators that sarcomere length was merely an indicator of the contractile state within a muscle fiber, and, although frequently coincidental with detectable declines in tenderness, was not usually causal to the degree of tenderness (Goll et al., 1964; Marsh and Leet, 1966; Busch et al., 1967; Parrish et al., 1973; Stromer et al., 1974; Dutson et al., 1975; Dutson, 1977). Lochner et al. (1980) reported that other effects of temperature on postmortem muscle are more important to tenderness than the reduction in the amount of muscle contraction (except in extreme cases of cold shortening).

High temperature storage, and the concomitant increased rate of pH decline that it causes (Busch et al., 1967; Dutson, 1977; Marsh et al., 1981) has been shown by several studies to affect the integrity of lysosomal membranes in postmortem muscle. Induced, early release of membrane bound lysosomal enzymes increases enzyme-substrate interaction time in postmortem tissue. Moeller et al. (1976) have shown significant differences in the distribution and total activity of β-glucuronidase and cathepsin C isolated from bovine longissimus dorsi muscle excised and stored at high temperatures when compared to the distribution and total activity of these cathepsins isolated from the excised contralateral longissimus dorsi stored at 2°C. The change in distribution of these cathepsins from membrane bound (sedimentable) to non-membrane bound (non-sedimentable) was indicative of the disruptive effect of high temperature storage on lysosomal or other membranes. While the total activity of both enzymes was lower in the proteolytic fraction isolated 12 hours post mortem from muscle stored at high temperatures, these differences were not significant when measured 18 and 24 hours post mortem. In a similarly designed experiment (Moeller et al., 1977), pH measurements in excised bovine muscle stored at 37°C were significantly lower at 4 and 12 hours post mortem than the pH measured in excised muscle stored at 2°C. The authors concluded, based on these results and those from studies of myofibril fragmentation, that favorable conditions of temperature and pH in bovine muscle stored at elevated temperatures leads to the early activation of lysosomal enzymes during postmortem storage. These results were also substantiated by Lochner et al., (1980)

who measured the effect of cooling rate on tenderness in bovine longissimus dorsi muscle.

While β-glucuronidase and cathepsin C have not been shown to utilize myofibrillar proteins as substrates, other lysosomal enzymes which do have been isolated from skeletal muscle. Cathepsins B and D have been isolated from bovine skeletal muscle and have been shown to degrade purified myosin, actin and troponin-T (Okitani et al., 1976; Schwartz and Bird, 1977; Bird et al., 1978) at low pH and high temperature. Penny (1980) emphasized that the pH range within which these enzymes are active does not preclude them from being active in postmortem muscle, especially at high temperatures (Arakawa et al., 1976; Penny and Dransfield, 1979; Ouali and Valin, 1981). For example, cathepsin B and D exhibit optimum activity at pH 5.2 and 4.0, respectively. At ultimate postmortem muscle pH (5.4 - 5.6), cathepsin B has 50% and cathepsin D has 30% of their optimum activity (Bird et al., 1978).

The low pH and high temperature conditions of muscle have been found to be quite favorable for certain other lysosomal enzymes which catabolize mucopolysaccharides of the ground substance (Dutson and Lawrie, 1974) and some cross-linkages of collagen in the non-helical region (Etherington, 1976). Lysosomal collagenases have been shown to exhibit increased proteolytic activity at higher temperatures (Kopp and Valin, 1981; Wu et al., 1981) but these changes appear to be small relative to the increases in tenderness in high temperature aged muscle.

Proteases which are not lysosomally bound must also be considered as effectors of changes in tenderness associated with high temperature

conditioning. Stromer et al. (1967) showed that trypsin, an enzyme not found in muscle, removed Z lines from skeletal muscle myofibrils and caused fragmentation at the Z line. The structural changes induced by trypsin mimicked Z line changes seen during conditioning of bovine muscle. This was the first indication that the Z line was the first structure in the sarcomere that was degraded by proteolytic enzymes of unspecified origins.

Olson et al. (1976) showed an acceleration in the rate of fragmentation and tenderization in bovine longissimus and semitendinosus muscle stored at 25°C versus the same muscles stored at 2°C, as measured by myofibril fragmentation index (MFI) and Warner-Bratzler shear force. This confirmed previous work by Henderson et al. (1970) that showed increased myofibrillar fragmentation with increasing postmortem storage time. Phase contrast microscopy of myofibrils showed that myofibril fragmentation had occurred, in most instances, at the Z line, which was consistent with previous postmortem studies showing ultrastructural changes at the Z line during aging (Stromer et al., 1967; Davey and Gilbert, 1969; Henderson et al., 1970; Goll et al., 1971). In subsequent work by this group (Olson et al., 1977; Olson and Parrish, 1977; Cheng and Parrish, 1978) on the effects of high temperature aging on skeletal muscle, a calcium-activated protease (CAF) (Busch et al., 1972) active at near physiological pH values was implicated in causing breakdown of the Z line and regulatory proteins in high temperature aged and normally conditioned muscle. Biochemical characterization of purified CAF showed it to be maximally active at pH 7.5 (substantially active from pH 6.5 - 8.0) in the presence of 1mM Ca^{2+} and

2mM MCE at 25°C, and capable of removing Z lines from myofibrils and hydrolyzing purified tropomyosin, troponin I and T, and C-protein (Dayton et al., 1976). Indirect immunocytochemical localization of CAF with affinity purified antibodies against the 80,000 dalton subunit of CAF showed reaction product at the Z line and sarcolemma in fixed human muscle (Dayton et al., 1981). Purified CAF is currently known to also degrade the purified myofibrillar proteins, desmin (O'Shea et al., 1979), nebulin and titin (M. Zeece, Dept. of Food Technology, Iowa State University).

A serine or alkaline protease (Noguchi and Kandatsu, 1966; Sanada et al., 1978; Woodbury et al., 1978) capable of hydrolyzing native myosin, actin, troponin and tropomyosin has also been isolated from muscle but has been shown by immunofluorescent localization to be present not within muscle cells but in mast cells in the connective tissue surrounding muscle cells. The complete significance of this alkaline protease to postmortem breakdown in muscle is, at the present time, unknown.

Ultrastructural analysis of high temperature aged bovine muscle

Ultrastructural studies have documented the increased breakdown occurring in bovine skeletal muscle subjected to high temperature storage versus breakdown seen in conventional temperature (2°C) aged tissue. In a study on the effects of different aging temperatures on the ultrastructure of bovine semitendinosus muscle, Stromer et al. (1967) showed, at the electron microscope level of resolution, that shortening of sarcomeres was greatest in muscle stored 24 hours at 2°C, only slightly less at 37°C, and minimal at 16°C. Z lines were irregular in width, had lost their filamentous appearance, and were disrupted more

in muscle stored for 24 hours at 37°C when compared with Z lines in muscle stored at 16°C or 2°C. The structure of Z lines in muscle stored for 24 hours at 2°C most closely resembled that observed in at death samples. Muscle stored at 16°C for 312 hours contained Z lines that had both larger and a greater frequency of gaps or discontinuities than those seen in Z lines in muscle stored at 2°C for 312 hours. Henderson et al.(1970), in a follow-up electron microscope study, showed the rate of myofibrillar structural degradation to be faster in bovine semitendinosus muscle stored at 25°C or higher, than in muscle stored at 16°C or lower. Mitochondria and other membranes were disrupted and appeared as large vacuoles, in many instances, after four hours of storage at 37°C. After eight hours at 37°C, Z lines in bovine myofibrils were diffuse and amorphous, and discontinuities in the Z line were evident. After twenty-four hours at 25°C, in some cases, the Z line was completely removed and areas were seen where some breakdown of thin filaments had occurred. Similar increases in ultrastructural breakdown due to high temperature storage have also been reported in other electron microscope studies on bovine skeletal muscle (Stromer and Goll, 1967; Dutson, 1977). Structural degradations noticed in bovine muscle stored at 2°C or 16°C were generally less drastic than those detected in tissue stored at temperatures greater than 16°C (Henderson et al., 1970).

Epilogue

The preceding research has been conducted, in many instances, for the elucidation of mechanisms that increase tenderness during usual

temperature (2°C) postmortem storage of bovine skeletal muscle. High temperature aging of meat has, to date, not been utilized as a method of increasing tenderness due to problems such as bacterial growth, bone taint and excessive shrinkage (Newbold and Harris, 1972; Follett et al., 1974) which tend to be particularly noticeable after cutting the carcass into retail cuts. Some studies (Rey et al., 1970, 1978; Lochner et al., 1980; Marsh et al., 1981) indicated that high temperature storage for a short period after slaughter may, however, circumvent these problems and still increase tenderness over that in conventionally aged meat.

Electrical Stimulation and its Effects on Postmortem Muscle

The earliest workers to apply an electrical stimulus to large animal carcasses were Harsham and Deatherage (1951). Their intention was to hasten the decline in pH and the onset (and subsequent resolution) of rigor mortis for the purpose of decreasing postmortem storage time for beef carcasses. They discovered that electrical stimulation shortly after slaughter also had a tenderizing effect on the tissue, and postulated this to be due to a release of catheptic enzymes during the vigorous carcass contractions induced by application of an electrical stimulus.

The practical application of electrical stimulation for large animals was not utilized until researchers in New Zealand (Carse, 1973; Chrystall and Hagyard, 1975, 1976; Davey et al., 1976) investigated the potential for electrical stimulation to accelerate postmortem condition-

ing in lambs. The New Zealand lamb processing industry was looking for a means to shorten the time between slaughter and blast-freezing of lamb carcasses destined for overseas shipment. This work, designed for the needs of the lamb processing industry, showed that processing time before blast-freezing could be reduced without concern for cold shortening or thaw rigor if an electrical stimulus was applied to the carcass immediately after slaughter. Subsequent research focused on the biochemical mechanisms related to this reduction of cold shortening and increase in tenderness. As a consequence, changes in meat and carcass quality characteristics induced by electrical stimulation were categorized as well.

Consideration must be accorded various electrical parameters when comparing results of electrical stimulation studies. Voltage, the duration and type of current, pulse frequency and duration, and the mode of current application to the carcass are all parameters subject to change in a given electrical stimulation experiment. It is the focus of this review to consider generalized effects of electrical stimulation and not specific differences detected when these parameters vary. An excellent review of the range of conditions used during carcass stimulation and the effects that these variations can have on meat and carcass quality characteristics can be found in the review by Asghar and Henrickson (1982).

A major effect of electrical stimulation on carcasses is to increase the rate of postmortem glycolysis, as measured by the rate of pH decline. Measurements of pH monitor the accumulation of lactic acid,

the end product of anaerobic glycolysis, and thereby directly measure the rate of postmortem glycolysis. Asghar and Henrickson (1982) state that electrical stimulation increases the biochemical reactions of the glycolytic pathways by 100 to 150 times. While this rate increase seems somewhat overstated, agreement exists in the literature that substantial increases in postmortem glycolysis are induced by electrical stimulation (Carse, 1973; Bendall et al., 1976; Bouton et al., 1978; Sorinmade et al., 1978; George et al., 1980; Nichols and Cross, 1980).

The effects of electrical stimulation also improve scoring parameters used to grade meat and carcass quality characteristics. Smith et al. (1980) have summarized the effects of electrical stimulation of beef carcasses from a number of studies. This summary shows improvements in flavor, lean maturity, lean color, heat-ring, firmness, marbling and USDA quality grades from 4 - 23% over grades given to meat from unstimulated beef carcasses. Electrical stimulation does not affect water holding capacity (Morgan, 1979; George et al., 1980) or juiciness (Davey et al., 1976; Bouton et al., 1978; Elgasim et al., 1981) to any significant degree.

Proposed theories relating the effects of electrical stimulation to tenderness

Most researchers have agreed that postmortem electrical stimulation of bovine carcasses produces a tenderizing effect on the musculature (Smith et al., 1971, 1977, 1980; Davey et al., 1976; Savell et al., 1977, 1978a, 1978b, 1978c; Cross, 1979; Elgasim et al., 1981; McKeith et al., 1981a, 1981b). These conclusions have come from measurements of indi-

cators of tenderness such as shear force, myofibril fragmentation index and sensory panel ratings. The most prevalent theories on the mechanisms by which electrical stimulation improves carcass tenderness are as follows: 1) reduction in the intensity or effects of cold shortening (Davey et al., 1976; Chrystall and Hagyard, 1976); 2) physical disruption of myofibrils (Savell et al., 1978a); 3) increases in the activities of acid proteases (Savell et al., 1977; Dutson et al., 1980a). The remainder of this section of the literature review will consider, based on present knowledge, how adequately each theory explains the reported increases in tenderness that occur in electrically stimulated muscle.

When a carcass is chilled at low temperatures (below 0°C) immediately after dressing, when the pH and ATP levels are still high, muscles near the surface may cool below 15°C. Induced by the cold, certain sarcomeres within a muscle fiber will "cold shorten" up to 40% of their rest length (Marsh and Leet, 1966). Originally, it was thought that this cold shortening was induced by a general release of Ca^{2+} ions from the sarcoplasmic reticulum into the myofibrillar regions at low temperatures (Pearson et al., 1973; Davey and Gilbert, 1975). However, Buege and Marsh (1975) advanced the theory that the mitochondria release an overload of Ca^{2+} ions at low temperatures, and thus overload the sarcoplasmic reticulum so that an excess of free Ca^{2+} ions initiates shortening. This view is supported by the work of Cornforth et al. (1980). Muscle contraction induced by this additional release of Ca^{2+} ions during cold shortening causes a considerable subsequent toughening of the meat (Davey et al., 1976). It has been suggested that this tough-

ening is due to a combination of physical changes at the fiber level (mostly related to sarcomere length) and macromolecular changes at the level of the myofilaments (Voyle, 1969).

Researchers working with electrically stimulated lamb carcasses in New Zealand (Davey and Gilbert, 1975; Chrystall and Hagyard, 1976; Davey et al., 1976) have attributed increases in tenderness in electrically stimulated muscle to a prevention or mitigation of cold shortening. Sarcomeres in muscle fibers from stimulated muscle would therefore be expected to be longer than sarcomeres in unstimulated muscle when exposed to cold temperatures shortly after death. Smith et al. (1977) made comparisons of sarcomere lengths in bovine muscle fibers from control and electrically stimulated sides or carcasses. Electrical stimulation was associated with longer sarcomeres in only three of six comparisons, which suggested that the benefits of such treatment in bovine muscle were not simply related to cold shortening. Nichols and Cross (1980) evaluated the effect of excision time and storage method on sarcomere length in muscles from stimulated and unstimulated sides. Sarcomere length was not significantly affected by electrical stimulation, time after death of tissue excision from the carcass, or temperature at which the tissue was stored when sarcomere length measurements taken at the time of tissue excision were compared to measurements taken 120 hours post mortem. Savell et al. (1977, 1978a) showed no statistically significant difference in sarcomere length between myofibrils prepared from longissimus dorsi muscle excised 20 - 24 hours or 120 hours post mortem from both unstimulated and electrically stimulated sides. Elgasim

et al. (1981) state, on the basis of similar sarcomere lengths in myofibrils prepared 72 hours post mortem from longissimus dorsi muscle excised from unstimulated and electrically stimulated sides, that mechanisms other than or in addition to the prevention of cold shortening are responsible for the observed improvement in meat tenderness due to electrical stimulation. This statement is in agreement with the conclusions of Cross (1979), Will et al. (1979), and McKeith et al. (1981b).

Savell et al. (1978a) suggest that the physical disruption of muscle fibers resulting from massive contractions during electrical stimulation may provide an explanation for the improvement in tenderness associated with electrical stimulation. Marked structural differences can be detected between electrically stimulated and unstimulated muscle at the light microscopic level of resolution, particularly in the appearance of dark contracture bands in stimulated samples (Dutson, 1977; Savell et al., 1978a; Will et al., 1980; McKeith et al., 1980; Sorinmade et al., 1982). Similar irregularly distributed dark bands adjacent to superstretched or otherwise distorted sarcomeres have also been seen when longissimus dorsi muscle sampled from electrically stimulated sides was examined in an electron microscope (Dutson, 1977; Savell et al., 1978a; Will et al., 1980; McKeith et al., 1980; Sorinmade et al., 1982). The general interpretation of these bands is that they arise from an extreme shortening of sarcomeres within certain sections of the muscle fiber (Savell et al., 1978a). The concomitant tearing or rupture of muscle fibers which this shortening sometimes produces may be a factor related to the increased tenderness of electrically stimulated muscle

(Dutson, 1977).

Other interpretations of the significance of these contracture bands exist in the literature. McKeith et al. (1980) related that longissimus dorsi muscles isolated from electrically stimulated intact carcasses had less structural damage (as assessed by the fact that fewer contracture bands were noted) yet were as tender as longissimus dorsi muscle isolated from electrically stimulated sides. George et al. (1980) noted contracture bands at the light and electron microscope levels of resolution in bovine longissimus dorsi muscle excised and fixed 1.5 hours after electrical stimulation. This group's analysis, based on observations of cross and longitudinal sections of this muscle, was that the banding was caused by a deposition of denatured sarcoplasmic proteins on the myofilaments. Sorinmade et al. (1982) also considered this to be a possible explanation for the appearance of contracture bands seen in electrically stimulated muscle samples.

Many researchers have detected, at the electron microscope level of resolution, more specific ultrastructural changes in bovine longissimus dorsi muscle induced by electrical stimulation. These alterations include a reduction in the density, or transverse dimensional changes, in the I band and Z line (Dutson, 1977; Savell et al., 1978a; Elgasim et al., 1981; Sorinmade et al., 1982) and swollen T-tubules, triads and mitochondria (Will et al., 1980; Elgasim et al., 1981; Sorinmade et al., 1982). However, George et al. (1980) were unable to find any histological evidence of gross damage in electrically stimulated bovine longissimus dorsi on a scale sufficient to explain the detectable

increase in tenderness in electrically stimulated versus unstimulated longissimus dorsi muscle. It therefore appears that any increases in tenderness in electrically stimulated muscles may only be partially due to physical disruption of myofibrillar structure.

It seems that the tenderizing effects of electrical stimulation may be more directly related to increased proteolysis (due either to the release of acid cathepsins from lysosomes or to activation of enzymes such as CAF), than to possible reductions in cold shortening or increases in gross physical disruption of the stimulated muscle. Dutson et al. (1980a) noted that lysosomal membranes were less stable in electrically stimulated muscle, as measured by a decreased sedimentable specific activity of lysosomal enzymes when compared to the sedimentable (i.e. lysosomal bound) enzyme activity in unstimulated tissue. They suggested that the release of lysosomal enzymes was hastened by the rapid decline in pH, which disrupted lysosomal membranes. This conclusion was also substantiated by work done by Sorinmade et al. (1978).

Since the major effect of electrical stimulation is to increase postmortem glycolysis, the ultimate pH in postmortem tissue is reached more rapidly (and therefore at higher temperatures) in electrically stimulated than in unstimulated muscle. George et al. (1980) stated that the relationship of high temperature to low pH is necessary for the increase in tenderness noted in electrically stimulated versus unstimulated muscle. The increase in the rate of tenderizing at higher postmortem storage temperatures has been demonstrated by other researchers

(Busch et al., 1967; Davey and Gilbert, 1969; Davey and Gilbert, 1976; Dransfield et al., 1980; Marsh et al., 1981). Bendall (1980) stated that the accelerated natural tenderization of electrically stimulated meat was partly due to increased proteolytic enzyme activity at temperatures higher than those measured in unstimulated carcasses at a given pH. This conclusion is supported by the work of Elgasim et al. (1981) who detected more pronounced ultrastructural breakdown in meat conditioned by a combination of electrical stimulation and high temperature aging versus that in meat conditioned solely by electrical stimulation or high temperature aging. The altered pH/temperature relationship present in electrically stimulated muscle may also affect the activity of other non-lysosomally bound proteases, in particular that of calcium-activated factor (CAF) (Gunawardene, 1981).

Epilogue

The evidence discussed in this section leads to the conclusion that while electrical stimulation may have some direct degradative effect on the ultrastructure of muscle, improvements in tenderness are more consistently correlated to measurements showing an accelerated timecourse in the postmortem biochemical changes occurring in muscle. An accelerated pH decline affects the distribution and availability of proteolytic enzymes, particularly those that are membrane bound. While increases in postmortem glycolysis can also reduce the potential for a muscle to cold shorten, this may only be relevant to tenderness when the potential for dramatic cold shortening exists.

MATERIALS AND METHODS

All solutions were prepared from analytical reagent grade chemicals, using double-deionized glass distilled water and/or analytical grade chemicals as solvents. All preparations were done at 2°C, unless specified otherwise, for the purpose of reducing autolysis in protein samples.

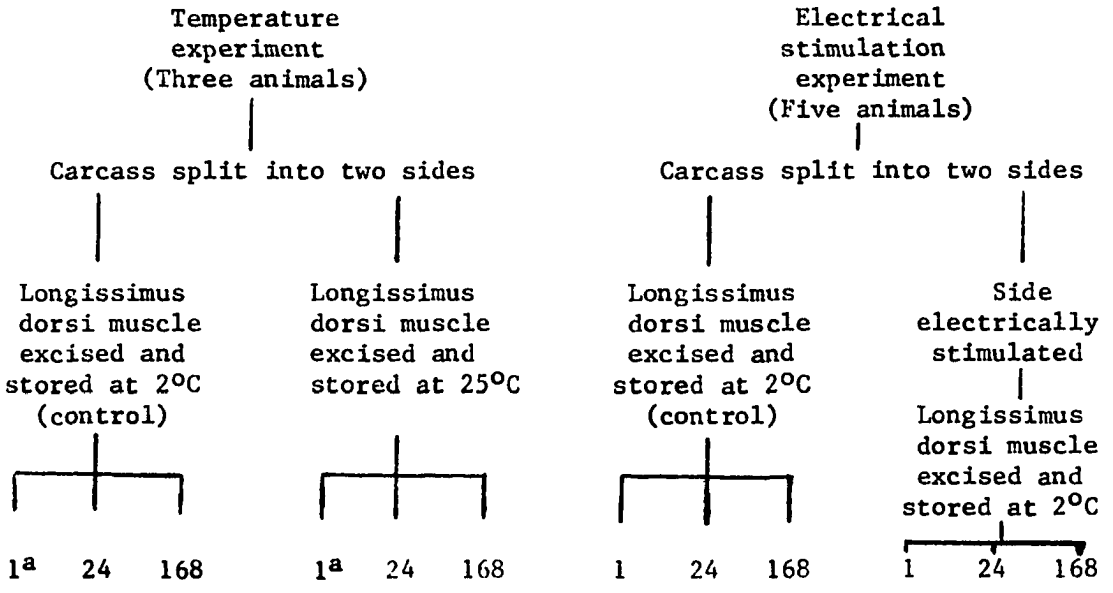
Animals and Tissue Samples

Crossbred steers or heifers 18 - 24 months of age were obtained from the Iowa State University beef herds. The animals were stunned with a captive bolt pistol, exsanguinated, dressed and split along the vertebral column by Iowa State University meat laboratory personnel. All tissue samples were taken from the longissimus dorsi muscle, adjacent and anterior to the 12th rib.

The experiments were designed to compare changes in desmin and the cytoskeleton at 1, 24 and 168 hours post mortem in muscles stored at 25°C versus a conventional storage temperature of 2°C and in muscles from electrically stimulated sides versus non-stimulated sides. Muscle samples from both the stimulated and unstimulated sides were stored at 2°C. In the experiments where temperature was the variable, the muscle sample from one side of the carcass was stored at 25°C while the muscle from the contralateral side was stored at 2°C and served as the control. Three animals were used for the temperature experiment and five were used for the electrical stimulation experiments. The experimental design is summarized in Figure 1.

A 3.2 kg sample was removed from each longissimus dorsi muscle, and

Figure 1. Experimental design of the study on the effects of temperature and electrical stimulation on the quantity of desmin isolated from bovine skeletal muscle at selected postmortem intervals. {A 3.2 kg portion of the longissimus dorsi muscle was excised from each side of a carcass and 600 gm samples were taken from this portion at 1, 24 and 168 hr postmortem for the preparation of desmin-enriched fractions. The amount of desmin in each desmin-enriched fraction was determined by densitometric scanning of one-dimensional tube gels as described in the Materials and Methods subsection - Quantitation of desmin from desmin-enriched fractions.}



[a]Because the intramuscular temperature of the one-hour samples was 29 ± 1°C, temperature differences would not have affected the amount of desmin extracted. Samples from five longissimus dorsi muscles with this treatment were analyzed and gave an indication both of the effect of temperature and of the reliability of the extraction method.

was wrapped in paper towels soaked with 100 ppm chloramphenicol, 10 ppm rifamycin, 1mM NaN_3 to limit bacterial growth. These towels were changed daily. All samples were also wrapped in Saran wrap and placed in a polyethylene bag to prevent moisture loss before being stored at the chosen temperature. Because the intramuscular temperature was virtually identical after one hour of storage at 2°C and at 25°C, the amount of desmin isolated from the samples (see Table 2 in Results) was not considered to have been affected by temperature. The very small error term associated with this value indicated that this assumption was valid. The small error term is also an indication of the repeatability of the isolation method because five one-hour samples were analyzed individually. If the isolation method had been unreliable, the error term would have been inflated.

Electrical Stimulation

The stimulator was constructed at the Research Equipment Assistance Program facility at Iowa State University from plans based on electrical specifications obtained from the Meat Research Institute, Langford, Bristol, England. Electrical specifications for the unit have been previously listed (Gunawardene, 1981).

One side of each carcass was stimulated and the other side served as the non-stimulated control. The carcass side to be stimulated was suspended, with a nylon rope through the Achilles tendon, from a ceiling-mounted hoist. This rope isolated the carcass electrically from the overhead rail system used to move carcasses through the slaughter facility. Power leads from the stimulator were attached by bulldog clips to 15 cm X 4.5 mm O.D. sharpened stainless steel pins inserted

into the trapezius and semitendinosus muscles. The carcass was electrically stimulated at 500 volts with a current of 360-420 milliamperes and a 12 Hz pulse frequency for three - 30 second periods. The direction of the current flow through the carcass was reversed during the thirty-second interval between periods of stimulation.

Isolation of Desmin-Enriched Fractions

Muscle fractions enriched in desmin were isolated from tissue excised at death from both carcass sides and sampled in all experiments at 1, 24 and 168 hours post mortem, according to the methods in the flowchart in Figure 2. A 600 gram muscle sample, trimmed free of all fat and connective tissue, was used for each isolation. The procedures were adapted from methods used by O'Shea et al. (1981) for isolating desmin-enriched fractions from porcine skeletal muscle. The sediment, after ammonium sulfate precipitation (Figure 2, Step XIII), was solubilized in 1% SDS, 20mM sodium phosphate, pH 7.80, 3mM MCE rather than in 6M urea, 1mM DTE, 10mM imidazole-HCl, pH 7.20 (O'Shea et al., 1981) to aviod losses of desmin due to incomplete protein solubility in the urea solution.

Electrophoresis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn (1969). A solution containing seven and one-half percent (v/v) polyacrylamide (75:1 acrylamide : methylenebisacrylamide), 0.1M sodium phosphate, 0.1% SDS, pH 7.0 was polymerized at room temperature to a length of 80 mm in 12 cm x 5 mm (I.D.) glass gel tubes and used as the support medium for protein

I. 600 grams ground bovine skeletal muscle
   a) Suspend in 6 v (v/w) of standard salt solution (SSS = 100mM KCl, 1mM sodium azide, 2mM EGTA, 2mM $MgCl_2$, 20mM K-phosphate, pH 6.8) by homogenization in a Waring blender as described below.
      i) Homogenize for 15 sec at 12,000 rpm and wait for 15 sec.
      ii) Homogenize for 15 sec at 12,000 rpm and wait for 15 sec.
      iii) Homogenize for 15 sec at 14,000 rpm and wait for 15 sec.
      iv) Homogenize for 15 sec at 18,000 rpm.
   b) Centrifuge at 2,000 x g_{max} for 10 min.

Supernatant

II. Sediment
   a) Resuspend in 5 v of SSS, 1% Triton X-100 and homogenize in Waring blender for 10 sec at 12,000 rpm.
   b) Centrifuge at 2,000 x g_{max} for 10 min.

Supernatant

III. Sediment
   a) Resuspend in 5 v of 50mM Tris-HCl, pH 7.65, 5mM EDTA, 1% Triton X-100, 1mM PMSF and homogenize in Waring blender for 10 sec at 12,000 rpm.
   b) Centrifuge at 2,000 x g_{max} for 10 min.

Supernatant

IV. Sediment
   a) Resuspend in 5 v of 0.15M KCl, 5mM EDTA, 1% Triton X-100, 1mM PMSF and homogenize in Waring blender for 10 sec at 12,000 rpm.
   b) Centrifuge at 2,000 x g_{max} for 10 min.

Supernatant

V. Sediment (washed myofibrils)
   a) Resuspend in 5 v of 500mM NaCl, 5mM EDTA, 5mM ATP, 1mM cysteine, 40mM imidazole-HCl, 1mM PMSF, pH 7.20, and homogenize in Waring blender for 10 sec at 12,000 rpm.

Figure 2. Flow chart of procedures used in the isolation of desmin-enriched fractions from bovine skeletal muscle (adapted from O'Shea et al., 1981)

b) Stir gently on magnetic stir-plate for 30 min.
c) Centrifuge at 13,800 x g_{max} for 30 min.

Supernatant

VI. Sediment
a) Resuspend in 5 v of 500mM NaCl, 5mM EDTA, 5mM ATP, 1mM cysteine, 40mM imidazole-HCl, 1mM PMSF, pH 7.20, and homogenize in Waring blender for 10 sec at 12,000 rpm.
b) Stir gently on magnetic stir-plate for 4 hr.
c) Centrifuge at 13,800 x g_{max} for 30 min.

Supernatant

VII. Sediment
a) Store at -5°C overnight.
b) Remove sediment from centrifuge tubes.
c) Resuspend in 5 v of 1M KI, 20mM imidazole-HCl, 1mM PMSF, pH 7.20, and homogenize in Waring blender for 10 sec at 12,000 rpm.
d) Stir gently on magnetic stir-plate for 2 hr.
e) Centrifuge at 13,800 x g_{max} for 30 min.

Supernatant

VIII. Sediment
a) Resuspend in 5 v of 1M KI, 20mM imidazole-HCl, 1mM PMSF, pH 7.20, and homogenize in Waring blender for 10 sec at 12,000 rpm.
b) Stir gently on magnetic stir-plate for 2 hr.
c) Centrifuge at 13,800 x g_{max} for 30 min.

Supernatant

IX. Sediment
a) Resuspend in 5 v of distilled water and homogenize in Waring blender for 10 sec at 12,000 rpm.
b) Centrifuge at 13,800 x g_{max} for

Figure 2 (continued)

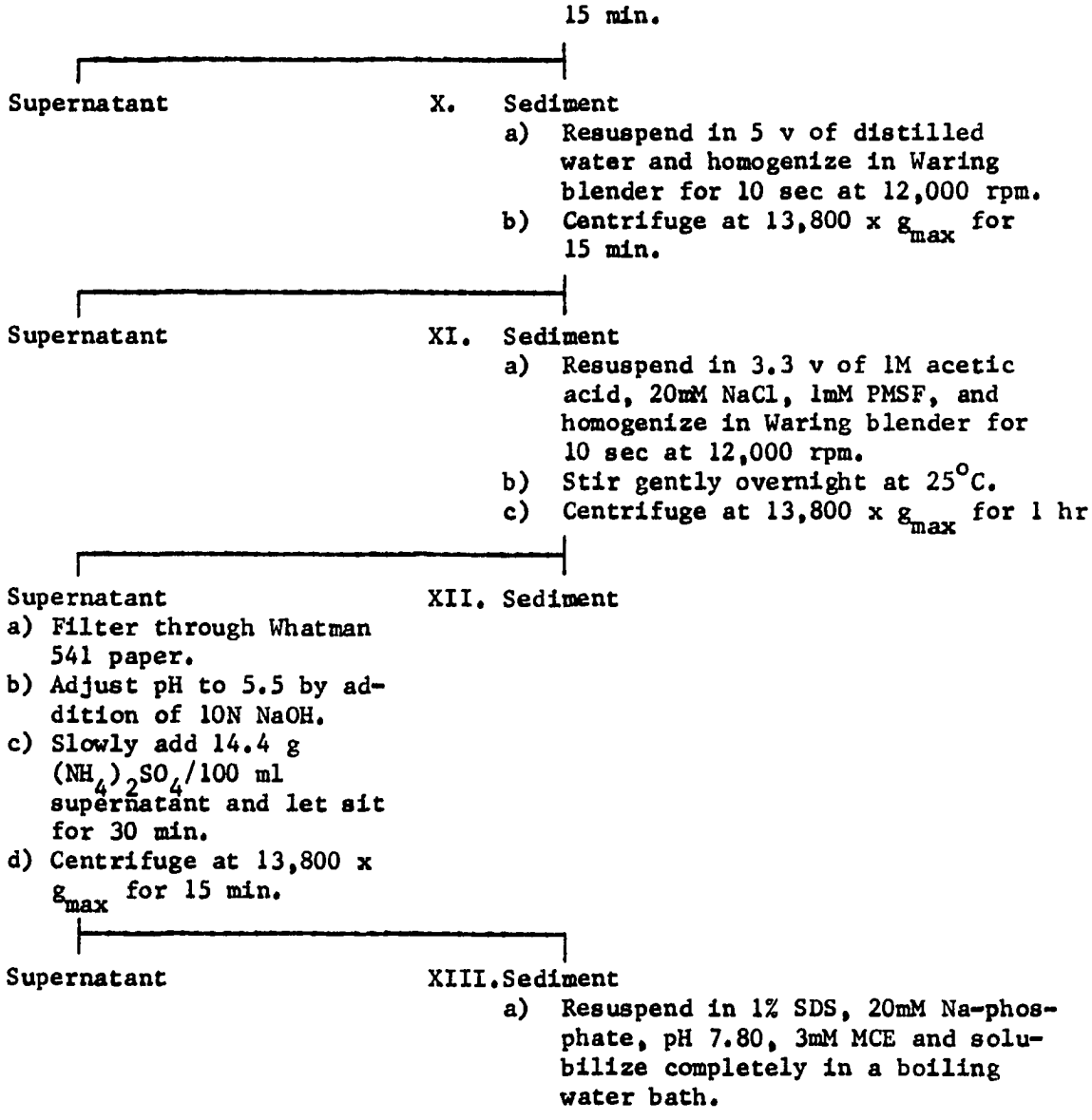


Figure 2 (continued)

separation. Soluble protein samples were prepared for electrophoresis by placing two parts of protein and one part of tracking dye (5.25% SDS, 4.6M MCE, 20% glycerol, 0.03% bromophenol blue, 60mM sodium phosphate, pH 7.0) in 12 x 75 mm test tubes and heating in a boiling water bath for five minutes. Insoluble samples were solubilized by heating in a boiling water bath in a solution of 1% SDS, 20mM sodium phosphate, pH 7.8, 3mM MCE, and prepared for electrophoresis as described. The gel tubes were placed in a Canalco electrophoresis tank (Canalco, Rockville, MD). Protein-dye mixtures were layered onto the tubes, the upper and lower reservoirs were filled with a gel buffer containing 0.1M sodium phosphate, 0.1% SDS, pH 7.0, and electrophoresis was done at a constant current of 8 milliamperes per gel until the tracking dye reached one centimeter from the bottom of the gel. The gels were then removed from the glass tubes and stained overnight in 0.1% Coomassie brilliant blue R (Sigma Chemical Co., St. Louis, MO), 50% methanol, 7% acetic acid. The gels were stored in a fresh change of this solution approximately one week before being photographed.

Two-dimensional electrophoresis of various desmin-enriched fractions was done according to the method of O'Farrell (1975). Isoelectric focusing in the first dimension was done on 4% acrylamide gels allowed to polymerize in 2.5 mm I.D. x 130 mm tubes to a length of 120 mm. Protein samples insoluble in non-denaturing solvents were solubilized in unbuffered 8M urea overnight at 2°C, clarified by centrifugation at 145,000 x g_{max} for one hour, and equilibrated in sample buffer as described for soluble samples (O'Farrell, 1975). The focused samples were then electrophoresed in the second dimension onto 10% polyacrylamide slab gels according to the method

of Allen et al. (1978) using the Laemmli (1970) buffer system, and stained and destained as previously described.

Chromatography

It was possible to achieve further purification of desmin from desmin-enriched fractions if the heterogeneous sediment after ammonium sulfate precipitation (Figure 2, Step XIII) was extracted in 6M urea, 1mM DTE, 10mM imidazole-HCl, pH 7.20 and maintained in this solution through subsequent column chromatography on hydroxyapatite and Sepharose CL-6B. Highly purified desmin was needed for preparing a standard curve for gel quantitation, for use as an antigen, for amino acid analysis and for filament formation studies. Desmin was purified by these methods in preparations separate from those where the sediment in Figure 2, Step XIII was solubilized in 1% SDS, 20mM sodium phosphate, pH 7.80, 3mM MCE and used for desmin quantification because SDS-solubilized protein was not suitable for further purification by column chromatography.

Urea solutions used throughout the chromatographic purification procedure were prepared from an 8M stock urea solution passed through a column of Amberlite MB-3 (Mallinckrodt, Inc., Paris, KY) to remove cyanate and metal ion contaminants. Column packing, equilibration flow rates and methods of elution were similar to those parameters used in the chromatographic purification of desmin isolated from porcine skeletal muscle (O'Shea et al., 1981).

Glass chromatography columns, flow adapters and Sepharose CL-6B used in this study were obtained from Pharmacia Fine Chemicals, Inc.

(Piscataway, NJ). Hydroxyapatite was purchased from Bio-Rad Laboratories (Richmond, CA). Columns were equilibrated and protein was loaded onto and eluted from columns at a constant flow rate by the use of LKB Multiperpex or Varioperpex pumps (LKB Instruments, Inc., Rockville, MD).

Electrophoretic Purification of Desmin

In certain instances, desmin was not completely separated from actin and other contaminants after column chromatography. A modification of the electrophoretic elution-concentration procedures of Stephens (1975) was used to separate desmin from these contaminants. The protocol involved separating desmin from other proteins in the presence of SDS in a 10% polyacrylamide slab gel and excising the portion of the gel to where desmin had migrated. This excised band was inserted into a glass cylinder (15 cm x 10 mm I.D.) that had been plugged with 2% agar. The tube was filled with 2% agar and the desmin was electrophoresed from this thick tube gel into a dialysis bag at 10 milliamperes for 20 hours. This procedure yielded highly pure desmin, as judged by tube gel electrophoresis, which was used in further experimental procedures after dialysis against 10mM Tris-acetate, pH 8.5, 1mM DTE.

Quantitation of Desmin from Desmin-Enriched Fractions

The amount of desmin present in desmin-enriched fractions was determined by densitometric scanning of SDS-polyacrylamide tube gels of these fractions. Peak area of the desmin band in a given fraction

was compared to a standard curve of peak area versus micrograms of desmin to extrapolate the amount of desmin in the gel of that fraction. By calculating the percent desmin on the gel and by knowing the total amount of protein in the desmin-enriched fraction, the yield of desmin was determined.

The standard curve for gel quantitation was obtained by using conditions for electrophoresis identical to those previously described, unless specified otherwise. Electrophoretically purified desmin was combined in a 2:1 ratio with tracking dye to final concentrations of 0.01 or 0.10 mg/ml (to keep load volumes between 10 and 80 µl) and boiled for precisely three minutes in loosely capped 12 mm x 75 mm test tubes. This protein was electrophoresed in quantities of 0.2, 0.4, 0.8, 1.0, 2.0 and 4.0 µg, in triplicate, on 7½% SDS-polyacrylamide gels at 8 milliamperes per tube until the tracking dye had migrated 6 centimeters into the gel. Gels were removed from the tubes and were stained overnight with Coomassie brilliant blue R staining solution. Destaining involved five minutes in the Canalco Quick Gel Destainer (Canalco, Rockville, MD) followed by a destain solution change and an additional 15 minutes in the Canalco Destainer. Gels were stored in the destain solution which was changed daily for one week. This procedure had previously been determined to be the best method for removing non-specific background staining before gel scanning (Allen, 1976).

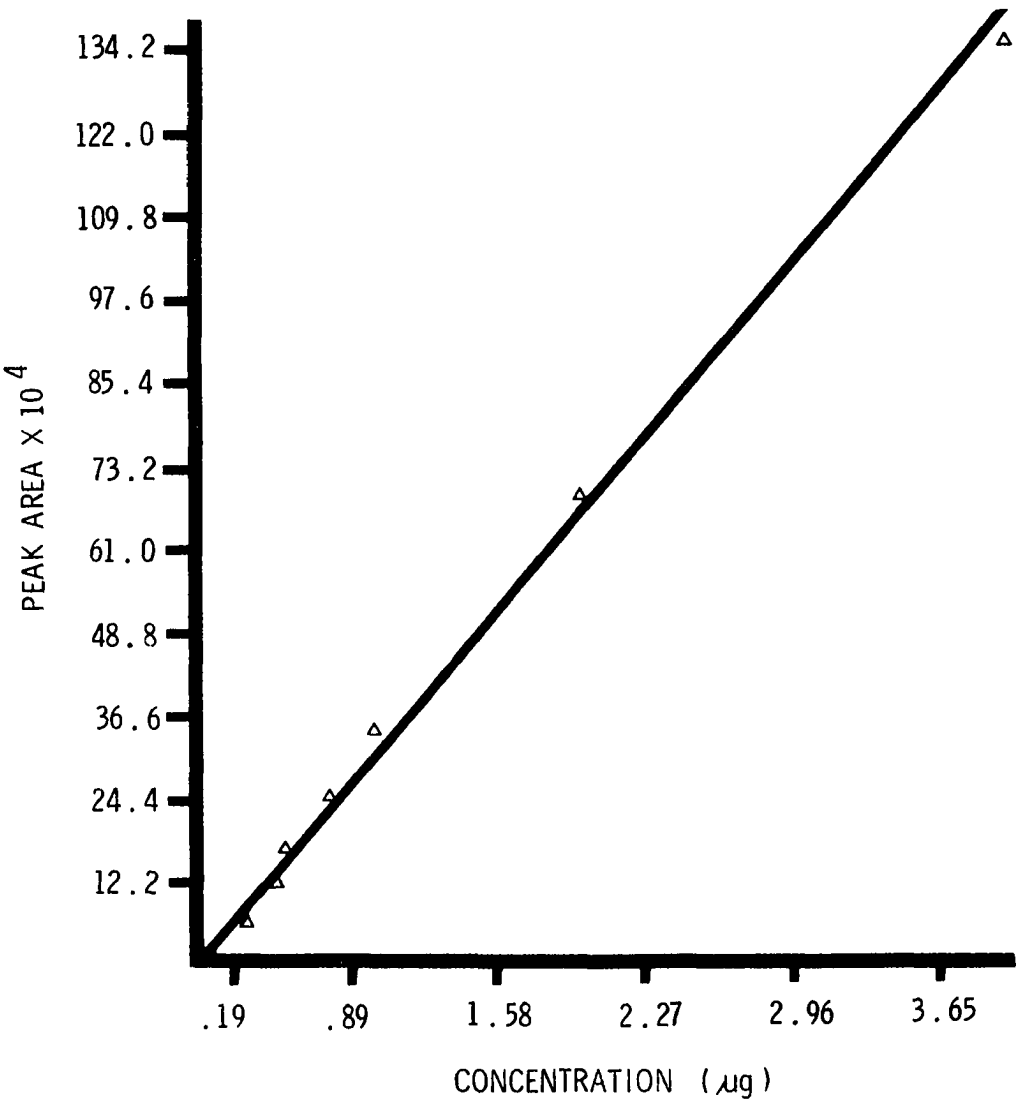
Each gel was scanned three times at 550 nm with a slit width of 0.02 nm on a Zeiss PMQ II Spectrophotometer (Carl Zeiss, West Germany) equipped with a thin layer chromatogram scanning attachment modified

to hold gels horizontally. Peak areas for the proteins were integrated by a System I Computing Integrator (Spectra-Physics, Santa Clara, CA) that received input directly from the spectrophotometer. The integrator was programmed to correct for baseline drift by restabilizing the baseline before and after each peak. A Honeywell Electronik 194 recorder (Honeywell, Inc., Fort Washington, PA) added in series with the spectrophotometer and integrator allowed visual monitoring of the gel scanning while in progress.

The nine data points for each concentration of purified desmin (triplicate scans of three gels) were averaged and were entered into a Commodore 2001 Computer (Commodore Business Machines, Inc., Santa Clara, CA) to obtain a plot of peak area versus desmin concentration (Figure 3). The regression coefficient for the standard curve was 0.998.

The following protocol was then used to determine the amount of desmin in a given desmin-enriched fraction. A ten µg load of the fraction was loaded on triplicate gels and each gel was scanned three times to yield nine peak areas for the desmin band in that fraction. The averaged peak area values corresponding to desmin for each crude desmin preparation were then entered into the Commodore 2001 computer along with data obtained for the standard curve to determine an estimated µg concentration of desmin in the 10 µg load. Multiplying the total milligrams of protein isolated in each desmin-enriched fraction times the percentage of desmin in the gel of that fraction allowed calculation of the total milligrams of desmin present at that stage of the extraction procedure.

Figure 3. Standard curve used for determining µg quantities of desmin in polyacrylamide tube gels of desmin-enriched fractions. {Each point is the average of nine peak area values (triplicate scans of three gels).}


134.2
122.0
109.8
97.6
85.4
73.2
61.0
48.8
36.6
24.4
12.2
PEAK AREA X 10^4
.19
.89
1.58
2.27
2.96
3.65
CONCENTRATION (μg)

Immunoautoradiography of Polyacrylamide Slab Gels

A variation of the technique of Towbin et al. (1979) was used for radioimmunological localization of desmin and its antigenically recognizable breakdown products isolated during various stages of extraction in the preparation of desmin-enriched fractions. The procedure involved electrophoretic transfer of proteins from unstained one-dimensional slab gels onto nitrocellulose paper, and subsequent radioimmunolabelling with specific desmin antibodies and ^{125}I Protein A.

Protein extracts from different steps in the preparation of desmin-enriched fractions were electrophoresed in duplicate on both halves of a 1.5 mm x 16 cm x 12 cm slab gel consisting of a 10% polyacrylamide separating gel and a 7½% polyacrylamide stacking gel, using the Weber-Osborn solutions previously described for tube gel electrophoresis. After division, one half of the gel was conventionally stained to assure that proper electrophoretic protein separation had occurred. The other, unstained half was backed with a moistened pure nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA) and placed into a water-cooled Bio-Rad Trans-Blot Cell containing a 4.8% Tris (w/v), 23% glycine (w/v) and 20% methanol (v/v) solution. Electrophoretic transfer of proteins for the gel onto the membrane was carried out for two hours at 90 volts and 6 amperes.

After electrophoresis, the nitrocellulose membrane was separated from the gel and washed with 2% BSA in water to saturate additional protein binding sites. The membrane was then rinsed in phosphate buffered saline (PBS) (0.01M sodium phosphate, 0.15M NaCl, pH 7.20)

and incubated for twelve hours in a 1:20 dilution of unfractionated serum, containing antibodies specific against desmin, in PBS. The membrane was then washed in three changes of PBS, reincubated for twelve hours with a 1:50 dilution of ^{125}I Protein A {iodinated by a modification of methods in Greenwood et al. (1963); separated by methods of Tuszynski et al. (1979)} in PBS, rewashed in five changes of PBS, and allowed to air dry. Loci of anti-desmin -- ^{125}I Protein A were then visualized by exposure to Kodak X - Omat AR sheet film (Eastman Kodak Co., Rochester, NY). After development, the exposed areas in the film were matched to their corresponding protein in the stained slab gel for interpretation.

Immunofluorescence Microscopy

Desmin was localized at the light microscope level in bovine longissimus dorsi myofibrils by using the indirect method of fluorescent antibody localization. This technique involved two distinct steps for specific localization of tissue antigens. The first step was to apply a specific, unlabelled antibody (primary antibody) that would recognize the antigen (in this case, desmin) to be detected. The second step involved the binding of a fluorescently-conjugated second antibody, which recognized gamma globulins from the same species used to raise the primary antibody, to the tissue antigen-primary antibody complex. This immunological complex was therefore a specific, detectable label for the tissue antigen.

Antibodies against electrophoretically purified desmin were produced in New Zealand white rabbits by two-multiple site, subcutaneous 500 µg injections of the purified desmin antigen. Each rabbit's

serum was prescreened against bovine myofibrils before injection to assure the absence of non-specific antibodies directed against myofibrillar proteins. For the initial injection, the antigen was combined in a 1:1 ratio with Freund's complete adjuvant (Cappel Laboratories, Cochranville, PA) to increase the formation and persistence of antibodies. The second, or booster, injection contained a 1:1 ratio of antigen with Freund's incomplete adjuvant and followed the initial injection by fourteen days. The rabbit was bled from the marginal ear vein 10 - 14 days after the booster injection. After clot retraction, whole blood cells were removed from the serum by clarification through three low speed (1,000 x g_{max} for 10 minutes each) centrifugations. This antiserum to desmin was tested for specificity in Ouchterlony double diffusion plates with purified porcine skeletal myosin, α-actinin, actin and tropomyosin and with porcine aortic vimentin and exhibited no cross reactivity. The desmin antiserum exhibited strong single precipitin lines against serial dilutions of electrophoretically purified bovine skeletal desmin antigen.

This unfractionated serum which contained monospecific antibodies to desmin was used to localize desmin in bovine longissimus dorsi myofibrils. Myofibrils were prepared by the method of Dayton et al. (1976) from these skeletal muscle samples: tissue stored at 2°C or 25°C for 1, 24 or 168 hours post mortem, and non-stimulated or electrically stimulated tissue stored at 2°C for 1, 24 or 168 hours post mortem. Myofibrils were stored in 50% (v/v) glycerol, 0.1M KCl, 0.02M sodium phosphate, 1mM NaN3, pH 7.10 at -20°C until required for use in labelling

procedures.

Glycerinated myofibrils were washed three times with ten volumes of phosphate buffered saline (PBS) before being labelled with mono-specific desmin antibodies. The labelling procedure (Richardson et al., 1981) involved incubating PBS-washed myofibrils in a 1:25 dilution of immune rabbit serum in PBS at 37°C for one hour. The myofibrils then were spun down and washed three times with ten volumes of 0.05M Tris-HCl, pH 9.50 to prevent non-specific staining. After the final wash cycle, the centrifuged myofibrils were incubated in a 1:25 dilution of FITC-conjugated, goat-anti-rabbit antiserum (Miles Laboratories, Inc., Elkhart, IN) in PBS at 37°C for one hour. Myofibrils were then spun down and washed three times with three ml aliquots of PBS, were resuspended in a medium of 90% (v/v) glycerol, 1mM sodium phosphate, pH 7.8 and were mounted on glass slides for viewing.

A serum blocking procedure (Richardson et al., 1981) was utilized to show that Z line labelling in myofibrils was specifically directed against desmin and not against other Z line proteins, namely α-actinin and vimentin (Figure 4).

All fluorescence samples were photographed with Tri-X film (Eastman Kodak Co., Rochester, NY) in a Zeiss Photomicroscope III equipped with a III RS epifluorescence attachment and a 63X planapochromat oil immersion objective (Carl Zeiss, West Germany).

Electron Microscopy

Muscle samples for transmission electron microscopy were excised from the carcass while clamped in a 9 mm I.D. modified Lambert Chalazion

Figure 4. Indirect immunofluorescent labelling of myofibrils prepared from 1 hour postmortem control bovine longissimus dorsi muscle with the serum blocking procedure. {The procedure is used to identify the antibodies in an antiserum. If Z line fluorescence after indirect labelling is reduced or eliminated after incubating the antiserum with an antigen, then this indicates that the antiserum contains antibodies to that antigen.

Five parts of immune rabbit serum containing monospecific antibodies to desmin (as assessed in Ouchterlony double diffusion plates) was preincubated for one hour at 37°C with one part of a "blocking" solution. The preincubated serum was then centrifuged and used in the indirect immunofluorescent labelling of myofibrils described in the Materials and Methods subsection - Immunofluorescence Microscopy. The following are phase contrast (left) and fluorescent (right) micrograph pairs of myofibrils labelled with immune serum preincubated with the following solutions:

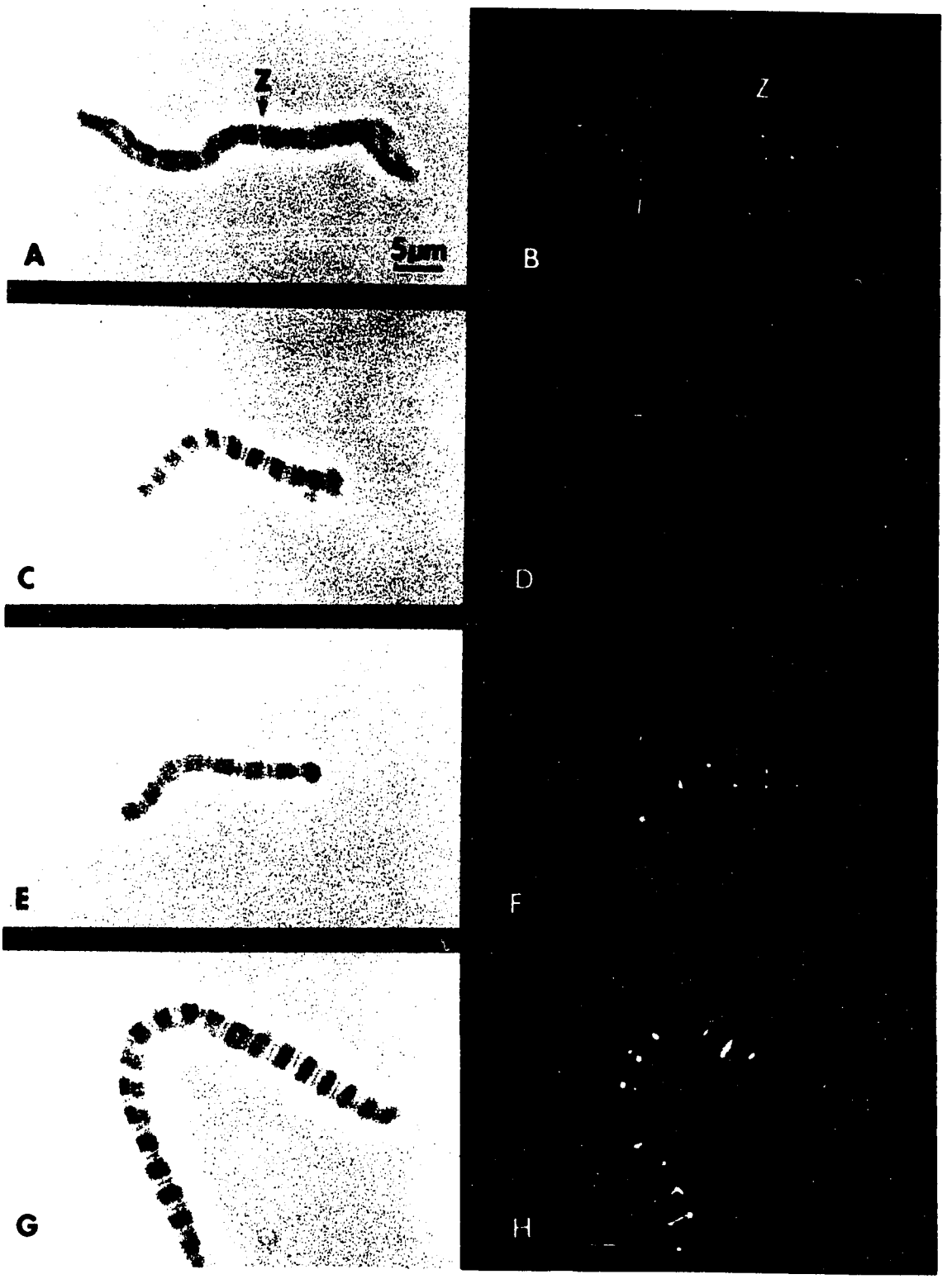
(A & B) 10mM Tris-acetate, 1mM DTE, pH 8.50 (control)

(C & D) 1.0 mg/ml bovine skeletal desmin in 10mM Tris-acetate, 1mM DTE, pH 8.50

(E & F) 1.0 mg/ml porcine skeletal α-actinin in 1mM $NaHCO_3$

(G & H) 1.0 mg/ml porcine aortic vimentin (courtesy of M. K. Hartzer) in 10mM Tris-acetate, 1mM DTE, pH 8.50

The micrographs show that desmin (C & D) was the only protein to block labelling of the myofibrils; none of the other proteins tested reduced or eliminated immunofluorescent labelling as compared to the 10mM Tris-acetate, 1mM DTE, pH 8.5 control (A & B)
1600X}


Z
A
5μm
B
Z
I
C
D
E
F
G
H

forceps (American Hospital Supply Corporation, McGaw Park, IL). The tissue was fixed three hours at 2°C in Karnovsky's fixative (Karnovsky, 1965), with a solution change at 90 minutes, rinsed in Millonig's phosphate buffer, removed from the clamps, diced into approximately 1 mm^3 pieces, and postfixed for 90 minutes at 2°C in 1% veronal-buffered OsO_4 with a solution change at 45 minutes. The tissue was then dehydrated in a graded series of acetones and infiltrated and embedded in a resin mixture which contained Epon, Araldite, DER, DDSA and DMP-30 (Stromer et al., 1967). After curing, the tissue blocks were thin sectioned on a LKB Ultrotome III ultramicrotome (LKB Instruments, Inc., Rockville, MD), picked up on bare 300 mesh copper grids, stained in methanolic 2% uranyl acetate and lead citrate (Reynolds, 1963) and viewed in an RCA EMU-4 electron microscope operated at 100 kV.

Glycerinated bovine longissimus dorsi myofibrils were labelled for immunoelectron microscopy by using a modification of the method reported by Richardson et al. (1981) for localization of horseradish peroxidase-labelled antibodies. The procedure involved fixing PBS-washed myofibrils (10 mg/ml) dropwise for twenty minutes in a six-fold final volume of picric acid-formaldehyde (PAF) fixative (Karlsson and Schultz, 1965). After fixation, the myofibrils were washed four times with ten volumes of PBS and incubated with a 1:20 dilution of immune rabbit serum at 37°C for two hours. The PBS contained 3% normal goat serum to reduce non-specific binding of the primary desmin antibodies to tissue components. After incubation, the myofibrils were washed four times in thirty-minute cycles in PBS and incubated with a 1:25 dilution of ferritin-conjugated, goat-anti-rabbit antibodies (Miles Laboratories, Inc., Elkhart, IN)

overnight at 2°C. The next day, after equilibration to room temperature, the myofibrils were washed three times in thirty-minute cycles with PBS and fixed for one hour in two changes of 4% glutaraldehyde in PBS. To remove excess fixative, the myofibrils were rinsed four times in PBS, then postfixed in 1% OsO_4 in PBS for thirty minutes. The myofibrils were then dehydrated in a graded series of ethanol and pure acetone and infiltrated, embedded, cured, sectioned and viewed as previously described.

Filament Formation

Purified desmin obtained after successive hydroxyapatite and Sepharose CL-6B column chromatography of a urea-solubilized, desmin-enriched fraction (isolated from one-hour postmortem control tissue) was diluted to 1 mg/ml and dialyzed against 10mM Tris-acetate, pH 8.5, 1mM DTE overnight to remove all denaturing urea. This soluble protein was then dialyzed against 150mM NaCl, 1mM $MgCl_2$, 10mM imidazole, pH 7.0 for eight hours to form synthetic 100 Å filaments. This method is similar to that used by Huiatt et al. (1980) for avian smooth muscle desmin and by O'Shea et al. (1981) for porcine skeletal muscle desmin filament formation.

After dilution with the dialysate to a concentration of 0.1 - 0.2 mg/ml, a drop of the protein suspension was placed on a glow-discharged, carbon-coated grid. The protein was allowed to settle for two minutes. The grid was then washed with 10 drops of water and was negatively stained for two minutes with 2% aqueous uranyl acetate. All solutions were kept at 0 - 2°C for negative staining. After removal of

excess stain by touching the stain drop with the edge of a piece of torn filter paper, the negatively stained 100 Å filaments were viewed in an RCA EMU-4 operated at 100 kV.

Amino Acid Analysis

Amino acid analyses were done on three samples of electorphoretically purified desmin with a Durrum D-400 Amino Acid Analyzer (Durrum, Palo Alto, CA). Duplicate samples were hydrolyzed *in vacuo* in 6N HCl for 22 hours at 110°C. Five µl of 10% phenol was added to the 1 ml samples to protect tyrosine. Cystine and cysteine were measured as cysteic acid after performic acid oxidation of duplicate samples by the method of Hirs (1967).

Determination of Protein Concentration

Protein concentrations were determined by the biuret method (Gornall et al., 1949) as modified by Robson et al. (1968), or by the Folin-Lowry method (Lowry et al., 1951) as modified by Goll et al. (1964). Trichloroacetic acid precipitation of proteins (Lowry et al., 1951) preceded protein concentration determinations by the Folin-Lowry procedure if interfering solvents were present.

Statistical Analysis

All statistical analyses of quantitative data from the isolation of desmin-enriched fractions were done with the Statistical Analysis System (SAS, 1982) of the Iowa State University Computation Center.

RESULTS

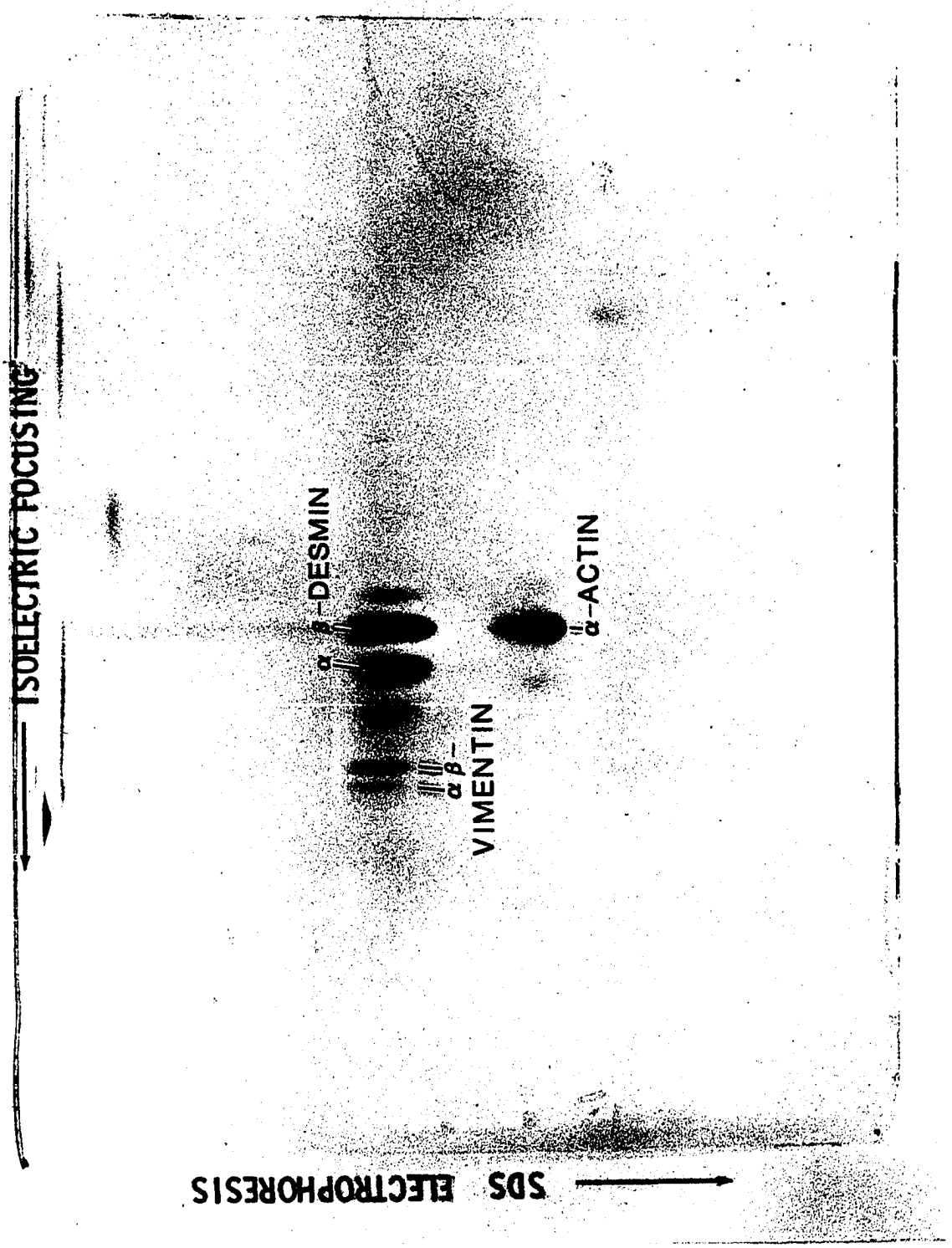
The objective of these experiments was to determine the changes that occurred in the bovine skeletal muscle cell cytoskeleton, with special emphasis on desmin, during postmortem aging of muscle that had either been stored at 2°C or 25°C or had been electrically stimulated before storage at 2°C. The results will be presented in four sections: 1) Isolation of desmin-enriched fractions and quantitation of desmin in these fractions; 2) Postmortem changes in desmin and other proteins in the desmin-enriched fractions; 3) Immunochemical localization of desmin-containing filaments in myofibrils; 4) Electron microscopy of electrically stimulated skeletal muscle.

Isolation of Desmin-Enriched Fractions and Quantitation of Desmin in These Fractions

Because desmin is the principal component of the 100 Å filaments that connect skeletal muscle myofibrils at the Z line and thereby is a major component of the muscle fiber cytoskeleton, it was first necessary to establish that the isolated protein fraction was enriched in desmin. The first method used to determine that the protein fraction contained no other components that would migrate with a molecular weight corresponding to 55,000 daltons on tube gels was to electrophorese the protein in two dimensions (Figure 5). The migration of desmin was compared with the migration of an aliquot of smooth skeletal muscle α-actin which was added to the sample as a marker. The result shown in Figure 5 was then compared with the pattern reported by O'Shea et al. (1981) for the two-dimension electrophoresis of chromatographically purified porcine skeletal

Figure 5. Two-dimensional gel electrophoresis of a desmin-enriched fraction prepared from bovine longissimus dorsi muscle sampled after one-hour storage at 2°C. {The acidic end of the pH gradient in the isoelectric focusing dimension is to the left. Spots corresponding to the isoelectric variants of desmin and vimentin and α-actin are labelled. Vimentin is present in amounts less than 1 - 2% of the total protein and appears due to heavy protein loading of the gel.

Two-dimensional gel electrophoresis was done by Mary Bremmer, Muscle Biology Group, Iowa State University.}


ISOELECTRIC FOCUSING
α β-DESMIN
αβ-VIMENTIN
α-ACTIN
SDS ELECTROPHORESIS

muscle desmin. Figure 5 contains a pattern that is very similar to that reported by O'Shea et al. (1981) and shows that the α and β desmin variants are the major protein components. Small amounts of α and β vimentin, comprising an insignificant amount (<1 - 2%) of the total protein were also detected on this heavily loaded gel.

The only known functional test for desmin is that the purified protein will form 100 Å diameter filaments at pH and ionic strength conditions that are near physiological conditions (Huiatt et al., 1980). This in vitro function was a second means by which the 55,000 dalton protein in the desmin-enriched fractions was shown to be desmin. Highly purified 55,000 dalton protein obtained after successive chromatography on hydroxyapatite and DEAE-Sepharose CL-6B columns was dialyzed against 150mM NaCl, 1mM $MgCl_2$, 10mM imidazole-HCl, pH 7.0 at 2°C. The filaments (Figure 6) which formed were long, had an average diameter of 100 Å and were very similar in appearance to filaments formed in vitro from porcine skeletal desmin (O'Shea et al., 1981) and from avian smooth muscle (Huiatt et al., 1980; Stromer et al., 1981) under similar conditions. Most filaments also show a characteristic helical substructure.

The third test was to compare the amino acid analysis of highly purified bovine skeletal muscle desmin (Table 1) with that reported for desmin from porcine skeletal muscle and porcine stomach (O'Shea et al., 1979) and from turkey gizzard (Huiatt, 1979). The values for bovine skeletal muscle desmin are plus or minus the standard error and were obtained from three separate preparations of highly purified desmin. Each preparation was analyzed in triplicate. The similarity in amino acid con-

Figure 6. Synthetic filaments formed by dialyzing highly purified bovine skeletal desmin against 150mM NaCl, 1mM $MgCl_2$, 10mM imidazole-HCl, pH 7.0. {The average filament diameter measured in this and other electron micrographs was 100 Å. 77,780X.}

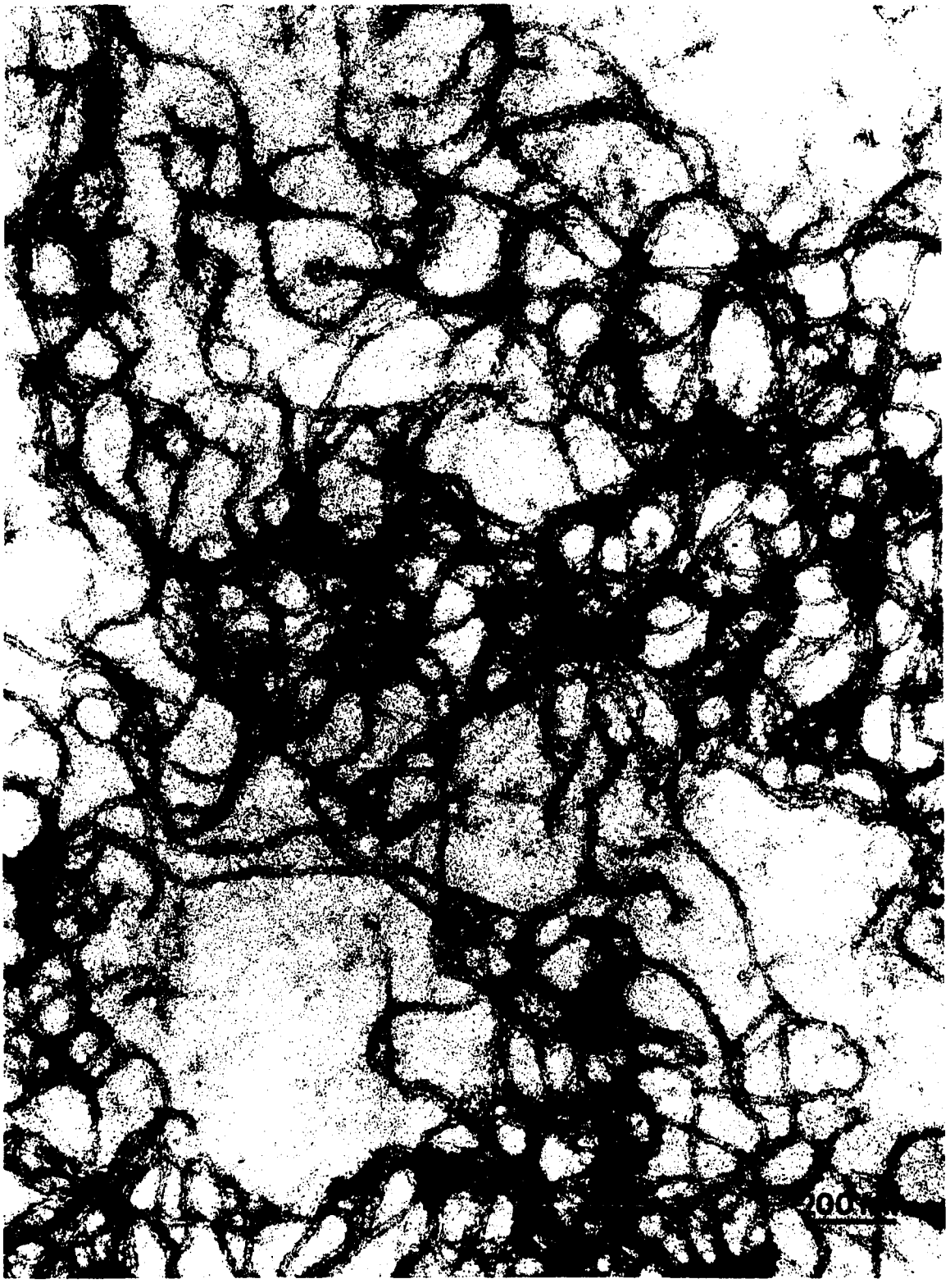


Table 1. Amino acid analysis of desmin[a]

Amino acid	Bovine skeletal desmin[b]	Porcine skeletal desmin[c]	Porcine stomach desmin[c]	Chicken gizzard desmin[c]
Asp	10.3±0.3	9.7±0.2	9.7±0.1	8.6±0.2
Thr	4.7±0.1	5.3±0.1	5.1±0.1	5.6±0.1
Ser	5.6±0.4	7.1±0.3	7.0±0.0	6.0±0.1
Glu	17.8±0.3	17.6±0.3	18.4±0.1	19.9±0.4
Pro	2.3±0.1	3.6±0.2	2.5±0.1	1.8±0.1
Gly	5.1±0.5	6.4±0.5	5.5±0.2	4.0±0.1
Ala	9.9±0.3	9.2±0.1	9.6±0.0	9.3±0.2
Cys	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0
Val	5.9±0.3	6.0±0.1	6.0±0.1	5.2±0.3
Met	2.3±0.1	1.8±0.1	1.8±0.1	2.6±0.1
Ile	5.0±0.2	4.1±0.1	4.1±0.1	4.4±0.1
Leu	12.1±0.2	9.4±0.2	10.1±0.1	10.8±0.4
Tyr	2.5±0.1	2.8±0.1	2.8±0.1	2.9±0.2
Phe	2.2±0.2	3.0±0.1	2.9±0.1	2.8±0.1
Lys	4.8±0.1	5.1±0.2	4.5±0.1	4.9±0.2
His	1.4±0.1	1.4±0.0	1.5±0.1	1.6±0.1
Arg	7.9±0.2	7.7±0.5	8.7±0.2	9.3±0.2

[a]All values are expressed as mole percent plus or minus standard error.

[b]Values are the averages plus or minus the standard error of triplicate analyses of three samples of electrophoretically purified desmin.

[c]From O'Shea et al. (1979).

tent of the purified 55,000 dalton protein prepared from bovine skeletal muscle and those of desmin from other muscles also demonstrated that the bovine skeletal muscle protein was desmin.

After identifying the desmin band in gels from the desmin-enriched fractions and determining that no other proteins were co-migrating with desmin, it was possible to quantitate the amounts of desmin present in the different fractions. Amounts of desmin present in desmin-enriched fractions isolated from 600 gram bovine skeletal muscle samples that had been stored at either 2° or 25°C for 1, 24 or 168 hours post mortem are summarized in Table 2. The desmin content of the one hour sample is the average of five separate determinations made on the left and right sides of the first two animals and one side of the third animal. These values are not separated by temperature because the intramuscular temperature at one hour was identical (29° ± 1°C) and, thus, would not have influenced the sample. Making five separate determinations on the one hour samples also provided confidence both in the repeatability of the extraction method and in the values for the starting amount of extractable desmin.

Significantly, more desmin was isolated from muscle stored at 2°C than at 25°C at both 24 and 168 hours post mortem (Table 2). The amounts of desmin isolated at 24 and 168 hours post mortem, however, show dissimilar patterns within a temperature. At 2°C, the amount of extractable desmin nearly doubles at 24 hours post mortem when compared with the one hour value and, at 168 hours, is 81% of the one hour value. In contrast to this, at 25°C, the amount of extractable desmin is nearly identical at

Table 2. Postmortem changes in quantities of desmin isolated from bovine longissimus dorsi muscle excised and stored at different temperatures[1]

	Temperature		
Postmortem time (hr)	2°C	25°C	29 ± 1°C
1			54.41 ± 1.86
24	103.73 ± 3.35[a]	53.74 ± 3.80[b]	
168	44.29 ± 4.25[c]	6.93 ± 2.19[d]	

[1]Values are the average yield in mg of desmin (plus or minus standard error) in desmin-enriched fractions isolated from 600 gm samples from each of three animals. The desmin content of the 1 hour sample is the control value and is the average of the desmin in one 600 gm sample from each of five samples. Values within a sampling time or within a temperature with different superscripts are significantly different at the 0.05 level as determined by the students' T-test.

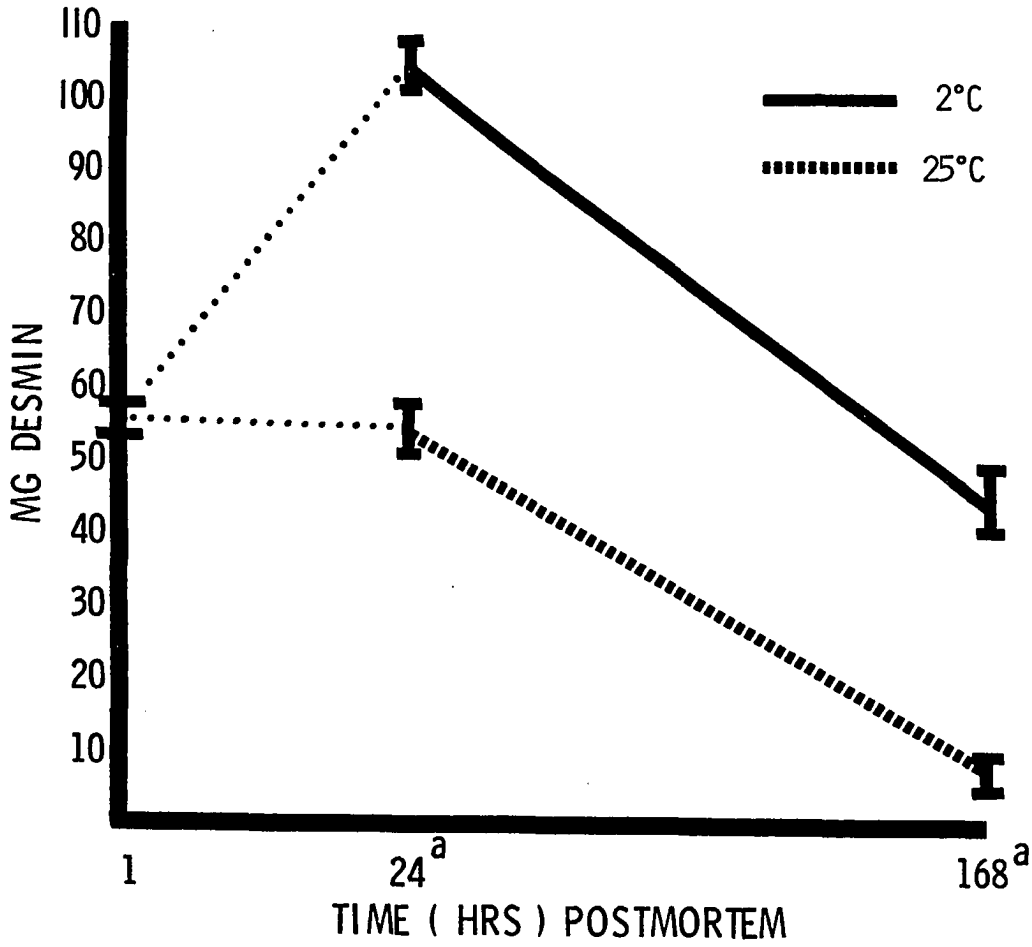
one and 24 hours but has decreased at 168 hours to only 13% of that in the one hour sample. These results are presented graphically in Figure 7. Detailed values of the total amount of protein and the desmin content of each sample are shown in Table 3.

Quantities of desmin extracted from non-stimulated and electrically stimulated bovine longissimus dorsi muscle are summarized in Table 4. Significantly more desmin was extracted from the electrically stimulated sample than from the non-stimulated sample after one hour storage at 2°C. At 24 hours, however, more desmin was extracted from the non-stimulated sample. At 168 hours, the extract from the electrically stimulated muscle again contained more desmin. The differences in desmin content between extracts from non-stimulated and stimulated samples after 24 and 168 hours storage were not significant. These results are shown graphically in Figure 8. The total amount of protein and the desmin content of each sample from all five animals are shown in Table 5.

Postmortem Changes in Desmin and Other Proteins in the Desmin-Enriched Fractions

Protein fractions from certain stages in the preparation of desmin-enriched fractions from both the temperature and electrical stimulation experiments were routinely electrophoresed on 7½% polyacrylamide tube gels to assess changes in protein content. An abbreviated flow chart of the procedure used and five typical gels of various fractions from this procedure are shown in Figure 9. The letters above the gels correspond to letters of the five different fractions listed in the flow chart. The unlabelled gel on the left is the standard and contains myosin, α-actinin,

Figure 7. Postmortem changes in quantities of desmin isolated from bovine longissimus dorsi muscle stored at 2°C or 25°C. {The one hour postmortem value is an average of the quantities of desmin isolated from 600 gm samples taken from five sides from three animals. Each value for 24 and 168 hours is an average (plus or minus standard error) of the quantities of desmin isolated from 600 gm samples from three animals. Amounts of desmin were determined from densitometric scans of 7½% polyacrylamide tube gels of desmin-enriched fractions.}


110
100
90
80
70
60
50
40
30
20
10
MG DESMIN
2°C
25°C
1
24a
168a
TIME (HRS) POSTMORTEM

$^{a}P < 0.005$

Table 3. Total protein, percent desmin[A], and amount of desmin in desmin-enriched fractions isolated from bovine longissimus dorsi muscle stored at 2° or 25°C for 1, 24 or 168 hours post mortem.

Animal	1 hr[B]			24 Hr		
	29 ± 1°C			2°C		
	a	b	c	a	b	c
1	648.6	7.5%	48.6	937.4	11.6%	108.8
	377.0	13.8%	46.5			
2	606.8	9.6%	58.3	585.2	16.3%	95.6
	655.2	8.9%	58.3			
3	390.5	14.9%	58.1	573.3	18.6%	106.8
$\bar{x}$ =	535.6	10.9%	54.4[C]	698.6	15.5%	103.7[C]

[A]Each value is the average of nine densitometric gel scans (triplicate gels each scanned three times).

[B]The two values for animals 1 and 2 were desmin-enriched fractions isolated from both sides of each animal.

[C]Averaged values of column c figures (plus or minus standard error) were presented in Table 2 and graphed in Figure 7.

[a]Total mg protein in desmin-enriched fraction.

[b]Percent desmin in desmin-enriched fraction.

[c]Total mg of desmin in desmin-enriched fraction

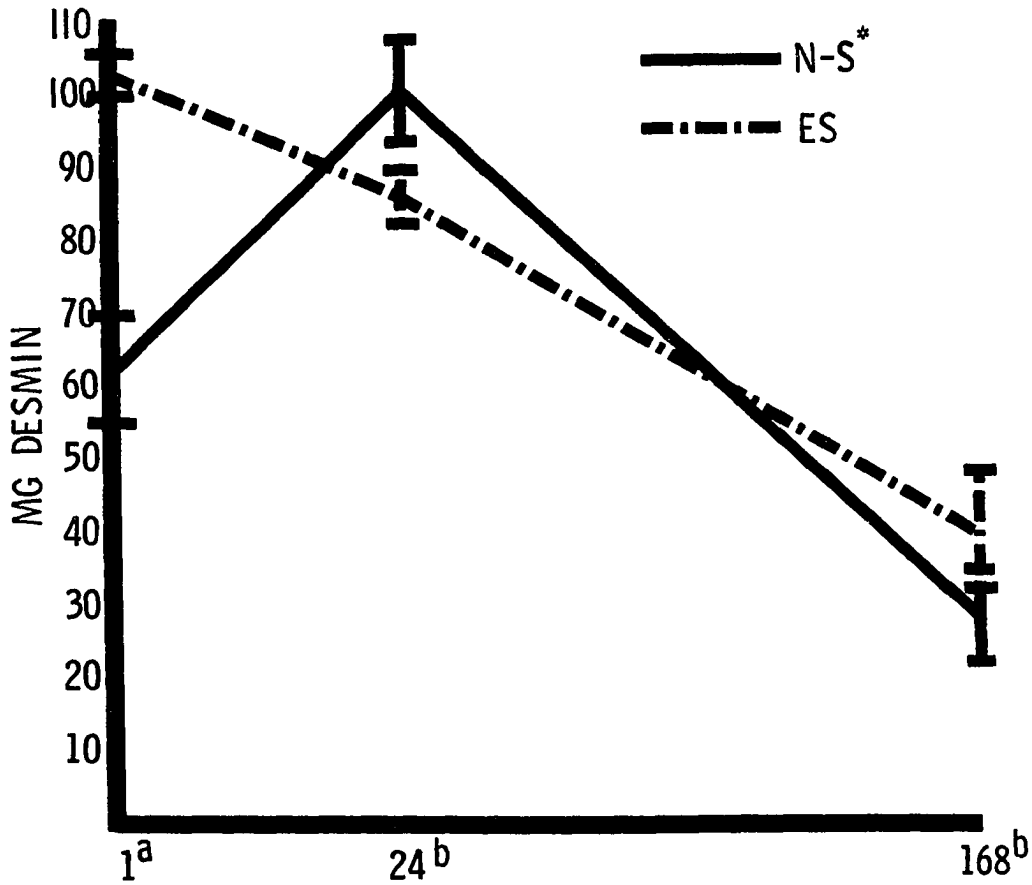
24 Hr			168 Hr			168 Hr		
25°C			2°C			25°C		
_a	_b	_c	_a	_b	_c	_a	_b	_c
883.2 x	6.0% =	53.1	938.7 x	4.0% =	37.1	1968.8 x	0.57% =	11.4
806.2 x	5.7% =	46.0	1392.4 x	2.9% =	41.4	3270.1 x	0.06% =	2.1
964.3 x	6.4% =	62.1	1118.0 x	4.8% =	54.4	4314.3 x	0.16% =	7.3
884.6	6.0%	53.7[C]	1149.7	3.9%	44.3[C]	3184.4	0.26%	6.9[C]

Table 4. Postmortem changes in quantities of desmin isolated from non-stimulated and electrically stimulated bovine longissimus dorsi muscle excised and stored at 2°C[1]

Postmortem time (hr)	Non-stimulated	Electrically stimulated
1	61.67 ± 6.91[a]	102.36 ± 5.17[b]
24	100.74 ± 6.67[b]	86.16 ± 3.93[b]
168	27.75 ± 5.88[c]	39.66 ± 7.82[c]

[1]Values are the average yield in mg of desmin (plus or minus standard error) in desmin-enriched fractions isolated from 600 gm samples from five animals. Values within a sampling time or within a treatment with different superscripts are significantly different at the 0.05 level as determined by the students' T-test.

Figure 8. Postmortem changes in quantities of desmin isolated from non-stimulated or electrically stimulated bovine longissimus dorsi muscle stored at 2°C. {Each value is an average (plus or minus standard error) of the quantities of desmin isolated from 600 gm samples from five animals. Quantities of desmin were determined from densitometric scans of 7½% polyacrylamide tube gels of desmin-enriched fractions.}



*NON-STIMULATED

a P < 0.001 b NSD

Table 5. Total protein, percent desmin[A], and amount of desmin in desmin-enriched fractions isolated from non-stimulated and electrically stimulated bovine longissimus dorsi muscle stored at 2°C for 1, 24 or 168 hours.

Animal	1 Hr			1 Hr			24 Hr		
	Non-stimulated			Electrically stimulated			Non-stimulated		
	a	b	c	a	b	c	a	b	c
4	520.5	14.1%	73.4	590.7	18.1%	106.7	846.2	10.6%	90.2
5	436.0	10.7%	46.7	580.6	14.3%	82.8	709.8	11.1%	79.7
6	719.4	11.3%	81.4	1115.2	10.0%	112.1	872.5	11.5%	100.4
7	583.7	11.2%	65.8	947.5	12.0%	114.0	782.6	15.4%	121.8
8	495.3	88.3%	41.0	801.0	12.0%	96.2	779.1	14.3%	111.5
$\bar{x}$ =	551.0	11.1%	61.7[B]	807.0	13.3%	102.4[B]	798.0	12.6%	100.7[B]

[A]Each value is the average of nine densitometric gel scans (triplicate gels each scanned three times).

[B]Averaged values of column c figures (plus or minus standard error) were presented in Table 4 and graphed in Figure 8.

[a]Total mg protein in desmin-enriched fraction.

[b]Percent desmin in desmin-enriched fraction.

[c]Total mg of desmin in desmin-enriched fraction.

24 Hr			168 Hr			168 Hr		
Electrically stimulated			Non-stimulated			Electrically stimulated		
a	b	c	a	b	c	a	b	c
966.4	7.4%	71.9	1435.6	2.9%	41.6	1198.3	4.5%	53.8
777.6	11.1%	85.6	1112.4	3.5%	39.0	867.2	4.3%	37.8
1588.5	6.1%	97.2	792.0	4.3%	34.2	1383.2	4.6%	64.7
929.0	10.4%	93.3	570.8	2.4%	13.9	781.2	2.6%	20.7
903.1	9.1%	82.8	840.0	1.2%	10.1	681.4	3.1%	21.3
1032.9	8.8%	86.2 [B]	950.2	2.9%	27.8 [B]	982.2	3.8%	39.7 [B]

Figure 9. Abbreviated flow chart of the procedure used to prepare desmin-enriched fractions and 7½% polyacrylamide tube gels of proteins contained in some of the fractions. {Capital letters used to identify the gels correspond to the same letters in the flow chart. The unlettered gel on the left is a standard which contains myosin, α-actinin, bovine serum albumin, desmin, actin and tropomyosin.}

600 grams longissimus dorsi muscle

Grind in standard salt solution (SSS) at 2°C

- Supernatant
- Pellet

Resuspend and extract 3X at 2°C

- Supernatants (discard)
- Washed myofibrils (A)

Resuspend and extract in HAMES (High ionic strength Actin and Myosin Extracting Solution) 2X at 2°C (4.5 hr total)

- HAMES Supernatant (B)
- HAMES Pellet (C)

Resuspend and extract in 1M KI, 2X at 2°C (4 hr total)

- KI Supernatant (D)
- KI Pellet (E)

Water wash 2X

Resuspend and extract in 1M HAc overnight at room temperature

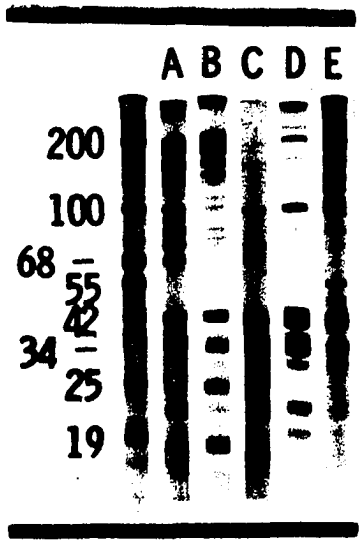
- Supernatant
- HAc Pellet (discard)

Filter and adjust pH to 5.5
Salt out at 14.4% $(NH_4)_2SO_4$ at 2°C

- Supernatant
- Pellet

Solubilize by boiling in 1% SDS 20mM PO_4, pH 7.8, 3mM MCE

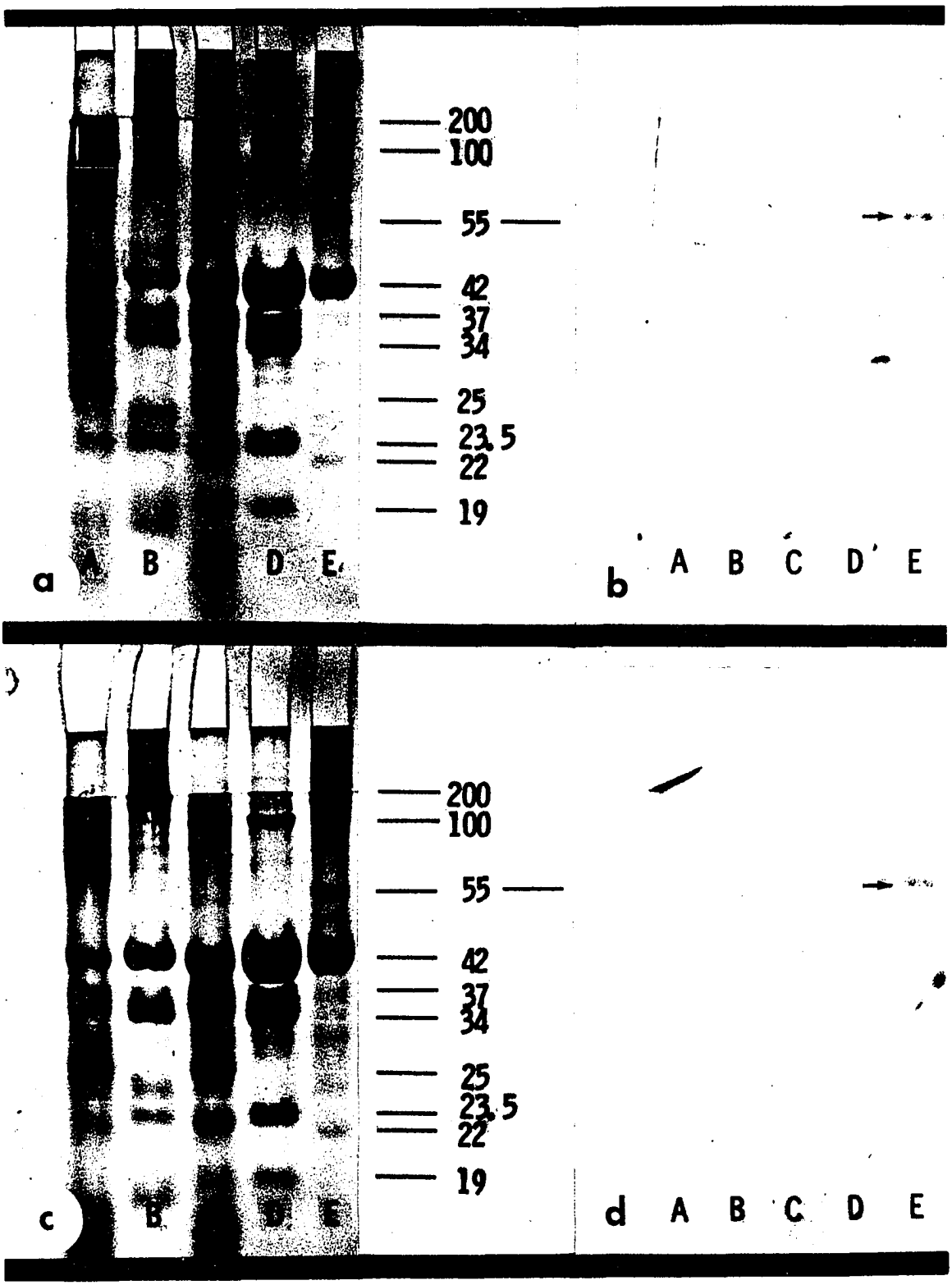
DESMIN ENRICHED FRACTION



bovine serum albumin, desmin, actin and tropomyosin. It is obvious, at the identical 25 µg protein loads used for these 7½% gels, that the 55,000 dalton desmin band first becomes visible in gel E from the KI pellet. Comparisons of proteins isolated at these same stages from either electrically stimulated samples stored at 2°C or non-stimulated samples stored at 2° or 25°C for 1, 24 or 168 hours showed differences only in amounts of desmin in the KI pellet (results not shown).

The possibility existed that changes were occurring in the protein composition, especially in desmin or its degradation products, which were not detectable in conventionally stained tube gels. If these same protein fractions obtained during the preparation of the desmin-enriched fraction (see Figure 9) were first separated on 10% polyacrylamide slab gels and then transferred to a nitrocellulose membrane ("Western Blot") for labelling with anti-desmin ^{125}I protein A, it might be possible to more completely separate the lower molecular weight bands and to detect as little as picogram quantities of desmin degradation products. The assumption would be that either these products were present in such small quantities or they co-migrated with other lower molecular weight proteins and thus escaped detection on conventional 7½% tube gels. A comparison of the electrophoretic patterns of polypeptides present at these same five stages in the preparation of desmin-enriched fractions (Figure 10, a and c) with the corresponding immunoautoradiograms (Figure 10, b and d) showed that only the 55,000 dalton desmin band was labelled. None of the fractions from either the sample stored at 2°C for 24 hours (Figure 10, a and b) or the sample stored at 25°C for 24 hours (Figure 10, c and d) showed any labelling

Figure 10. One-dimensional slab gels (a and c) of proteins extracted at five different stages in the preparation of a desmin-enriched fraction from bovine skeletal muscle and the corresponding immunoautoradiograms (b and d). {The gels in a and c were stained with Coomassie brilliant blue R. The gels were composed of a 7½% polyacrylamide stacking gel and a 10% polyacrylamide separating gel. Gel a and radiogram b are from muscle stored at 2°C for 24 hours. Gel c and radiogram d are from muscle stored at 25°C for 24 hours. Lane A = washed myofibrils; lane B = supernatant from HAMES; lane C = pellet after HAMES; lane D = supernatant after extraction with KI; lane E = pellet after extraction with KI.}


200
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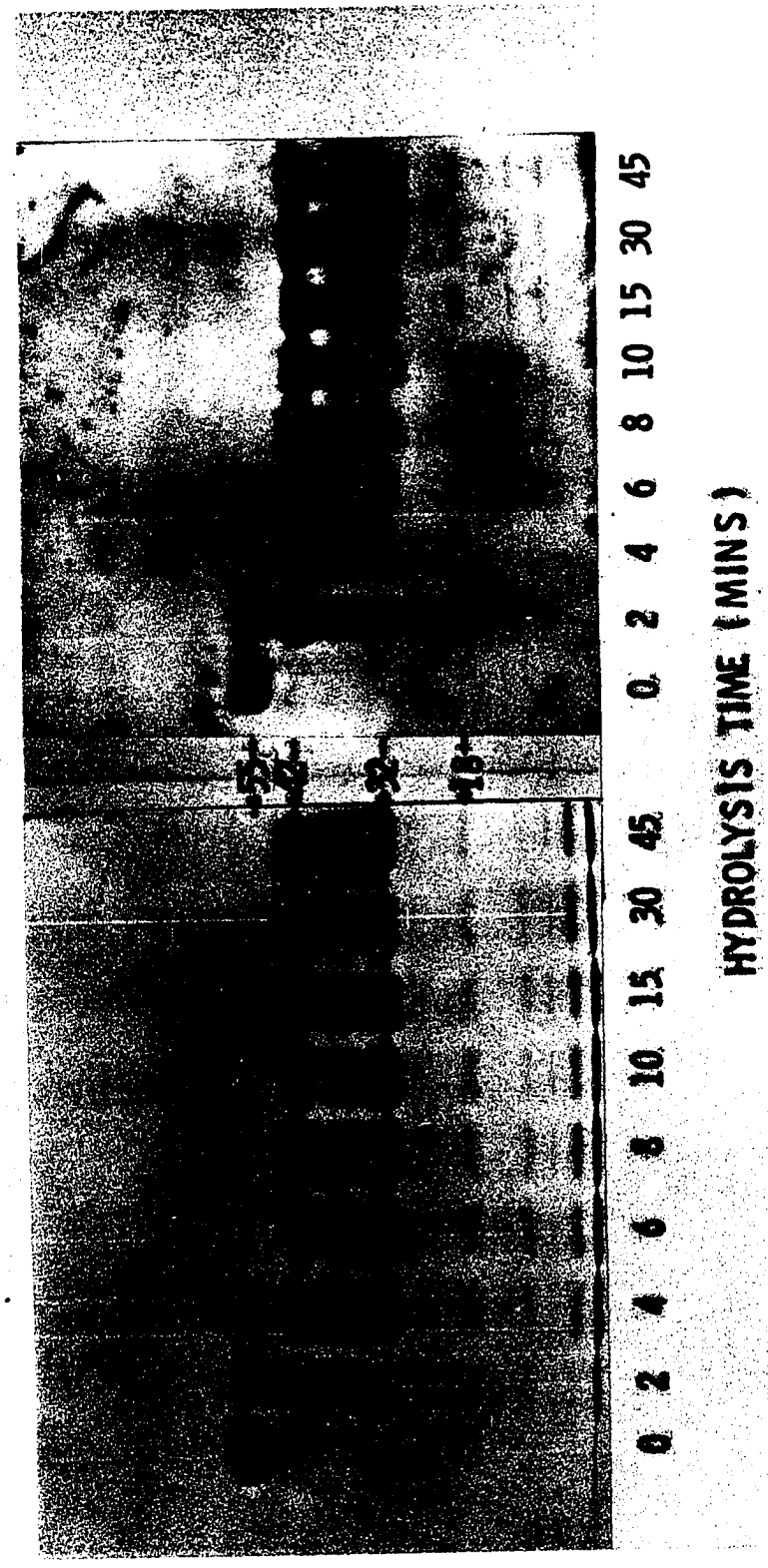
of bands other than the 55,000 dalton band. The desmin band was labelled only at the stage where it became visible in the conventionally stained gel (lane E), i.e. in the pellet remaining after extraction with 1M potassium iodide.

The feasibility of this method to improve the sensitivity of detecting degradation products, however, is dependent on showing that those products would be recognized by anti-desmin. The following approach was used to produce degradation products of desmin. Purified bovine skeletal muscle desmin was digested (1:400 by weight, enzyme to substrate) with the endogenous muscle protease, calcium-activated factor (CAF), which is known to utilize desmin as a substrate (O'Shea et al., 1979). Electrophoretic separation and immunoautoradiography of aliquots removed after 2, 4, 6, 8, 10, 15, 30 and 45 minutes during the 45 minute digestion showed that desmin and all lower molecular weight degradation products visible on the slab gel are also labelled in the autoradiogram (Figure 11). The majority of the degradation products ranged from just less than 55,000 daltons to 32,000 daltons, but minute quantities of an 18,000 dalton and smaller molecular weight components were also present. The pattern of degradation products was similar to that obtained when porcine skeletal muscle desmin was digested with CAF (1:50 by weight, enzyme to substrate) and was monitored by one-dimensional peptide mapping (O'Shea et al., 1979). The observation that anti-desmin would recognize proteolytic fragments of desmin permitted a more detailed examination of the desmin-enriched fractions prepared from both the temperature and the electrical stimulation experiments.

Figure 11. Digestion of purified bovine desmin with the endogenous muscle protease, calcium-activated factor (CAF). {Desmin was extensively dialyzed against 10mM Tris-acetate, pH 8.5, 1mM DTE, 0.1mM EDTA before digestion at 25°C at an enzyme to substrate ratio of 1:400 by weight. Hydrolysis was initiated by adding $CaCl_2$ to a final concentration of 5mM and was stopped by adding 10mM EDTA. Electrophoresis was done in duplicate 10% polyacrylamide slab gels by using the discontinuous Tris-glycine buffer system of Laemmli (1970). The gel on the left was stained with Coomassie brilliant blue R. The corresponding autoradiogram on the right was labelled with anti-desmin and ^{125}I protein A. Electrophoresis was done for a shorter time interval than subsequent immunoautoradiograms to assure inclusion of all low molecular weight polypeptides.

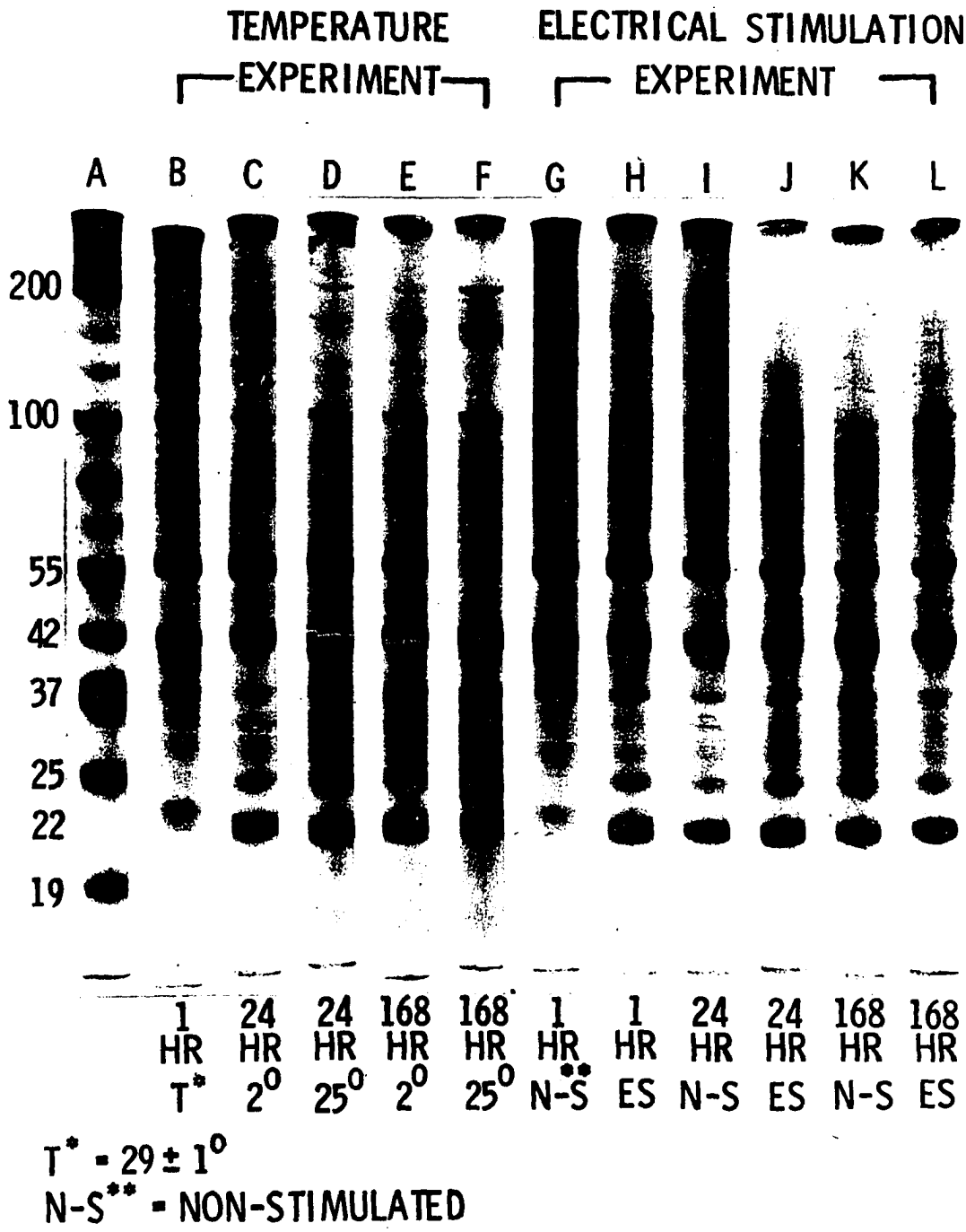
Preparation of purified porcine cardiac CAF and the digestion of purified bovine skeletal desmin was done by Michael G. Zeece, Muscle Biology Group, Iowa State University.}


0 2 4 6 8 10 15 30 45
HYDROLYSIS TIME (MINS)
0 2 4 6 8 10 15 30 45

Differences in protein composition were readily apparent when 7½% polyacrylamide gels of desmin-enriched fractions from the temperature experiment were examined and compared with gels from the electrical stimulation experiment (Figure 12). In the temperature experiments (gels B - F), two differences are clear: 1) the amount of desmin extracted decreases more rapidly in tissue stored at 25°C than at 2°C (see also Table 2); 2) sometime after one hour post mortem, a band with a molecular weight of approximately 22,000 daltons appears. The density of this band is greatly diminished in the sample stored for 168 hours at 25°C (gel F). In the electrical stimulation experiments (gels G - L), the 22,000 dalton band appears first in the stimulated sample taken one hour post mortem (gel H) and persists in both the non-stimulated and stimulated 24 hour and 168 hour samples (gels I - L). The desmin band is diminished but still present in both the non-stimulated (gel K) and the stimulated (gel L) 168 hour samples (see also Table 4).

Identification of desmin and its degradation products in the desmin-enriched fractions was done by separating the polypeptides on 10% slab gels and then, after transferring the proteins to nitrocellulose membranes, using immunoautoradiography to mark the location of desmin and related peptides. Figure 13a shows, in the Coomassie brilliant blue R-stained gel, that the 55,000 dalton band diminishes with increasing storage time and temperature and is nearly absent in the fraction from the sample stored at 25°C for 168 hours (lane E). In addition, a 22,000 dalton band is readily seen in the fractions from the samples stored at 2°C for 24 hours (lane B), at 25°C for 24 hours (lane C) and at 2°C for

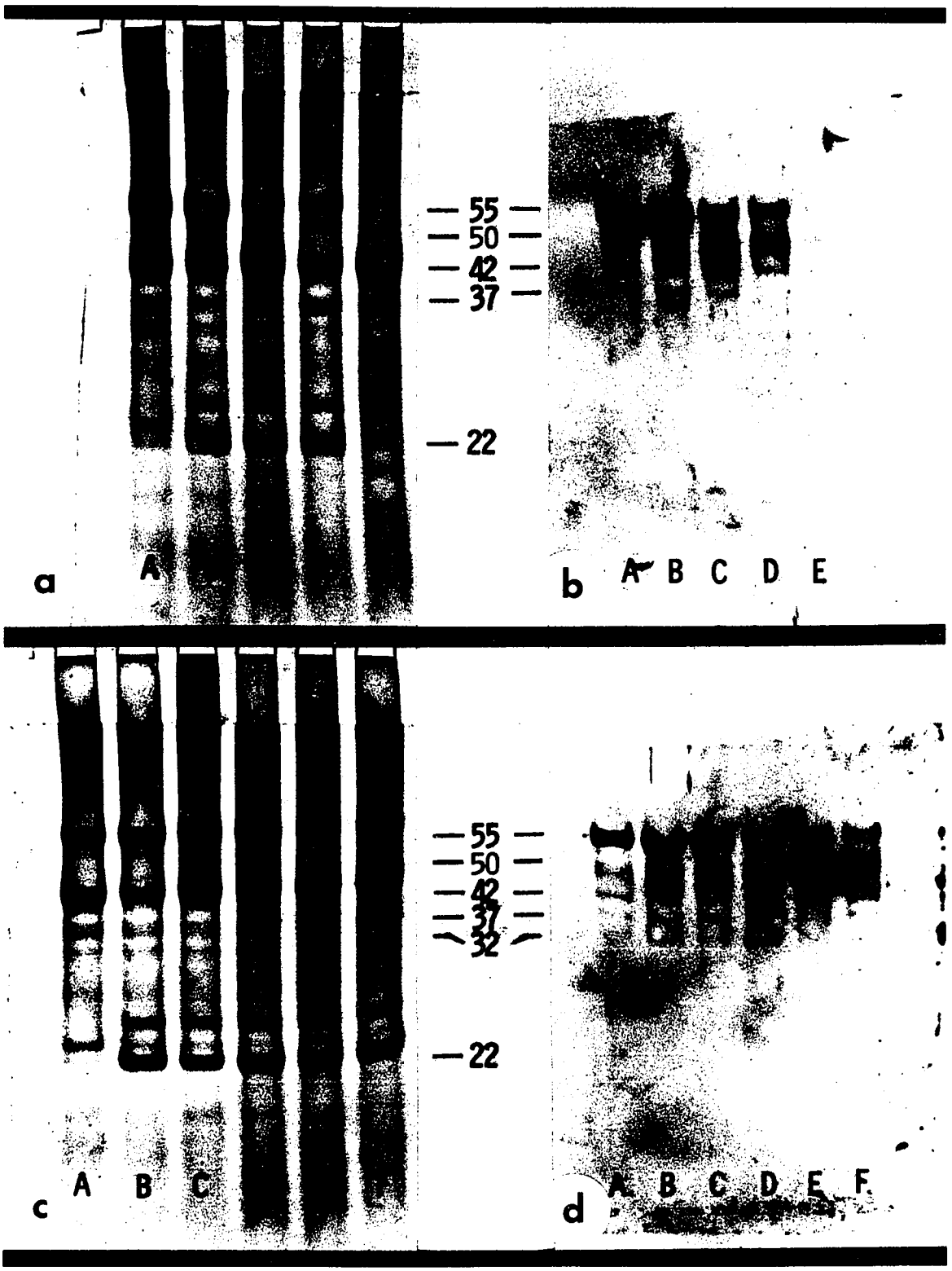
Figure 12. Desmin-enriched fractions prepared at 1, 24 and 168 hours post mortem from tissue from the temperature (lanes B - F) and electrical stimulation (lanes G - I) experiments. {Gel standard (lane A) contained myosin, α-actinin, desmin, actin and tropomyosin. Gels are 7½% polyacrylamide, and each was loaded with 25 µg of protein.}



$T^{*} = 29 \pm 1^{\circ}$

$N\text{-}S^{**}$ = NON-STIMULATED

Figure 13. One-dimensional slab gels of desmin-enriched fractions prepared at all sampling times from the temperature experiments (a) and from the electrical stimulation experiments (c) and the corresponding immunoautoradiograms (b and d). {The gels in a and c were composed of a 7½% polyacrylamide stacking gel and a 10% polyacrylamide separating gel and were stained with Coomassie brilliant blue R. In a and b, lane A = one hour, 29 ± 1°C; lane B = 24 hours, 2°C; lane C = 24 hours, 25°C; lane D = 168 hours, 2°C; lane E = 168 hours, 25°C. In c and d, lane A = one hour, non-stimulated; lane B = one hour, stimulated; lane C = 24 hour, non-stimulated; lane D = 24 hour, stimulated; lane E = 168 hour, non-stimulated; lane F = 168 hour, stimulated. The "Western Blots" were labelled with anti-desmin and ^{125}I protein A (b and d).}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c
A B C
d
A B C D E F

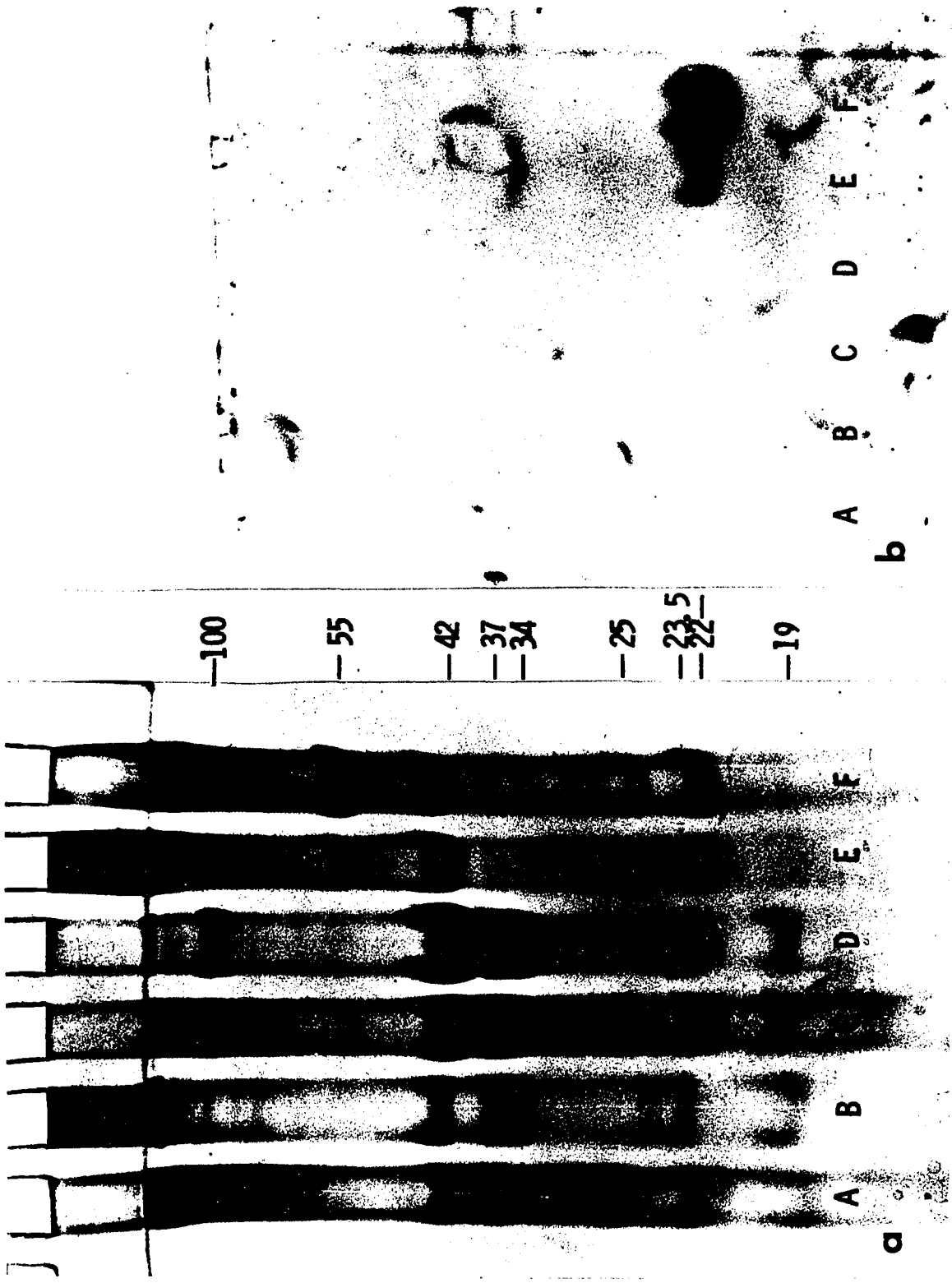
168 hours (lane D). The immunoautoradiogram of this gel is shown in Figure 13b. The desmin band is heavily labelled, and the major degradation products at 50,000, 42,000 and at 37,000 daltons are also clearly labelled in lanes A, B, C and D. All three of these bands are essentially absent from the fraction prepared from muscle stored at 25°C for 168 hours (lane E). A very lightly labelled 32,000 dalton band is present in lanes A, B and C but is absent from fractions stored at either 2°C or 25°C for 168 hours (lanes D and E). Immunolabelling of the 22,000 dalton band does not occur at any temperature or time.

The desmin band decreased with increasing storage time in the electrical stimulation experiments (Figure 13c). The intensity of labelling of this band also showed a similar decrease (Figure 13d). The 50,000, 42,000, 37,000 and the 32,000 dalton bands are labelled comparably to those in Figure 13b. The 22,000 dalton band again remains unlabelled.

The labelling pattern of the bands between desmin and the 32,000 dalton band is not similar to the observed labelling of products of the CAF digestion of purified bovine skeletal muscle desmin (Figure 11). It seems evident, however, that the 22,000 dalton band in the desmin-enriched fractions (Figure 13) is distinct and immunologically unrelated.

An antibody to the 22,000 dalton polypeptide isolated from one-hour post mortem electrically stimulated tissue exhibited strong recognition of its antigen and no cross-reactivity with any myofibrillar proteins (Figure 14). When used for immunofluorescent localization, this antibody did not label glycerinated bovine skeletal myofibrils, cryostat

Figure 14. One dimensional slab gel (a) of proteins extracted at six stages in the preparation of a desmin-enriched fraction from bovine skeletal muscle stored at $2^{o}C$ for 24 hours. {The desmin-enriched fraction is also included (lane F). The corresponding immuno-autoradiogram (b) has been labelled with anti-22,000 dalton polypeptide and ^{125}I protein A. The slab gel was composed of a 7½% polyacrylamide stacking gel and a 10% polyacrylamide separating gel and was stained with Coomassie brilliant blue R. Lane A = washed myofibrils; lane B = supernatant from HAMES; lane C = pellet after HAMES: lane D = supernatant after extraction with KI; lane E = pellet after extraction with KI; lane F = desmin-enriched fraction. Note that both lane E and F in a show a stained band migrating at 22,000 daltons and that these two bands are labelled in b.}


a
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F

sections of bovine longissimus dorsi muscle or 9 day old chicken skeletal myotubes. A preliminary amino acid analysis of the 22,000 dalton polypeptide is presented in Table 6.

Desmin was also monitored by two-dimensional polyacrylamide gel electrophoresis to assess whether changes were occurring in either the charge or the ratio of the isoelectric variants as a result of treatment (Figure 15). It was apparent, by comparison with the migration of skeletal muscle α-actin, that the charge of the major isoelectric variants (α and β) of desmin in desmin-enriched fractions isolated from non-stimulated and electrically stimulated muscle is not affected by electrical stimulation and does not change during postmortem storage. Minor isoelectric variants present in all samples also appear not to change their charge distribution. The only detectable difference among identically loaded samples was a decrease in the quantity of desmin in both the non-stimulated and the stimulated samples after 168 hours of storage and the concomitant appearance of lower molecular weight degradation products. The appearance of two-dimensional gels of desmin fractions isolated from non-stimulated and stimulated muscle after 24 hours storage was consistent with these observations.

Immunochemical Localization of Desmin-Containing Filaments in Myofibrils

Myofibrils were prepared from bovine longissimus dorsi muscle at the same times that samples were taken for the preparation of the desmin-enriched fractions. These samples included one-hour post mortem tissue which had an intramuscular temperature of 29 ± 1°C; 24 hours stored at either 2°C

Table 6. Amino acid analyses of the 22,000 dalton polypeptide and of other myofibrillar of cytoskeletal proteins.[a]

Amino acid	22,000 dalton polypeptide[b]	Bovine skeletal muscle desmin[c]	Rabbit skeletal red muscle α-actinin[d]	Actin[e]
Asp	7.7	10.8	11.7	9.4
Thr	4.6	4.9	4.7	7.5
Ser	8.6	5.8	5.0	6.4
Glu	10.0	18.6	16.8	10.9
Pro	10.0	2.5	3.7	5.0
Gly	5.7	6.7	5.6	7.5
Ala	6.3	10.3	9.2	8.1
Cys	0.7	0.4	1.2	1.3
Val	5.9	6.2	3.5	5.0
Met	1.4	2.5	2.7	4.4
Ile	5.3	5.3	4.3	7.3
Leu	8.3	12.7	9.5	6.9
Tyr	1.2	2.8	2.4	4.3
Phe	6.8	2.4	3.1	3.1
Lys	5.2	5.1	5.7	5.0
His	4.8	1.5	2.4	2.0
Arg	7.3	8.3	6.6	4.9

[a]Results are expressed in mole percent.

[b]Values are the average of triplicate analyses of one sample of the 22,000 dalton polypeptide prepared from electrically stimulated bovine longissimus dorsi muscle that was stored for one hour at 2°C.

[c]From Table 1.

[d]From Suzuki et al., (1973).

[e]From Carsten (1963).

Figure 15. Two-dimensional gel electrophoresis of desmin-enriched fractions prepared from control and electrically stimulated bovine longissimus dorsi muscle sampled after 1 and 168 hours storage at 2°C. {The acidic end of the pH gradient in the isoelectric focusing dimension is to the left. Spots corresponding to the isoelectric variants of desmin and vimentin and α-actin are labelled. Vimentin is present in amounts less than 1 - 2% of the total protein and appears due to heavy protein loading of the gel.

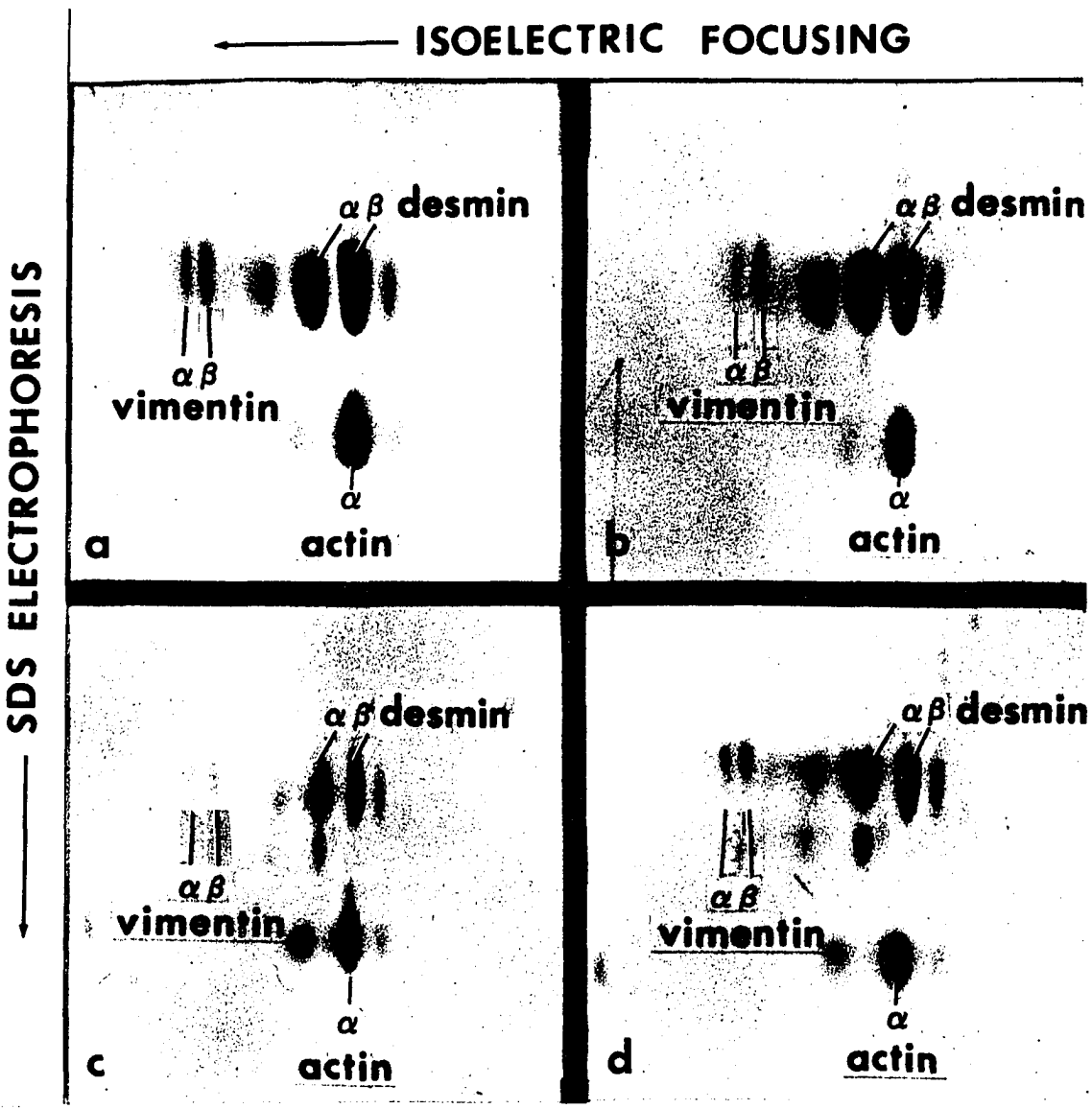
(a) Two-dimensional gel of a desmin-enriched fraction prepared one hour post mortem from non-stimulated bovine longissimus dorsi muscle

(b) Two-dimensional gel of a desmin-enriched fraction prepared one hour post mortem from electrically stimulated bovine longissimus dorsi muscle. Note the similarity in the appearance of desmin and its isoelectric variants to those in non-stimulated tissue (a)

(c) Two-dimensional gel of a desmin-enriched fraction prepared 168 hours post mortem from non-stimulated bovine longissimus dorsi muscle. Note the reduction in the quantities of the desmin isoelectric variants and the concomitant appearance of lower molecular weight breakdown products, particularly below α-desmin, in comparison to the one hour samples (a and b)

(d) Two-dimensional gel of a desmin-enriched fraction prepared 168 hours post mortem from electrically stimulated bovine longissimus dorsi muscle. The appearance of the polypeptides is similar to that seen in the 168 hour non-stimulated sample (c)

Two-dimensional electrophoresis was done by Mary Bremner, Muscle Biology Group, Iowa State University.}


ISOELECTRIC FOCUSING
SDS ELECTROPHORESIS
αβ desmin
αβ vimentin
α actin
a
b
c
d

or 25°C; 168 hours stored at either 2 or 25°C; non-stimulated and stimulated muscle stored at 2°C for 1, 24 or 168 hours. Myofibrils were chosen as the experimental material for two reasons. First, myofibrils are small organelles that can be readily penetrated by immunochemical reagents. Most myofibril preparations do, however, contain fragments of two or more myofibrils that are still connected laterally. These areas of lateral connection are of particular relevance to studies such as this on the cytoskeleton. Second, myofibrils can be studied directly with the light microscope, i.e. phase contrast and/or fluorescence and can readily be prepared for this sectioning and examination with the electron microscope. Present indications are that our laboratory is able to achieve slightly greater consistency of antibody labelling, especially for the electron microscope, when myofibrils rather than intact tissue are used. The purpose of these antibody localization experiments was to determine if there was a reduction in antibody binding which paralleled changes in amount of desmin present in desmin-enriched fractions and if there was a change in the localization pattern that could be related either to the treatment (temperature or electrical stimulation) or to the storage time.

Fluorescence microscope localization of desmin

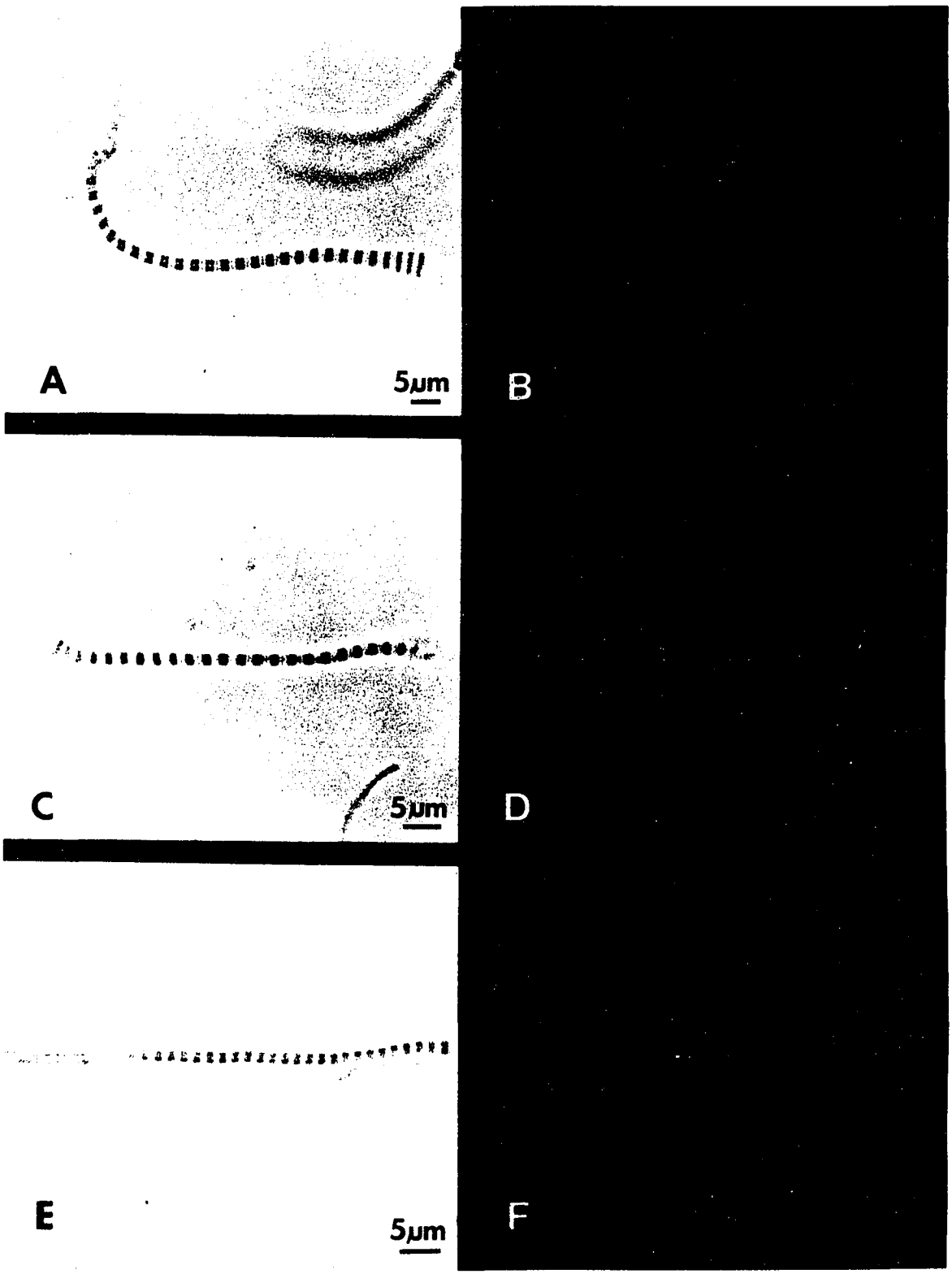
Myofibrils prepared from bovine longissimus dorsi muscle that had been stored for one hour have sarcomere lengths which average 2.52 μm and the banding pattern of normal, rest-length myofibrils (Figure 16, A and C). Treatment of these myofibrils with pre-immune rabbit serum (Figure 16B) instead of the specific desmin antiserum indicates that the pre-immune serum contains no antibodies against desmin or any of the myofibrillar

Figure 16. Myofibrils prepared from bovine longissimus dorsi muscle one hour post mortem or after storage at 25°C for 168 hours.

(A & B) Phase contrast and fluorescence microscope appearance of myofibrils prepared from tissue one hour post mortem and incubated with pre-immune rabbit serum and FITC-conjugated goat-anti-rabbit antibodies. The absence of fluorescence (B) demonstrates the lack of antibodies directed against desmin or other myofibrillar proteins in the pre-immune serum. 1000X

(C & D) Phase contrast and fluorescence microscope appearance of myofibrils prepared from tissue one hour post mortem and incubated with immune rabbit serum (containing monospecific desmin anti-bodies) and FITC-conjugated goat-anti-rabbit antibodies. Intense fluorescence is present between or at the periphery of individual myofibrils at the level of the Z line. 1250X

(E & F) Phase contrast and fluorescence microscope appearance of myofibrils prepared from tissue stored at 25°C for 168 hours and labelled for desmin as previously described. No differences in the intensity or distribution of desmin labelling could be detected in these samples when compared to myofibrils prepared from muscle stored for one hour. 1250X


A
5µm
B
C
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E
5µm
F

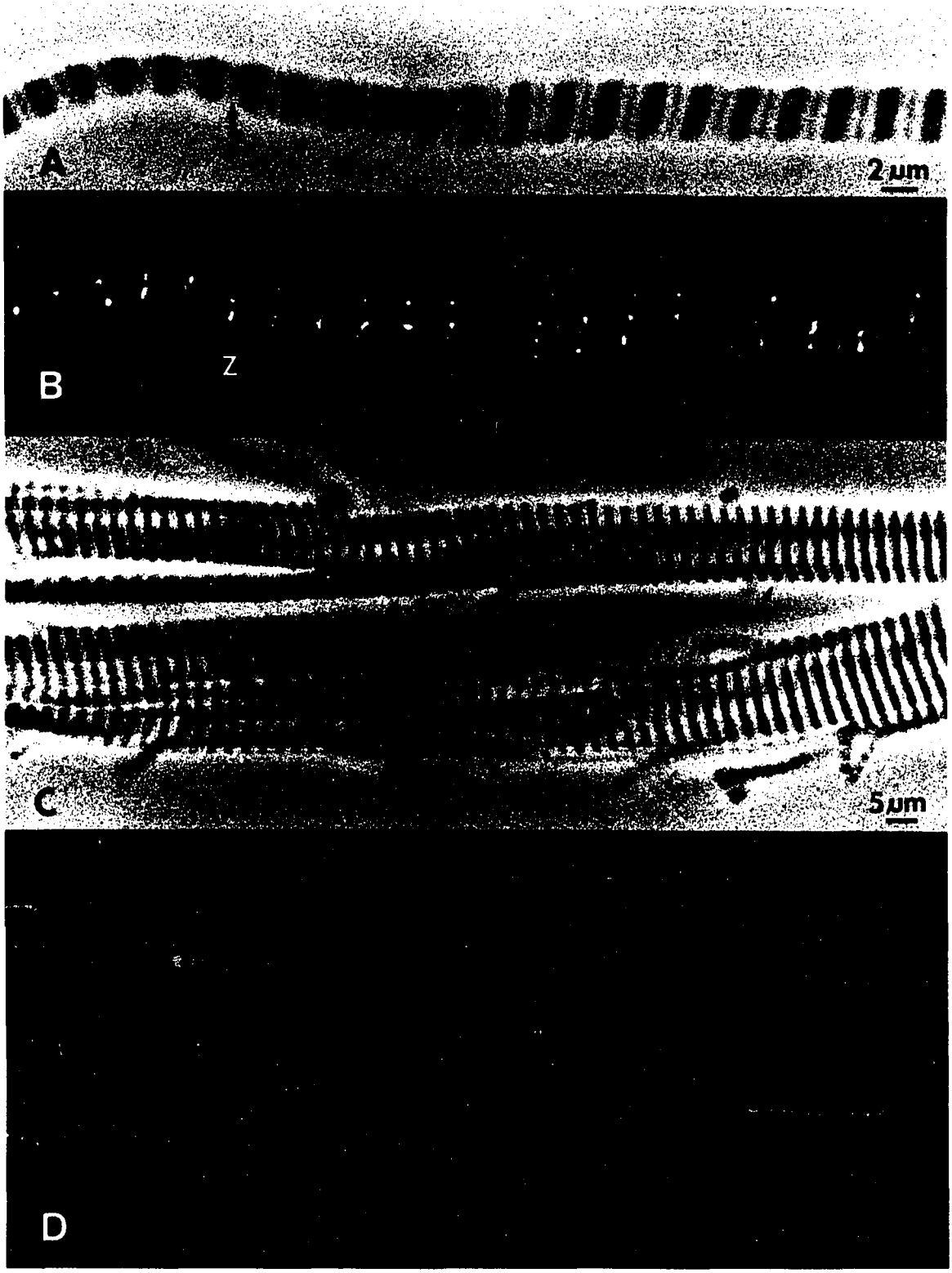
proteins. Substituting anti-desmin for the pre-immune serum in the fluorescence labelling technique results in a punctate arrangement of fluorescence at the edge of the myofibril near the Z line (Figure 16D). Some of the Z lines have a lower intensity of fluorescence between the edge dots. This is probably due to other fluorescent areas that are above or below the plane of focus at which this micrograph was taken. Myofibrils from muscle stored 168 hours at 25°C (Figure 16E) have shorter sarcomere lengths ($\bar{x}$ = 1.81 μm) and tend to fragment into pieces with fewer sarcomeres per myofibril. The intensity and distribution of anti-desmin fluorescence, however, was unchanged from its location at the edge of the myofibril (Figure 16F).

Higher magnification micrographs of a myofibril bundle isolated from muscle stored for one hour are shown in phase contrast (Figure 17A) and in fluorescence (Figure 17B). The bright dots of fluorescence indicate there are probably three myofibrils in this bundle. Two large bundles of myofibrils are shown in phase contrast (Figure 17C) and in fluorescence (Figure 17D). Because only limited areas of any one myofibril are in the plane of focus, image clarity is reduced (Figure 17C). The appearance in fluorescence (Figure 17D), however, shows two interesting features. First, the left half of the field again shows the punctate pattern of bright dots between myofibrils. Second, the right half of the micrograph at the arrow shows myofibrils that are no longer in register with the adjacent myofibrils (cf. Figure 17C). The fluorescence micrograph of this region (Figure 17D) shows oblique fluorescent intensity between the brighter dots which could be attributed to a distortion of the encircling network of desmin-

Figure 17. Myofibrils prepared from bovine longissimus dorsi muscle one hour post mortem.

(A & C) Appearance of myofibril bundles in the phase contrast microscope. The thin dark bands indicated by arrows are skeletal muscle Z lines (Z). A = 3000X; C = 1050X

(B & D) Myofibrils were labelled with desmin antibodies produced in a rabbit and FITX-conjugated goat-anti-rabbit. The small bundle of myofibrils shown in B shows a punctate pattern of fluorescence between and at the edge of the myofibrils at the level of the Z line. Although the resolution is lower in D, this larger myofibril bundle shows both the punctate pattern in the left side of the field and a three-dimensional appearance (arrow) where myofibrils that are off-set from each other may have distorted the encircling network of desmin-containing filaments. B = 3000X; D = 1050X


A
2 µm
B
Z
C
5 µm
D

containing filaments. Although sarcomere lengths usually were shortest ($\bar{x}$ = 1.53 μm) in myofibrils prepared from muscle stored for 24 hours at either 2°C or 25°C (not shown), there was no difference in the intensity of or the distribution of desmin labelling in myofibrils prepared from muscle stored at either 2°C or 25°C for 1, 24 or 168 hours post mortem.

Myofibrils prepared from electrically stimulated muscle had shorter sarcomere lengths at all sampling times when compared with myofibrils from non-stimulated muscle but no other differences were seen with the phase contrast technique (results not shown). Differences in the intensity or the distribution of desmin labelling could not be detected in myofibrils prepared from either stimulated or non-stimulated muscle stored at 2°C for 1, 24 or 168 hours post mortem (results not shown).

An attempt was made in a pilot experiment to cytophotometrically quantitate the intensity of Z line fluorescence in labelled myofibrils prepared from non-stimulated muscle that was sampled at 1 hr or that had been stored at 25°C for 168 hours. This pair of samples was chosen because the 168 hour sample had the lowest amount of extractable desmin (Table 2), presumably due to postmortem degradation, and the one-hour sample presumably should have the least amount of degradation. The fluorescence from twenty Z lines in each sample was measured at 492 nm with a Leitz cytophotometer (E. Leitz, Inc., West Germany). No differences in the average amount of Z line fluorescence were detected in the two samples. Attempts to make measurements from a larger area within a field were unsuccessful because other factors such as myofibril or sarcomere length, number of laterally associated myofibrils and plane of focus along the myofibril axis could

not be adequately standardized.

Electron microscope localization of desmin

A typical example of the low-level, diffuse, non-specific distribution of ferritin which occurs when pre-immune serum is used as a control is shown in Figure 18A. The ferritin-conjugated goat anti-rabbit label is uniformly distributed over the myofibrils with this pre-embedding labelling technique. If serum containing antibodies to desmin is used instead, dense clusters of ferritin granules are present between myofibrils adjacent to the Z line (Figure 18B). These dense clusters are absent when pre-immune serum is used. At slightly higher magnification (Figure 19), the ferritin granules seem to be associated with filaments that are between myofibrils at the level of the Z line. The ferritin-associated filaments in both Figures 18B and 19 have average diameters of 100 Å. This is the first report of desmin localization with electron-immunocytochemistry in bovine skeletal muscle.

Electron Microscopy of Electrically Stimulated Skeletal Muscle

One of the objectives of this study was to compare the effects that postmortem storage had on electrically stimulated bovine skeletal muscle with those on non-stimulated muscle. Samples were taken from stimulated and non-stimulated muscles that had been stored at 2°C for one, 24 or 168 hours. Conventional sample preparation for thin sectioning and for examination in the electron microscope was used.

A survey micrograph (Figure 20A) of non-stimulated muscle sampled

Figure 18. Indirect immunoelectron microscope labelling of desmin in myofibrils prepared from bovine longissimus dorsi muscle stored for one hour at 2°C.

(A) Unstained section of myofibrils incubated with pre-immune rabbit serum and ferritin-conjugated goat-anti-rabbit antibodies. Note that the ferritin-conjugated antibodies non-specifically adhere to protein throughout the myofibrils. Z = Z line. 34,400X

(B) Unstained section of myofibrils incubated with immune rabbit serum (containing monospecific desmin antibodies) and ferritin-conjugated goat-anti-rabbit antibodies. Although non-specifically bound ferritin-conjugated antibodies are present throughout the section, discrete, specifically bound clusters of ferritin (small arrows) can be seen at the periphery of myofibrils in conjunction with the filamentous structures (large arrows) having mean diameters of 100 Å. Z = Z line. 35.450X

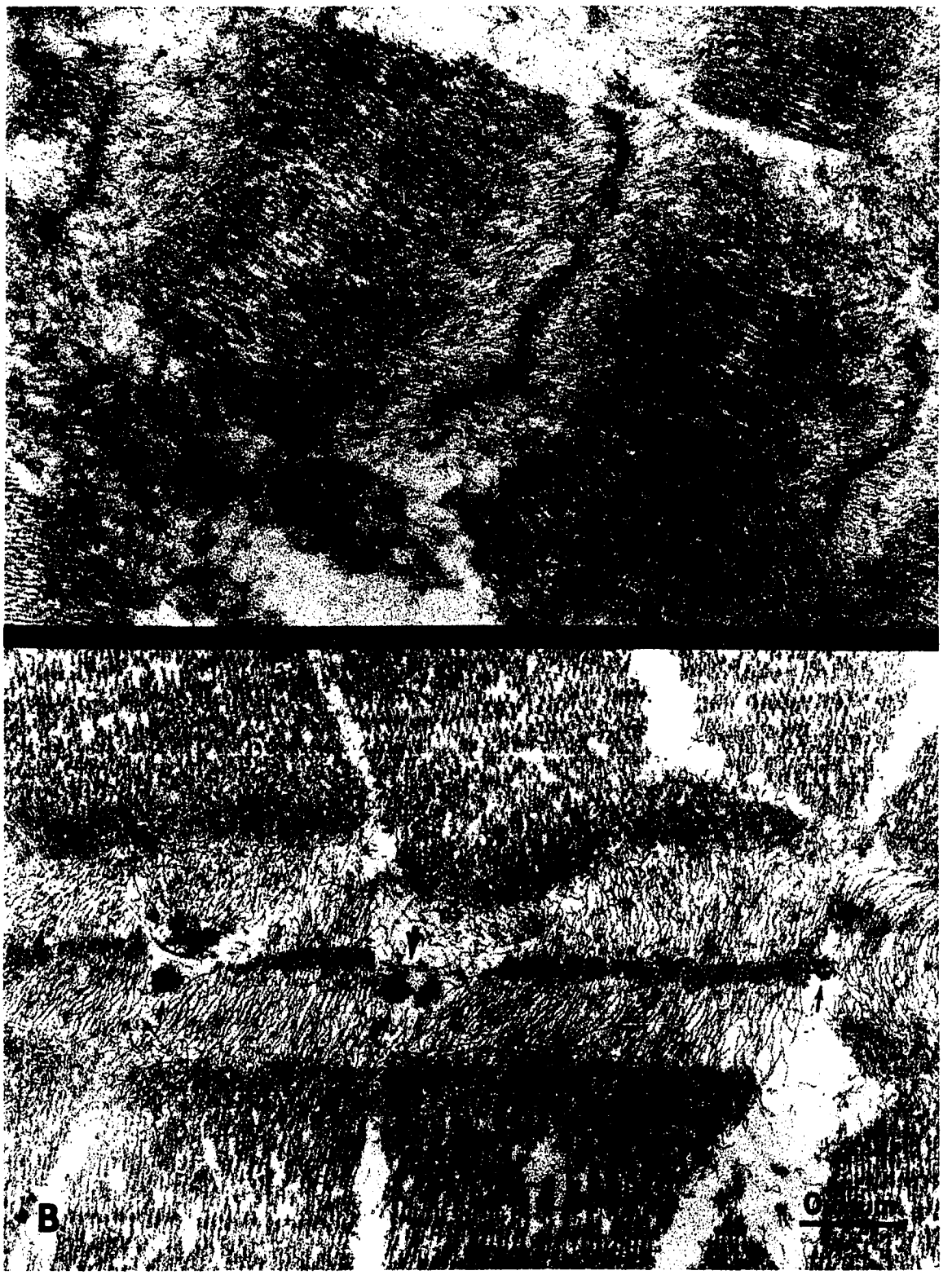

B

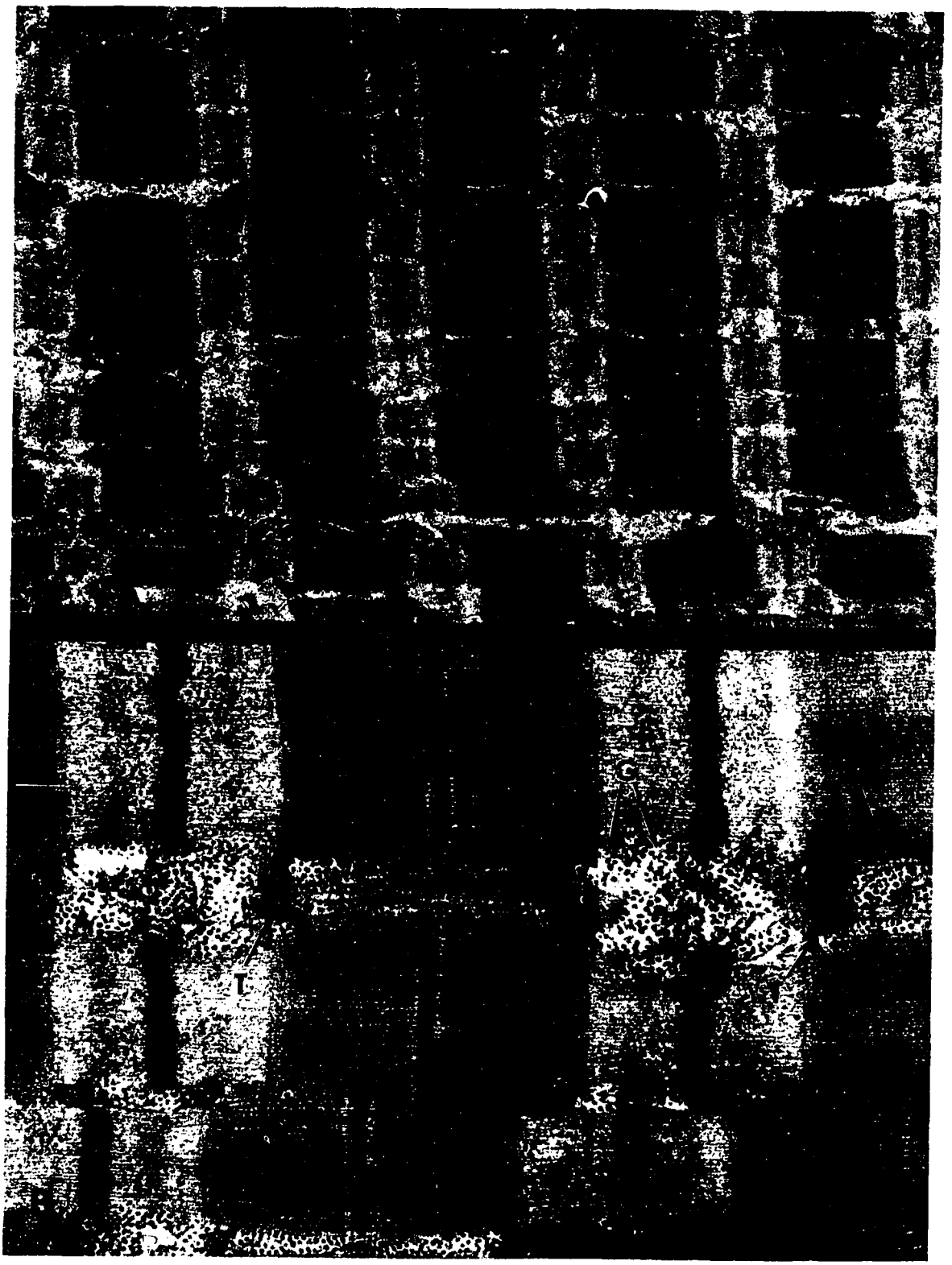
Figure 19. Intermediate (100 Å) filaments in myofibrils prepared from bovine longissimus dorsi muscle that was stored for one hour at 2°C. {Myofibrils were incubated with immune rabbit serum and ferritin-conjugated goat-anti-rabbit antibodies. 100 Å filaments (arrows) can be seen coursing between myofibrils at the level of the Z line (Z). Sections were lightly stained with methanolic 2% uranyl acetate and lead citrate. 48,100X }


Z
Z
0.2 µm

Figure 20. Bovine longissimus dorsi muscle excised from a non-stimulated carcass after one hour at 2°C.

(A) Note the tight packaging and lateral alignment of myofibrils within a given cell. Mitochondria (mi) are not expanded and exhibit a normal internal structure. Z = Z line. 14,550X

(B) Well-preserved details of a relaxed muscle fiber (sarcomere length = 2.49 μm) are apparent at higher magnification. T = T-tubule; SR = sarcoplasmic reticulum; M = M-line; G = glycogen granules. 37,250X


C
I
P

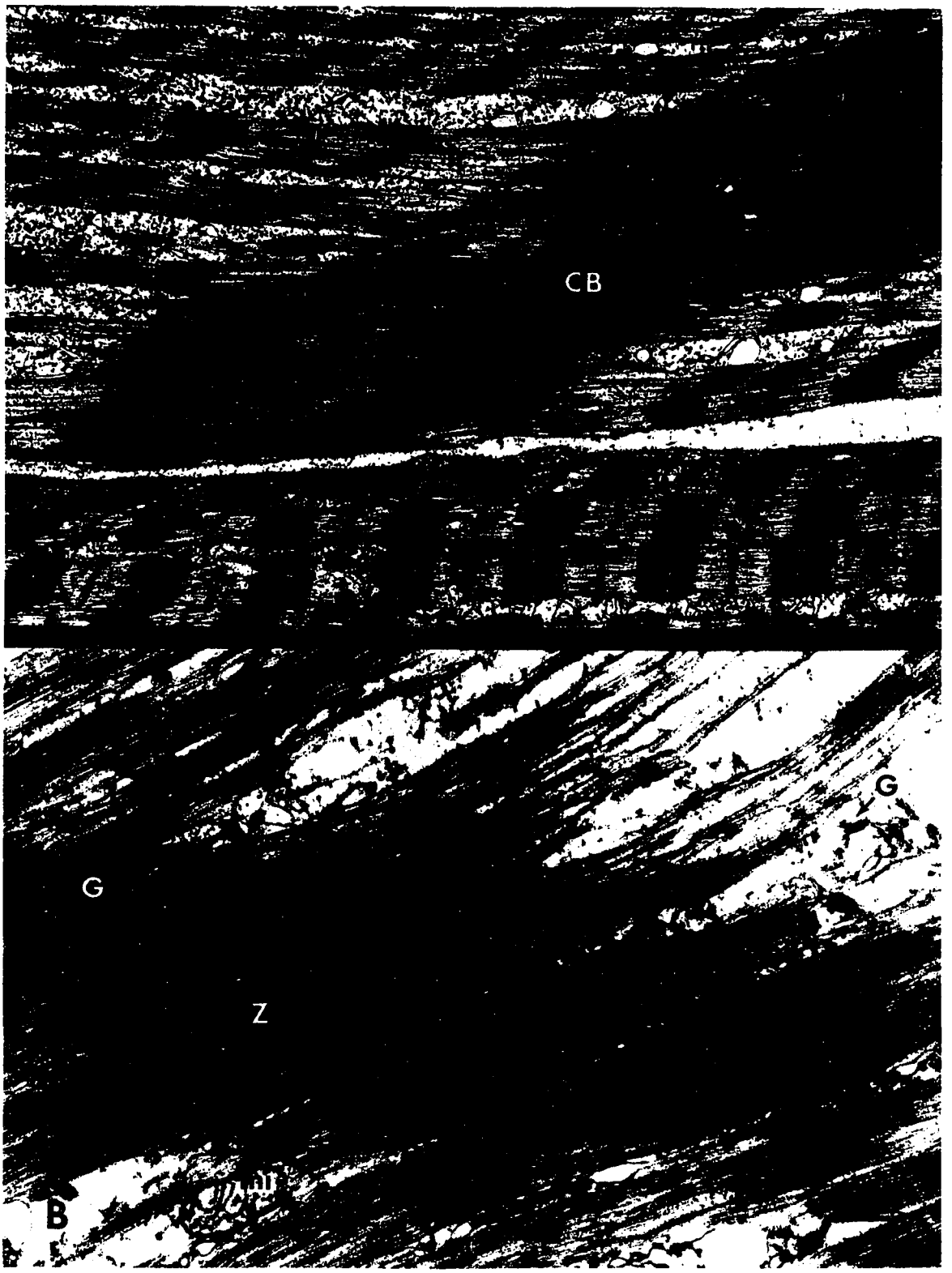
after one hour at 2°C shows the tightly packed myofibrils in lateral register. Mitochondria and sarcoplasmic reticulum are interspersed between the myofibrils and have typical structure. Three adjacent sarcomeres in a sample of non-stimulated muscle stored for one hour at 2°C are shown at higher magnification in Figure 20B. Thick and thin filaments can be clearly identified in these relaxed sarcomeres (sarcomere length 2.49 μm). Five transverse striations which form the M-lines and the wide, dense Z lines which are characteristic of red mammalian skeletal muscle are also evident. Sarcoplasmic reticulum membranes and T-tubules have an array of regularly spaced connections ("feet") at their junctions and are not dilated. Glycogen granules are abundant in the space between myofibrils.

This typical morphology is radically altered in bovine skeletal muscle that was electrically stimulated and sampled after one hour (Figure 21A). The cell in the upper portion of this field contains a dense contraction band which is formed by an accumulation of myofilaments from several adjacent sarcomeres. Although all cells do not exhibit contracture bands, when the bands occur, they alternate with highly stretched sarcomeres of various lengths. Membrane-bounded vesicles exist between myofibrils but mitochondria and sarcoplasmic reticulum membranes are no longer recognizable. An adjacent cell in the lower portion of this field contains stretched myofibrils, disorganized but recognizable mitochondria and triads. Sarcomere lengths in electrically stimulated samples are variable and usually exceed the normal rest length of 2.3 - 2.6 μm. A higher magnification of a portion of a contraction band shows

Figure 21. Bovine longissimus dorsi muscle excised from an electrically stimulated carcass after one hour at 2°C.

(A) In the upper cell, extreme contraction during electrical stimulation induced the formation of a region of densely packed, supercontracted myofibrillar protein called a contraction band (CB) which is surrounded by areas of extreme myofibrillar stretching. Note the mitochondrial expansion and loss of myofilament lateral registration in both cells. All sarcomeres in the lower cell are stretched. Mitochondria and triads are distorted but can be easily identified. 7950X

(B) Higher magnification of a region within a contraction band. Myofibrillar details are still apparent but the components of the sarcomere are extremely distorted. Z = Z line; G = glycogen granules; mi = mitochondria. 23,500X


CB
G
G
Z
B

the accumulation of thick and thin filaments and the intermixing of transverse dark bands which are segments of Z lines. A disrupted mitochondrion and a few glycogen granules can be seen at the edge of the contraction band.

The appearance of non-stimulated bovine longissimus dorsi muscle excised after 24 hours storage at 2°C is shown in Figure 22. Parts of two adjacent cells (Figure 22A) show sarcomeres which average 1.76 μm long and an increase in the space between myofibrils. Triads and T-tubules are recognizable but the sarcoplasmic reticulum has vesiculated and mitochondria can, at best, only be tentatively identified. M-lines have lost most of their substructure (Figure 22B). Z lines are less dense and have some gaps or discontinuities across some myofibrils. Electrically stimulated muscle excised after 24 hours storage at 2°C has more extensive myofibrillar changes (Figure 23) than were observed in the non-stimulated sample. Contraction bands are still present (Figure 23A) but adjacent sarcomeres, although variable in length, have shortened to approximately 1.5 μm. Space between myofibrils was frequently greater than that shown in Figure 23A. Swollen mitochondria which contain small remnants of cristae can be located near the edge of the contraction band. Many spaces are also seen between filaments and in the Z line. An indication that there has been a greater loss of lateral connections between myofibrils in the stimulated sample is that myofibrils in this intact tissue have a greater tendency to course in and out of the plane of section than in the non-stimulated sample. The M-line is highly disorganized and is difficult to see, even at the

Figure 22. Bovine longissimus dorsi muscle excised from a non-stimulated carcass that was stored for 24 hours at 2°C.

(A) Two adjacent cells both show similar postmortem changes and have sarcomere lengths which average 1.76 μm. Intermyofibrillar space is increasing and the side-by-side alignment of myofibrils is less precise than in the one hour sample (Figure 20). Glycogen granules (G) are still present in both cells. T-tubules (T) and terminal cistemae (TC) are still recognizable. 10,900X

(B) Loss of density in Z (Z) and M-lines (M) and an increasing frequency of gaps or discontinuities in Z lines is apparent at higher magnification. mi = mitochondria. 45,700X

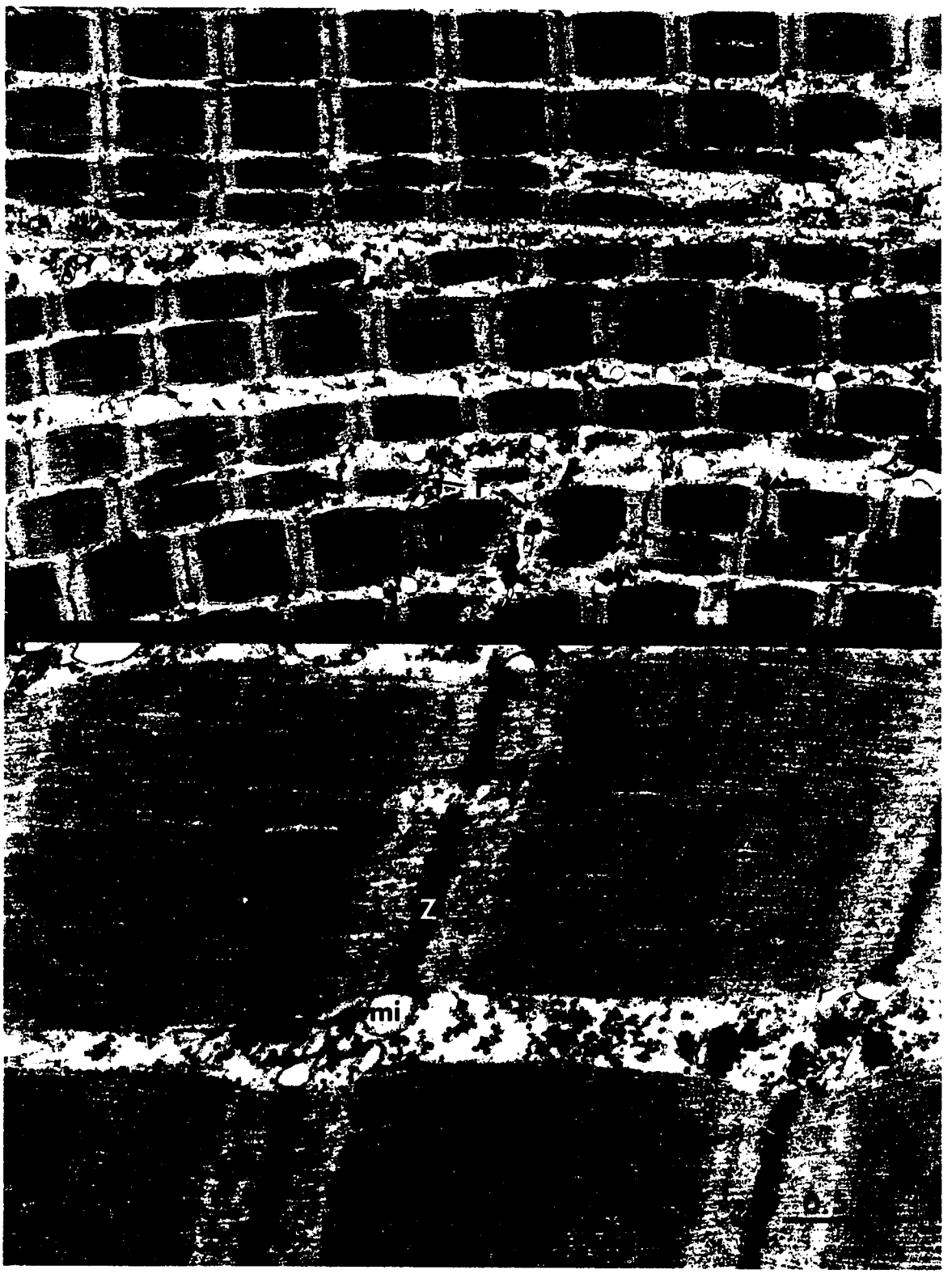
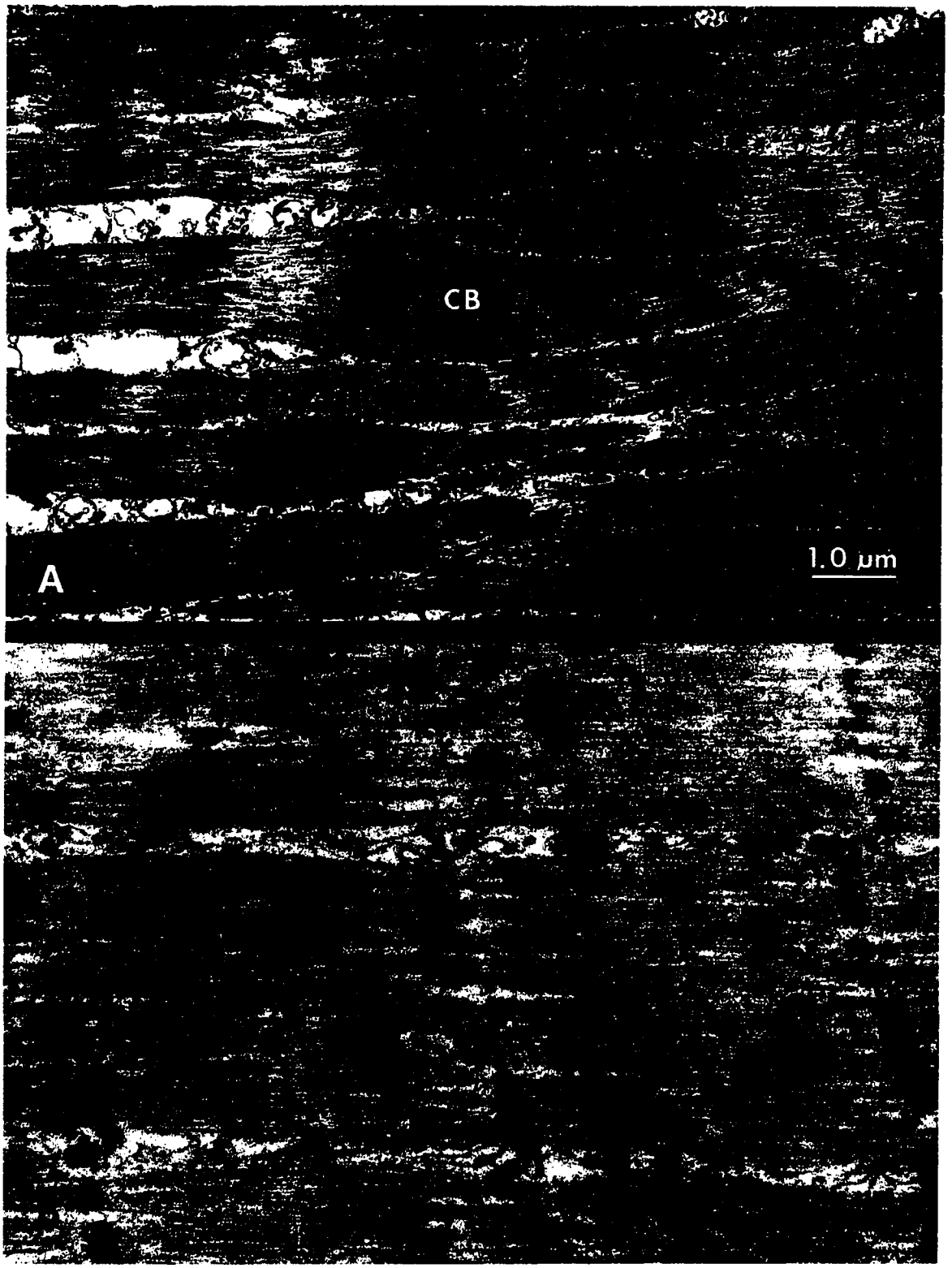

Z
mi

Figure 23. Bovine longissimus dorsi muscle excised from an electrically stimulated carcass that was stored for 24 hours at 2°C.

(A) Ultrastructural damage induced by severe contractions during electrical stimulation is still apparent at 24 hours post mortem. CB = contraction band. 14,400X

(B) A marked decrease in the densities of Z and M-lines (M) and an increase in the number of Z line gaps or discontinuities is readily apparent in comparison to those features in tissue excised 24 hours post mortem from a non-stimulated bovine carcass side (Figure 22). 48,100X


CB
A
1.0 µm

higher magnification of Figure 23B. The loss of material from and discontinuities in Z lines are particularly clear. When Figure 22 is compared with Figure 23, it is very certain that after 24 hours storage at 2°C, electrically stimulated bovine skeletal muscle shows greater disruption of filament-anchoring or connecting structures such as Z lines and M-lines. Increased space between myofibrils may indicate that cytoskeletal elements (100 Å desmin filaments) that laterally connect myofibrils have been weakened more in the stimulated sample.

Non-electrically stimulated muscle stored at 2°C for 168 hours (Figure 24A and B) contains Z lines that are less dense, more diffuse and have more discontinuities than at 24 hours (cf. Figure 22). M-lines can be easily identified and some membranous vesicles remain between myofibrils. Electrically stimulated muscle stored at 2°C for 168 hours retains contraction bands (Figure 25A) but has lost additional density from the Z line (Figure 25B). The disruption in the I band is evident. Fragments of the M-line are very difficult to identify.

While observations on one hour samples indicated that the additional disruption seen in the stimulated samples was mainly due to sever contraction which resulted in the formation of contraction bands and alterations in adjacent sarcomeres and myofibrils, it is apparent, when comparing non-stimulated and electrically stimulated muscles that have been stored for either 24 or 168 hours, that factors other than severe contraction must be operating in the stimulated muscle. This was evidenced by the greater loss of density from Z lines, the greater disruption of M-lines, Z lines and I bands, and the indication of reduced cyto-

Figure 24. Bovine longissimus dorsi muscle excised from a non-stimulated carcass that was stored for 168 hours at 2°C.

(A & B) Typical appearance of bovine skeletal muscle 168 hours post mortem. Z and M-lines (M) are still apparent although densities are diminished in comparison to those features in one hour and 24 hour postmortem non-stimulated tissue (Figures 20 and 22). mi = mitochondria. A = 31,500X; B = 41,050X

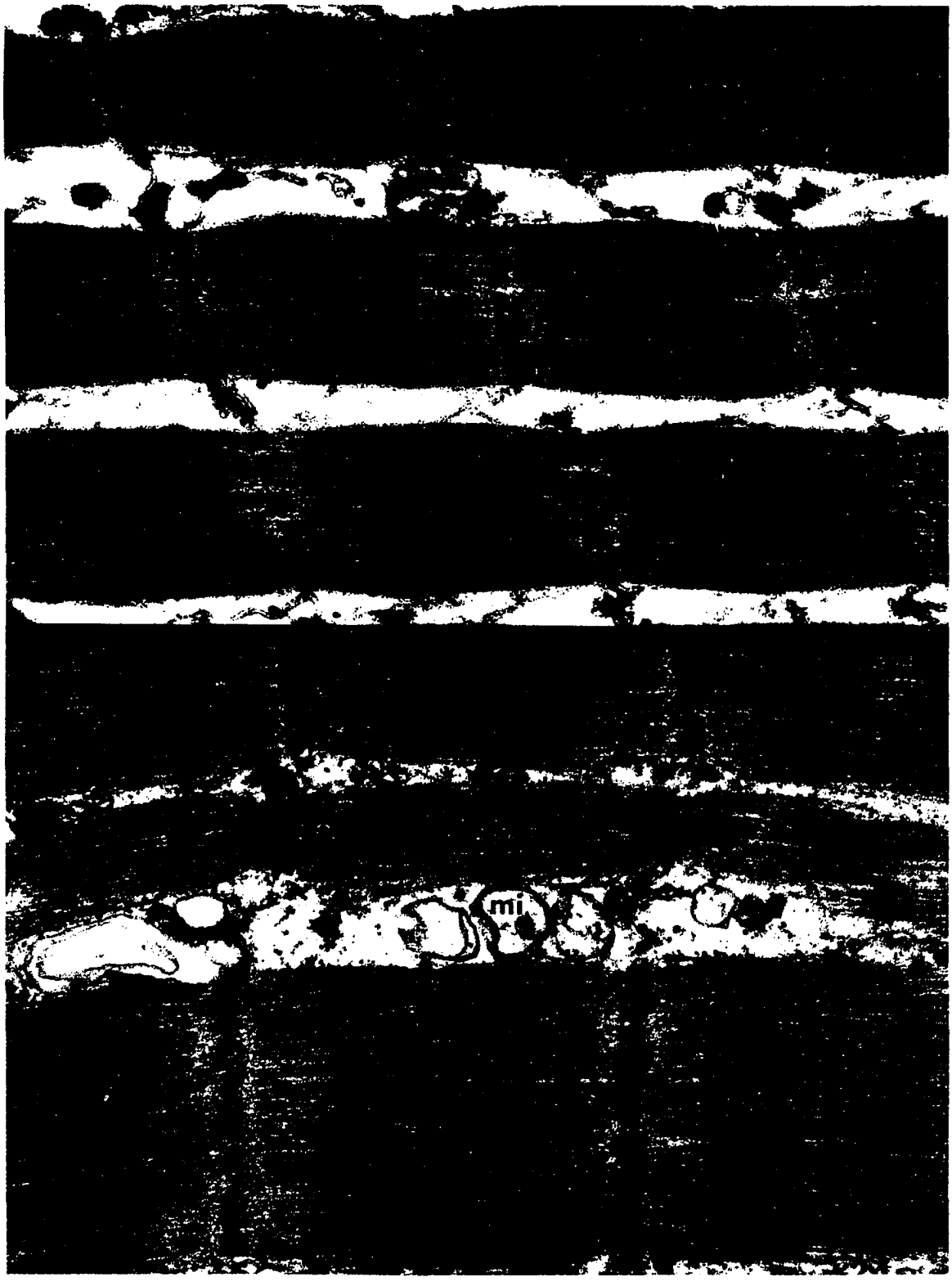
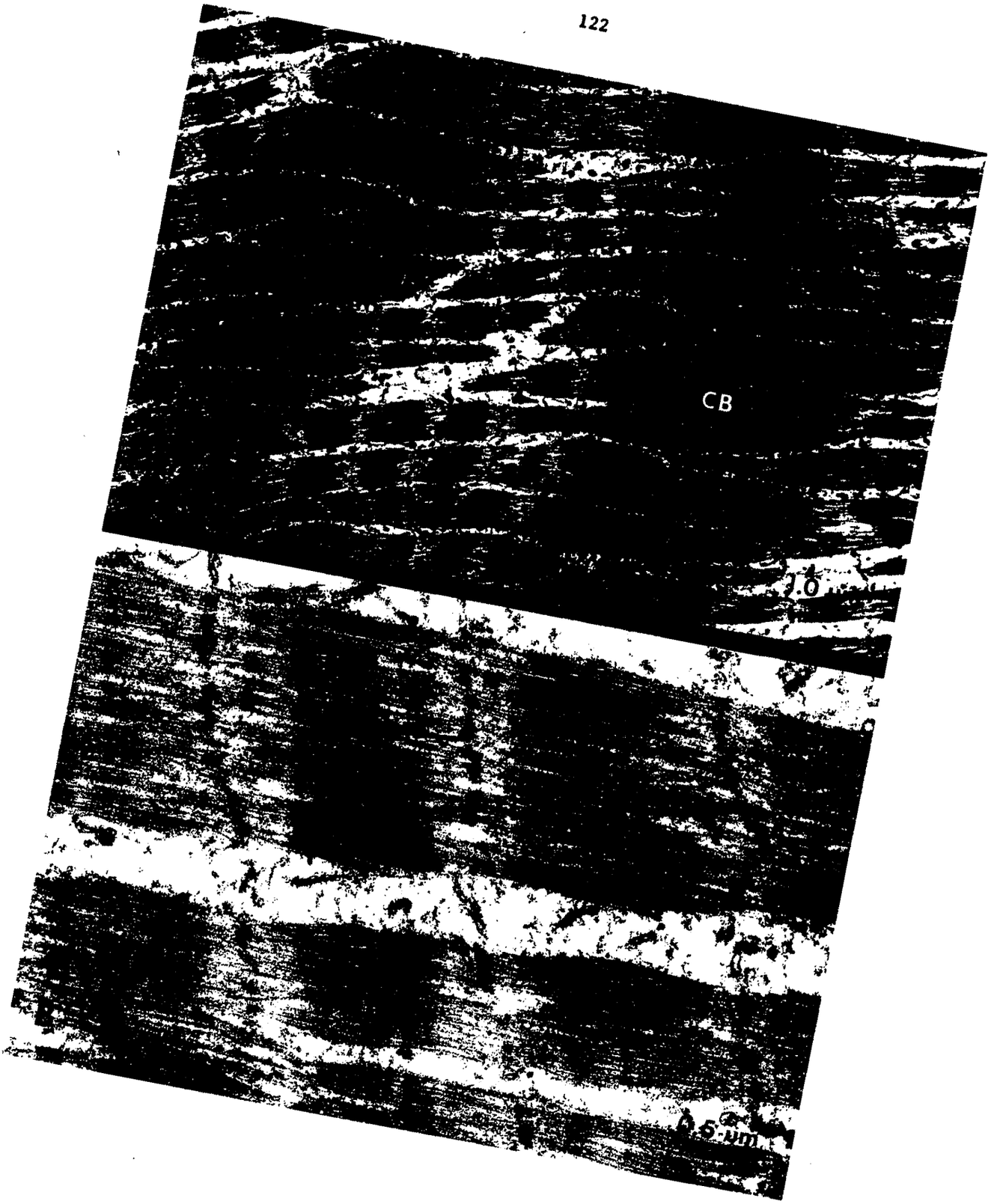

mi

Figure 25. Bovine longissimus dorsi muscle excised from an electrically stimulated carcass that was stored for 168 hours at 2°C.

(A) Effects of electrical stimulation are still apparent in this tissue sample although structures within contraction bands (CB) are no longer recognizable. 9,200X

(B) Typical appearance of myofibrils at higher magnification than in (A). Z lines (Z) have many discontinuities within a single fibril; M-lines are no longer apparent. Thick and thin filament densities are also drastically reduced in comparison to those structures in non-stimulated 168 hour postmortem tissue (Figure 23). 34,200X


CB
1.0
5 µm

skeletal integrity evidenced by increased intermyofibrillar spacing in the stimulated samples that had been stored for either 24 or 168 hours.

DISCUSSION

Mature skeletal muscle cells are comprised of subcellular contractile protein units called myofibrils. The arrangement of these subcellular units permits the generation of coordinated cellular contractile forces in living tissue. This subcellular arrangement is due in part to a network of 100 Å diameter filaments which surround and interconnect myofibrils at the level of the Z line in a three-dimensional cytoskeletal array. Results of previous research indicated that desmin was the major (and likely only) component of this network of skeletal muscle 100 Å filaments.

The postmortem integrity of the 100 Å filament network has been implicated as a factor which contributes to the degree of tenderness in bovine tissue (Young et al., 1981; Robson et al., 1981, 1983). It was the objective of this study to determine the extent of specific alterations in the cytoskeleton and its constituent protein, desmin, during aging of bovine skeletal muscle. Comparing quantitative and qualitative changes in desmin and the cytoskeleton in bovine longissimus dorsi muscle stored under a conventional aging regime (2°C for 168 hours) to changes occurring in similar tissue subjected to procedures known to increase tenderness (i.e. high temperature aging and electrical stimulation) was the approach used to assess the extent to which the cytoskeleton changed while tenderness was increasing.

A less detailed study of the postmortem changes in desmin was done by Young et al. (1981) who showed a progressive decline in the percentage of guanidine-HCl soluble desmin isolated at 0, 1 and 6

days post mortem from ox sternomandibularis muscle stored at 15°C. No attempts were made in their study to quantitate the amounts of desmin or determine the specific alterations in the molecule at any of the three sampling times. It was therefore difficult to assess the impact that the postmortem reduction in the percentage of desmin had on the cytoskeleton in tissue stored at this temperature. This has been the only study to date reporting on changes in desmin in postmortem bovine muscle.

For this dissertation, desmin-rich fractions were prepared from conventionally aged, high temperature aged and electrically stimulated bovine longissimus dorsi muscle at 1, 24 and 168 hours post mortem. Changes in the protein composition of fractions isolated during the preparation of each desmin-rich fraction were assessed with one- and two-dimensional electrophoresis and slab gel immunoautoradiography. Desmin was quantitated in the desmin-enriched fractions at all times for all experimental conditions by replicated densitometric scans of polyacrylamide tube gels. Changes in the cytoskeleton were also studied by using fluorescent antibody localization of desmin in myofibrils prepared at the same postmortem times that samples were taken for desmin quantitation. In addition, structural changes in control and electrically stimulated tissue were compared at the electron microscope level of resolution at these same times.

The ability to isolate and purify desmin and elicit a monospecific antibody against it was requisite to the majority of techniques just outlined for studying desmin and the cytoskeleton. As reported in the Results section, the amino acid content, filament forming ability and

two-dimensional gel appearance of desmin isolated for this dissertation are similar to previous reports in the literature.

Results from the biochemical and ultrastructural analyses used in this dissertation will be discussed together by treatment. This approach allows a more substantial assessment of the effect that each treatment had on desmin and the cytoskeleton, and gives more confidence in comparisons made between the treatments.

Effects of Postmortem Treatment on Desmin and the Cytoskeleton

Control tissue

Analysis of the effects of postmortem treatment on desmin and the cytoskeleton will begin with the condition of usual postmortem handling of bovine tissue, i.e. aging for 168 hours at 2°C. This aging protocol was the control for studies on tissue subjected to accelerated aging changes resulting from high temperature storage or electrical stimulation.

Changes in the amounts of desmin in desmin-enriched fractions isolated from control tissue over 168 hours of postmortem storage at 2°C are presented in Tables 2 and 4 and graphically depicted in Figures 7 and 8. Both graphs depict the almost twofold increase in the amount of desmin isolated at 24 hours versus one hour post mortem, and show a return by 168 hours post mortem to levels lower than those existing at one hour post mortem.

It is important to note that the reported values of soluble desmin are the products of values of total soluble protein and percentage of desmin in the desmin-enriched fractions (Tables 3 and 5). Studying these two factors which yield the values of soluble desmin aids in analyzing

the mechanisms behind postmortem changes in desmin in each of the treatments. The increased amount of desmin extracted at 24 hours versus 1 hour post mortem was due to increases in both the amount of total soluble protein and the percentage of the total soluble protein which was desmin. In Table 3 it can be noted that this trend of increased protein release from the cytoskeleton in control tissue continued at 168 hours post mortem, but was accompanied by substantial decreases in the percentage and amount of desmin. The decrease in the amount of desmin isolated at 168 hours post mortem could be explained by one or a combination of several possibilities: 1) desmin released before 168 hours post mortem was sufficient to substantially reduce the percentage of desmin remaining; 2) desmin was no longer extractable from the cytoskeleton; 3) desmin was preferentially degraded and present in smaller quantities in the insoluble cytoskeleton before extraction at 168 hours. It seems unlikely that release of desmin prior to its extraction from the cytoskeleton with acetic acid could be operative, based on the observations that a) desmin, or any of its breakdown products, never appeared in extraction fractions before the final acetic acid extraction, as shown with polyacrylamide gel electrophoresis (Figure 9) and immunoautoradiography (Figure 10). This observation of the insolubility of desmin in steps before acetic acid extraction is consistent with the results from prior research (Gard and Lazarides, 1980; Young et al., 1981); and b) immunofluorescent localization of desmin occurred solely at the level of the Z line in 168 hour postmortem control tissue (results not shown). The second possibility is very unlikely because it would require a reversal of desmin

solubility from tissue sampled 24 hours post mortem, where desmin was more extractable than from one-hour postmortem tissue. The third possibility, that desmin is preferentially degraded and present in smaller quantities in the cytoskeleton, was supported by the two-dimensional electrophoretic appearance of desmin-enriched fractions prepared from control tissue at one and 168 hours post mortem (Figure 15, a and c). Decreases in the amount of desmin and the appearance of desmin degradation products at 168 hours were noted in comparison to similar desmin-enriched fractions prepared one hour post mortem.

The increased rate of release of desmin from the cytoskeleton and its concomitant degradation during postmortem storage at $2^{o}C$ can be correlated with decreases in myofibrillar packing density and slight loss of lateral register noted in electron micrographs of postmortem control tissue (cf. Figures 20, 22 and 24). The weakening of interfibrillar linkages with increasing postmortem storage has also been observed with the light and electron microscope by other researchers (Stromer et al., 1967; Davey and Gilbert, 1969; Davey et al., 1976; Young et al., 1981).

The increase in the amount of desmin extracted from the cytoskeleton also coincided with the appearance of a 22,000 dalton polypeptide not seen at one hour post mortem (Figure 12, cf. gels B and G with gel C). It is uncertain whether this polypeptide is derived from desmin which yielded more degradation products at 24 hours (Figure 13b, lane B) than at one hour (Figure 13b, lane A) or if this peptide is associated directly or coincidently with the release of desmin. The lack of recognition of the 22,000 dalton band by anti-desmin (Figure 13, b and d) indicated that the

peptide either is not a fragment of desmin or, if it is a desmin fragment, it contains no antigenic sites which the antibody can recognize. Although a 45 minute digestion of pure bovine skeletal desmin (Figure 11) or of porcine skeletal, porcine smooth and avian smooth desmins (O'Shea et al., 1979) with pure calcium-activated factor (CAF) produced no 22,000 dalton polypeptide, Geisler et al. (1982) have shown that a 21,000 dalton polypeptide was generated by the proteolytic breakdown of chicken gizzard desmin protofilaments with chymotrypsin. As was the case with desmin, the 22,000 dalton polypeptide was not extracted by HAMES or KI but seemed to be extractable only with acetic acid (Figure 14). Therefore, in an attempt to clarify the significance of the 22,000 dalton polypeptide in postmortem bovine tissue, the polypeptide was purified and used as an antigen for monospecific antibody production.

The specific anti-22,000 dalton antibodies were used for immunoautoradiographic analysis and <u>in situ</u> localization of the polypeptide. The antibody labelled the 22,000 dalton polypeptide in immunoautoradiograms of fractions isolated during the preparation of desmin-enriched fractions (Figure 14b, lanes E and F). It is notable in this figure that the anti-22,000 dalton antibody did not label other myofibrillar proteins that migrate to this region of the gel {such as the A-1 light chain of myosin (25,000 daltons), troponin-I (23,500 daltons), or troponin-C (19,000 daltons) or the DTNB-light chains of myosin (18,000 daltons)}. Desmin was also not labelled. It should also be noted that the 22,000 dalton polypeptide was not labelled in immunoautoradiograms of similar protein fractions incubated with anti-desmin antibodies (Figure 10, b and d).

Several attempts at labelling nine day old chicken myotubes, adult bovine skeletal myofibrils and cryostat sections of adult bovine longissimus dorsi muscle with the anti-22,000 dalton polypeptide antibody yielded negative results. The hypothesis that the polypeptide is a protein which somehow is associated with or controls the release of desmin from the cytoskeleton could explain the lack of in situ labelling with the anti-22,000 dalton antibody if the antigenic determinants of the protein are inaccessible until it is extracted with acetic acid. At the present time, therefore, the immunological data does not clarify the origin or function of the 22,000 dalton polypeptide which was present in all samples except in the one hour, 29 ± 1°C and in the one hour, non-stimulated samples.

It therefore seems that desmin is preferentially released from the cytoskeleton at or before 24 hours post mortem in control tissue and is subsequently broken down at a rate greater than that occurring in one-hour postmortem tissue. A 22,000 dalton polypeptide appears in the acetic acid extracts of the highly insoluble cytoskeleton at the same time as the increased rate of desmin release is detected. The significance and origin of the polypeptide are unclear at the present time.

High temperature aged tissue

A somewhat different interpretation can be applied to the events involving cytoskeletal breakdown in high temperature (25°C) aged tissue. While total soluble protein released from muscle cells increased with time (Table 3), as was the case in control tissue, the percentage of that total protein which was desmin steadily declined over 168 hours of postmortem storage. This reduction in the percentage of soluble protein which was

desmin decreased the amount of desmin extracted from tissue stored for 168 hours at 25°C to 15% of that extracted from tissue stored for 168 hours at 2°C. The amount of desmin at 168 hours in tissue stored at 25°C was, however, sufficient to cause usual Z line patterns of antibody labelling (Figure 16, cf. E and F).

While the rate of desmin degradation was greater in tissue stored at 25°C than in tissue stored at 2°C for 24 hours post mortem, the rates after 24 hours of storage were nearly identical as indicated by the similar slopes of the respective lines between 24 and 168 hours (Figure 7).

These results of increased desmin breakdown during high temperature storage were not unexpected in light of the following facts regarding bovine muscle aged at high temperatures: 1) raising the temperature of storage increases the rate of tenderization in beef muscle (Busch et al., 1967; Parrish et al., 1973; Davey and Gilbert, 1976; Lochner et al., 1980; Penny, 1980; Dransfield et al., 1980) and is a factor which increases enzymatic activity in, and protein release from, postmortem muscle (Penny, 1980; Asghar and Pearson, 1980); 2) the rate of appearance of a 30,000 dalton polypeptide, a possible indicator of postmortem autolysis in bovine skeletal muscle (Penny, 1980), was faster in tissue stored at 25°C than in tissue stored at 2°C (Olson et al., 1977). This 30,000 dalton polypeptide may be a degradative product of troponin-T (Dayton et al., 1975) and appeared when myofibrils were incubated with purified CAF, a calcium-activated endogenous muscle protease which removes Z lines when incubated with myofibrils (Busch et al., 1972) and also degrades purified bovine skeletal muscle desmin (Figure 11); 3) increased fragmentation of muscle at the Z line, which correlates to increases in tenderness (Culler et al., 1978),

has been shown to occur more readily in muscle stored at temperatures above 16°C than below 16°C (Stromer et al., 1967; Henderson et al., 1970; Davey and Gilbert, 1976; Olson et al., 1976; Moeller et al., 1977); 4) lysosomal enzymes are liberated from their membranes more rapidly and have higher specific activities in tissue stored at 37°C for 12 hours than enzymes in tissue stored at 2°C for 12 hours (Moeller et al., 1977).

Based on the evidence in this dissertation and from previous related research it seems that desmin is being degraded earlier in tissue stored at 25°C than in the control tissue stored at 2°C. The increased breakdown accounts for the declining amounts of desmin in tissue over 168 hours of postmortem storage, even though total protein extractability increased with time. It is of interest that the 22,000 dalton polypeptide is present at all sampling times after 24 hours in amounts which parallel the amount of desmin. The parallel decline of the 22,000 dalton polypeptide and desmin indirectly suggests that the polypeptide is not a breakdown product of desmin. The lack of immunological cross reactivity supports this hypothesis.

Electrically stimulated tissue

Electrical stimulation accelerated the release of protein, particularly desmin, from the insoluble cytoskeleton in bovine skeletal muscle. This increased release of total protein and of desmin can be noted by comparing values obtained from the one-hour postmortem samples from control and electrically stimulated tissue in Table 5. The increased amounts of desmin and total protein released from electrically stimulated muscle are probably due to an accelerated onset of mechanisms which also function in

non-stimulated tissue. This is corroborated by the observations that 1) the percentage of desmin and amount of total protein (and therefore the amount of desmin) isolated one-hour post mortem from electrically stimulated tissue closely parallel these same values at 24 hours from non-stimulated tissue (Table 5); 2) breakdown products of desmin in one-hour post mortem electrically stimulated tissue are detectable on immunoautoradiograms (Figure 13b, lane B). These degradation products were present in only very small quantities in the one-hour non-stimulated sample (Figure 13b, lane A). Not until 24 hours in the non-stimulated samples (Figure 13b, lane C) was there evidence of desmin degradation that was similar to that observed after one hour in the stimulated samples; 3) the 22,000 dalton polypeptide, that appeared coincidental with the selective release of desmin, was first detected at one-hour post mortem in electrically stimulated tissue (Figure 12, gel H and Figure 13b, lane B). This was not noted in non-stimulated tissue until 24 hours post mortem (Figure 12, gel I and Figure 13b, lane C).

The accelerated release of protein in electrically stimulated tissue was still apparent 24 hours post mortem. The amount of protein extracted from electrically stimulated samples 24 hours post mortem was nearly 130% of that extracted from stimulated muscle after one hour of storage. The percentage of desmin in these extracts, however, had decreased from 13.3% after one hour to 8.8% after 24 hours (Table 5). This can possibly be explained by the earlier onset of desmin degradation in the one-hour and 24 hour stimulated samples (Figure 13d, lanes B and D). The appearance of more breakdown products of desmin in extracts of the electrically stimu-

lated tissue, when considered with the decline in the percentage of desmin over the 24 hour storage interval circumstantially argues that the initial rate of desmin breakdown is greater in electrically stimulated versus non-stimulated tissue. Quantitation of all proteins in the desmin-enriched fractions would be necessary to correlate these observations to an actual decline in amounts of desmin. As assayed, the amounts of desmin extracted from control and electrically stimulated tissue were not significantly different at 24 or 168 hours.

The data obtained from this study clearly indicate that electrical stimulation caused accelerated release of cytoskeletal and other myofibrillar proteins from bovine skeletal muscle. A plausible explanation for these observations exists in the studies done by Gunawardene (1981) who found that proteolytic activity of the endogenous, calcium-activated enzyme was significantly higher in electrically stimulated bovine muscle immediately after stimulation and after six hours storage. This enzyme is known to utilize desmin from several species as a substrate (O'Shea et al., 1979; Figure 11), to degrade troponin-T, troponin-I, tropomyosin and C-protein and to release α-actinin from myofibrils (Dayton et al., 1975). The more active protease in stimulated muscle would be expected to weaken both the myofibrillar and the cytoskeletal structures and, thus, make the cytoskeleton easier to separate from the myofibrils. The rapid digestion of purified desmins by this enzyme in vitro suggests that, if desmin was accessible to the more active protease in situ, degradation of desmin would occur.

The role of other intracellular proteases in the release or degrada-

tion of muscle proteins, particularly in response to electrical stimulation, is virtually unknown. Dutson et al. (1980a) reported a significant increase in the percent free activity of two lysosomal enzymes, β-glucuronidase and cathepsin-C. No data have been published which shows that β-glucuronidase is inside muscle cells; this would be a useful attribute if proteins inside muscle cells are to serve as substrates. Although cathepsin-C is inside muscle cells, there is no evidence that it hydrolyzes any myofibrillar proteins. For these reasons, the role of lysosomal enzymes in accelerated proteolysis after electrical stimulation is unknown and is open to conjecture. If cathepsin-C is considered to be a reliable indicator of the behavior of other lysosomal enzymes in electrically stimulated muscle, it is possible that cathepsins A, B, D, H and L, which are all inside muscle cells and hydrolyze one or more of the myofibrillar proteins, could have a role in accelerated proteolysis. Gunawardene (1981) has shown that the intramuscular pH of electrically stimulated bovine skeletal muscle is 6.4 immediately after stimulation and decreases to 5.75 six hours after stimulation. This pH range, which is consistently lower than that found in non-stimulated muscle, would be a more favorable environment for lysosomal enzyme activity. Cathepsins A, B, H and L all have pH optima that range down from either 6.0 or 6.5.

The contraction bands which form in electrically stimulated muscle by one hour after stimulation (Figure 21) and persist at both 24 hours (Figure 23) and 168 hours (Figure 25) were the most prominent structural change seen when these samples were compared with non-stimulated samples. Contraction band formation and a similar loss of myofibrillar alignment

in electrically stimulated bovine skeletal muscle have been reported by others (Dutson, 1977; Savell et al., 1978a; Will et al., 1980; Sorinmade et al., 1982). Contraction bands obviously contain sarcomeres so drastically shortened that only with close scrutiny can structural elements of the sarcomere be recognized. Contraction bands are bounded by sarcomeres that have been stretched to 4.02 μm (Figure 21A), which is well beyond normal rest length for mammalian muscle. If the 100 Å desmin-containing filaments that are located between myofibrils at the level of the Z line (Lazarides and Granger, 1978; Figure 19) participate in forming a structural cytoskeleton which is attached to Z lines and if these attachments are broken or weakened when sarcomere lengths become highly variable as is the case in electrically stimulated muscle, then the formation of contraction bands could increase the cytoskeletal extractability. Confirmation of this suggestion must await a better understanding of the mechanism of contraction band formation and of the link between the cytoskeleton and the Z line, if one exists.

Based on quantities of desmin isolated from electrically stimulated and non-stimulated muscle at 1, 24 and 168 hours post mortem, ultrastructural analysis of muscle fixed at these same times, and previous research on the effects of electrical stimulation on postmortem muscle, it seems clear that electrical stimulation accelerated the usual mechanisms of protein release in postmortem bovine skeletal muscle. The increased rate of protein released from muscle cells may be due to a combination of accelerated proteolytic activity and physical damage to the fibers.

Intermediate (100 Å) Filament Localization in Postmortem Bovine Skeletal Muscle

Intermediate filaments were located at the level of the Z line in bovine skeletal myofibrils prepared at the three sampling times in each of the three storage conditions by indirect immunofluorescent antibody labelling with monospecific desmin antibodies. The pattern of fluorescence, similar to those reported in the literature (Lazarides and Hubbard, 1976; Campbell et al., 1979; Richardson et al., 1981), did not change due to sampling time or storage temperature. The intensity of the fluorescence was also apparently not altered by increased postmortem storage temperature or time (Figure 16). An attempt to quantitate this qualitative observation by cytophotometry was not successful due to the inability to control factors such as myofibril or sarcomere length, numbers of myofibrils in lateral association and plane of focus along the myofibril axis.

Results from the immunoelectron microscope localization of desmin indicated that the 55,000 dalton protein was present in bovine skeletal muscle in the form of 100 Å filaments between and mostly perpendicular to myofibrils at the level of the Z line (Figures 18 and 19). This observation is corroborated by the results of other immunoelectron microscopic studies of desmin localization in chicken skeletal muscle (Richardson et al., 1981; Tokuyasu et al., 1983). Tokuyasu (1983) has also described, based on anti-desmin labelling and electron microscope observations, the existence of both longitudinally and transversely oriented intermediate filaments between chicken cardiac myofibrils. This dissertation, however, is the first published report of immunoelectron

microscope localization of desmin in bovine skeletal muscle and shows that 100 Å filaments exist between Z lines of adjacent myofibrils and that these filaments can be decorated (labelled) by using immunoelectron microscope techniques.

Amino Acid Analysis

Bovine skeletal muscle desmin has an amino acid profile that closely resembles the profiles obtained by others from porcine skeletal or stomach desmin and chicken gizzard desmin (Table 1). There is little doubt that bovine skeletal muscle desmin is the bovine cell analog to the desmins from the other muscle cells. It is interesting to note, however, that the amino acid composition of bovine skeletal muscle desmin more closely resembles that from porcine skeletal and smooth muscle than it does from chicken gizzard. This trend is most noticeable for proline, glycine and valine. This would seem to indicate that the amino acid composition of mammalian skeletal and smooth muscle desmins are slightly more similar to each other than to avian desmin.

Although the amino acid analysis of the 22,000 dalton peptide (Table 6) must be considered preliminary, differences seem to exist when this peptide is compared with other muscle proteins. The glutamic acid content of the 22,000 dalton peptide is much lower than that of bovine skeletal muscle desmin and rabbit skeletal α-actinin but the proline content is much higher than the other three proteins. Phenylalanine and histidine are present in much greater amounts in the 22,000 dalton peptide, but serine is only slightly higher and aspartic acid and alanine slightly lower than the other three proteins. Based on the

triplicate analysis of one sample of the peptide, it is not possible to tentatively identify the origin of the peptide. If these differences can be confirmed on additional samples, then it seems doubtful that this peptide is derived from desmin, α-actinin or actin.

CONCLUSIONS

The following points summarize the major findings of this dissertation:

1) Quantitation of desmin at 1, 24 and 168 hours from muscle held under control conditions of usual postmortem storage (2°C for 168 hours) showed desmin release from the cytoskeleton to increase between 1 and 24 hours post mortem. Proteolytic breakdown of desmin corresponded to this initial release of protein in control tissue. Continued proteolysis reduced the amounts of soluble desmin isolated from control tissue at 168 hours to less than 50% the amount isolated after 24 hours of postmortem storage.

2) High temperature aging increases the release and breakdown of desmin from the cytoskeleton. The rate of desmin breakdown up to 24 hours post mortem in high temperature (25°C) aged tissue was greater than in control tissue while the rate of desmin breakdown after 24 hours of postmortem storage was not significantly different from that in control tissue during the same time. Quantities of desmin isolated from tissue stored for 24 and 168 hours at 25°C were significantly lower than in control tissue while quantities of total extractable protein increased in relation to the control, indicative of more extensive proteolysis in the high temperature aged tissue.

3) Desmin release from the cytoskeleton was accelerated in electrically stimulated tissue. The amount of desmin released from the cytoskeleton and percentage of total protein comprised by desmin

at one hour was equivalent to respective values in 24 hour control tissue. This increased rate of protein release from muscle cells may be due to a combination of accelerated proteolytic activity and physical damage to the fibers. The amount of desmin extracted 24 or 168 hours post mortem from electrically stimulated tissue was not significantly different from that extracted from control tissue. The accelerated release of myofibrillar protein from electrically stimulated tissue was mitigated by 168 hours of storage.

4) In all three storage regimes the concurrent release and breakdown of desmin corresponded to the appearance of a highly insoluble 22,000 dalton polypeptide. Immunological data did not clarify whether the polypeptide was a breakdown product of desmin or a protein functioning in some other capacity within the cell. The amino acid content of the polypeptide indicates non-identity with desmin, actin or α-actinin.

5) Desmin is present in bovine skeletal muscle in the form of 100 Å filaments which interconnect myofibrils at the level of the Z line. This was proven with indirect immunofluorescence and immunoelectron microscope labelling of bovine skeletal myofibrils with monospecific desmin antibodies. Purified desmin was shown by amino acid analyses to be the bovine cell analog to desmins from other muscle cells.

6) No change from the Z line location of intermediate filaments was noted in immunofluorescently labelled bovine myofibrils prepared

at 1, 24 and 168 hours post mortem from tissue subjected to all three experimental aging conditions. An effort to quantitate fluorescent intensity for possible correlation to quantitative data on soluble desmin proved not to be successful.

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