

1984

The effect of calcium activated protease (CAF) and cathepsin D on bovine muscle myofibrils under varying conditions of pH and temperature

Michael George Zeece
Iowa State University

Follow this and additional works at: <https://lib.dr.iastate.edu/rtd>

 Part of the [Agriculture Commons](#), and the [Food Science Commons](#)

Recommended Citation

Zeece, Michael George, "The effect of calcium activated protease (CAF) and cathepsin D on bovine muscle myofibrils under varying conditions of pH and temperature " (1984). *Retrospective Theses and Dissertations*. 7744.
<https://lib.dr.iastate.edu/rtd/7744>

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.

INFORMATION TO USERS

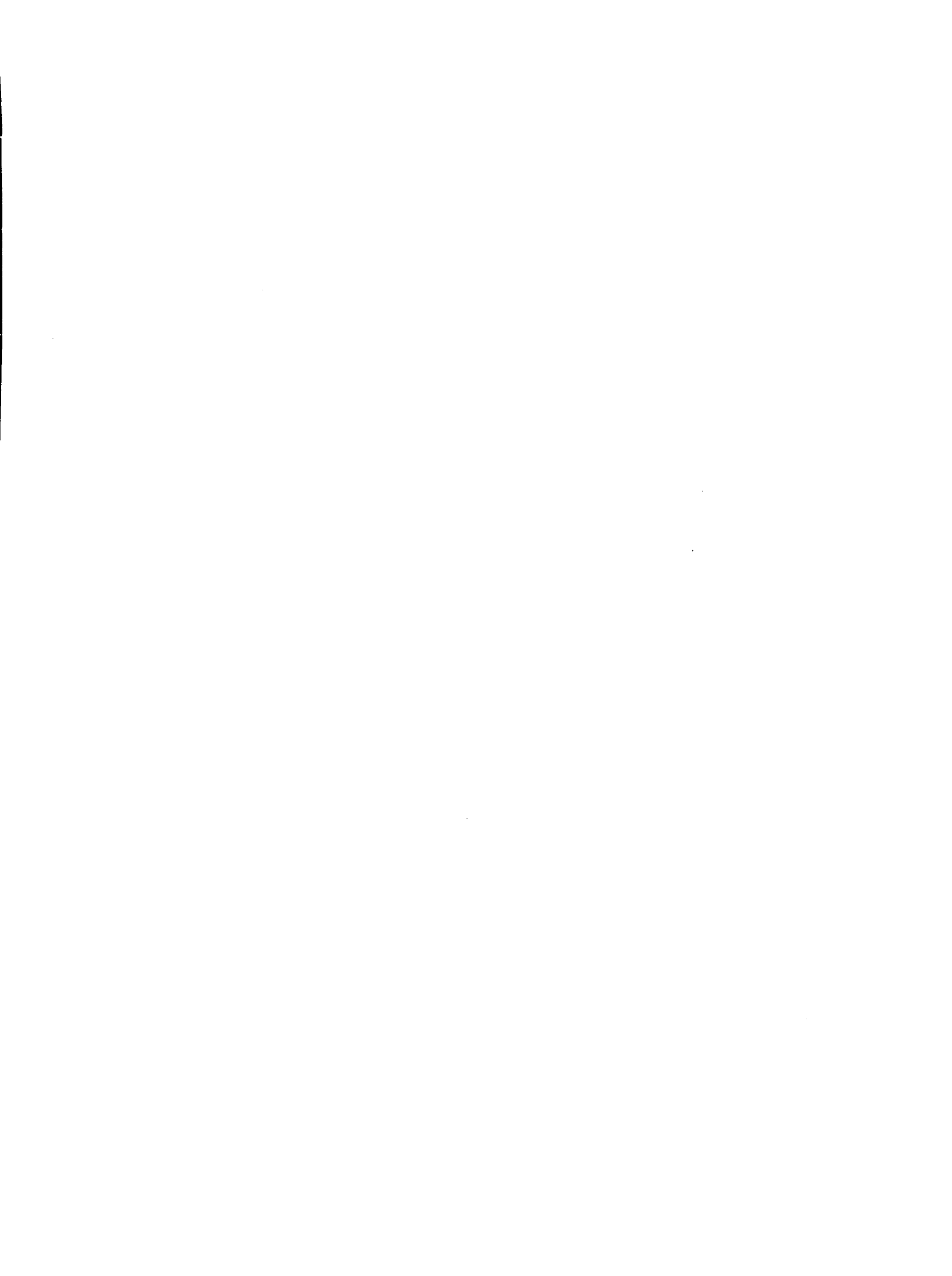
This reproduction was made from a copy of a document sent to us for microfilming. While the most advanced technology has been used to photograph and reproduce this document, the quality of the reproduction is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help clarify markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure complete continuity.
2. When an image on the film is obliterated with a round black mark, it is an indication of either blurred copy because of movement during exposure, duplicate copy, or copyrighted materials that should not have been filmed. For blurred pages, a good image of the page can be found in the adjacent frame. If copyrighted materials were deleted, a target note will appear listing the pages in the adjacent frame.
3. When a map, drawing or chart, etc., is part of the material being photographed, a definite method of "sectioning" the material has been followed. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.
4. For illustrations that cannot be satisfactorily reproduced by xerographic means, photographic prints can be purchased at additional cost and inserted into your xerographic copy. These prints are available upon request from the Dissertations Customer Services Department.
5. Some pages in any document may have indistinct print. In all cases the best available copy has been filmed.

**University
Microfilms
International**

300 N. Zeeb Road
Ann Arbor, MI 48106



8423690

Zeece, Michael George

THE EFFECT OF CALCIUM ACTIVATED PROTEASE (CAF) AND CATHEPSIN
D ON BOVINE MUSCLE MYOFIBRILS UNDER VARYING CONDITIONS OF PH
AND TEMPERATURE

Iowa State University

PH.D. 1984

University
Microfilms
International 300 N. Zeeb Road, Ann Arbor, MI 48106

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy.
Problems encountered with this document have been identified here with a check mark ✓.

1. Glossy photographs or pages ✓
2. Colored illustrations, paper or print _____
3. Photographs with dark background ✓
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages _____
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Other _____

University
Microfilms
International

The effect of calcium activated protease (CAF)
and cathepsin D on bovine muscle myofibrils
under varying conditions of pH and temperature

by

Michael George Zeece

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major: Food Technology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1984

TABLE OF CONTENTS

	<u>Page</u>
LIST OF ABBREVIATIONS	xi
I. INTRODUCTION	1
II. LITERATURE REVIEW	3
A. Skeletal Muscle Structure, Composition, and Proteins	3
1. Skeletal muscle structure	3
2. Composition of muscle	8
3. Proteins of muscle	8
B. Effect of Sarcomere Length, Myofibril Fragmentation, and Other Selected Postmortem Changes on Meat Quality	17
1. Sarcomere length	17
2. Myofibril fragmentation	18
3. Some postmortem changes in muscle	19
C. Proteolytic Enzymes in Muscle	28
1. Proteases of lysosomal origin	30
2. Proteases of nonlysosomal origin other than CAF	39
3. Identification and properties of the protease CAF	40
III. MATERIALS AND METHODS	49
A. Myofibril Preparation	49
B. Extraction and Purification of CAF	49
C. Preparation of Antipain-Sepharose 4B Affinity Matrix Used in the CAF Purification	52
D. Assays of CAF Activity	53
1. CAF activity against casein (tube assay)	53
2. CAF activity by the casein agarose plate method	55
E. Treatment of Myofibrils with CAF	56

	<u>Page</u>
F. Extraction and Purification of Cathepsin D	57
1. Extraction of cathepsin D and preparation of crude enzyme	57
2. Purification of cathepsin D by chromatography	58
3. Preparation of pepstatin-Sepharose 4B for cathepsin D chromatography	59
G. Assays of Cathepsin D on Hemoglobin and on Benzyl-DL-arginine 2-Naphthylamide (BANA) and Benzyloxycarbonylarginylarginine 2-Naphthylamide (Z-arg-arg)	60
1. Hemoglobin assay	60
2. BANA and Z-arg-arg assays	61
H. Treatment of Myofibrils with Cathepsin D	62
I. Fragmentation of Myofibrils Following Protease Digestions	63
J. Electrophoretic Methods	63
1. SDS-polyacrylamide electrophoresis	63
2. Electrophoresis in nondenaturing buffers	64
3. Two-dimensional gel electrophoresis	65
4. Immunoblotting	65
IV. RESULTS	67
A. CAF: Purification and Properties	68
B. The Effect of Purified CAF on Myofibrils	85
1. Protein released from myofibrils by CAF treatment	85
2. SDS-PAGE analysis of myofibril fractions after CAF treatment	90
3. Titin immunoblot of myofibril fractions after CAF treatment	132
C. Cathepsin D: Purification and Properties	158
D. The Effect of Purified Cathepsin D on Myofibrils	167
1. Protein released from myofibrils by cathepsin D treatment	167

	<u>Page</u>
2. SDS-PAGE analysis of myofibril fractions after cathepsin D treatment	172
3. Fragmentation of myofibrils following cathepsin D digestion	198
V. DISCUSSION	211
A. Purification and Characterization of CAF	211
B. Examination of the Effect of Purified CAF on Myofibrils	215
C. Purification and Characterization of Cathepsin D	220
D. Effect of Cathepsin D on Myofibrils	223
VI. SUMMARY AND CONCLUSIONS	227
A. Studies with CAF	227
B. Studies with Cathepsin D	229
VII. BIBLIOGRAPHY	232
VIII. ACKNOWLEDGEMENTS	249

LIST OF FIGURES

	<u>Page</u>
Figure 1. Flow sheet for the preparation of P ₀₋₄₀ crude CAF from bovine cardiac muscle	69
Figure 2. Elution profile of the P ₀₋₄₀ crude CAF fraction from a 5.0 x 45 cm DEAE-cellulose column	71
Figure 3. Detection of CAF activity by the spot assay method	73
Figure 4. Elution profile of the DEAE-cellulose purified CAF from a 2.5 x 40 cm phenyl-Sepharose column	76
Figure 5. Elution profile of the phenyl-Sepharose purified CAF from a 1.0 x 8.0 cm antipain-Sepharose column	79
Figure 6. Polyacrylamide gel electrophoretic examination of purified CAF under nondenaturing and denaturing conditions	81
Figure 7. Two-dimensional PAGE analysis of purified CAF	84
Figure 8. Protein released from myofibrils after incubation with purified CAF at 25° C and pH 7.5, 6.5, or 5.5	87
Figure 9. Protein released from myofibrils after incubation with purified CAF at 15° C and pH 7.5, 6.5, or 5.5	89
Figure 10. Protein released from myofibrils after incubation with purified CAF at 5° C and pH 7.5, 6.5, or 5.5	92
Figure 11. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 25° C and pH 7.5	94
Figure 12. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 25° C and pH 7.0	97

	<u>Page</u>
Figure 13. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 25° C and pH 6.5	100
Figure 14. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 25° C and pH 6.0	103
Figure 15. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 25° C and pH 5.5	106
Figure 16. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 15° C and pH 7.5	109
Figure 17. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 15° C and pH 7.0	111
Figure 18. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 15° C and pH 6.5	114
Figure 19. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 15° C and pH 6.0	116
Figure 20. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 15° C and pH 5.5	119
Figure 21. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 5° C and pH 7.5	122

	<u>Page</u>
Figure 22. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 5° C and pH 7.0	124
Figure 23. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 5° C and pH 6.5	127
Figure 24. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 5° C and pH 6.0	129
Figure 25. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 5° C and pH 5.5	131
Figure 26. Identification of titin and/or titin fragments by an immunoblot procedure	134
Figure 27. Phase contrast micrographs of myofibrils after incubation with purified CAF at 25° C and pH 7.0 and a brief homogenization	137
Figure 28. Phase contrast micrographs of myofibrils after incubation with purified CAF at 25° C and pH 6.0 and a brief homogenization	140
Figure 29. Phase contrast micrographs of myofibrils after incubation with purified CAF at 15° C and pH 7.5 and a brief homogenization	142
Figure 30. Phase contrast micrographs of myofibrils after incubation with purified CAF at 15° C and pH 6.5 and a brief homogenization	145
Figure 31. Phase contrast micrographs of myofibrils after incubation with purified CAF at 15° C and pH 5.5 and a brief homogenization	147
Figure 32. Phase contrast micrographs of myofibrils after incubation with purified CAF at 5° C and pH 7.5 and a brief homogenization	149

	<u>Page</u>
Figure 33. Phase contrast micrographs of myofibrils after incubation with purified CAF at 5° C and pH 6.5 and a brief homogenization	152
Figure 34. Phase contrast micrographs of myofibrils after incubation with purified CAF at 5° C and pH 5.5 and a brief homogenization	154
Figure 35. Summary of degree of myofibril fragmentation after incubation with CAF at selected pH values and temperatures and a brief homogenization	157
Figure 36. Flow sheet for the preparation of crude P ₄₅₋₆₅ cathepsin D from bovine cardiac muscle	159
Figure 37. Elution profile of the crude P ₄₅₋₆₅ cathepsin D fraction from a 2.5 x 30 cm concanavalin A-Sepharose 4B column	161
Figure 38. Elution profile of the concanavalin A-Sepharose 4B purified cathepsin D from a 2.5 x 12 cm pepstatin-Sepharose column	163
Figure 39. Polyacrylamide gel electrophoretic examination of purified cathepsin D under nondenaturing and denaturing conditions	166
Figure 40. Two-dimensional PAGE analysis of purified cathepsin D	169
Figure 41. Protein released from myofibrils after incubation with purified cathepsin D at 37° C and pH 5.5, 6.5, or 7.5	171
Figure 42. Protein released from myofibrils after incubation with purified cathepsin D at 25° C and pH 5.5, 6.5, or 7.5	174
Figure 43. Protein released from myofibrils after incubation with purified cathepsin D at 15° C and pH 5.5, 6.5, or 7.5	176
Figure 44. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified cathepsin D at 37° C and pH 5.5	178

	<u>Page</u>
Figure 45. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified cathepsin D at 37° C and pH 6.5	181
Figure 46. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified cathepsin D at 37° C and pH 7.5	184
Figure 47. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified cathepsin D at 25° C and pH 5.5	186
Figure 48. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified cathepsin D at 25° C and pH 6.5	188
Figure 49. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified cathepsin D at 25° C and pH 7.5	191
Figure 50. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified cathepsin D at 15° C and pH 5.5	193
Figure 51. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified cathepsin D at 15° C and pH 6.5	195
Figure 52. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified cathepsin D at 15° C and pH 7.5	197
Figure 53. Phase contrast micrographs of myofibrils after incubation with purified cathepsin D at 37° C and pH 5.5 or 6.5 and a brief homogenization	200
Figure 54. Phase contrast micrographs of myofibrils after incubation with purified cathepsin D at 37° C and pH 7.5 or at 25° C and pH 5.5 and a brief homogenization	202

	<u>Page</u>
Figure 55. Phase contrast micrographs of myofibrils after incubation with purified cathepsin D at 25° C and pH 6.5 or 7.5 and a brief homogenization	205
Figure 56. Phase contrast micrographs of myofibrils after incubation with purified cathepsin D at 15° C and pH 5.5 or 6.5 and a brief homogenization	207
Figure 57. Summary of degree of myofibril fragmentation after incubation with cathepsin D at selected pH values and temperatures and a brief homogenization	210

LIST OF ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
ATPase	adenosine triphosphatase
BANA	benzyl-DL-arginine 2-naphthylamide
BSA	bovine serum albumin
CAF	calcium activated protease
CANP	calcium activated neutral protease
CBZ	carbobenzoxy
DEAE	diethylaminoethyl
°C	degree centigrade
DFP	disopropylphosphofluoridate
DMSO	dimethyl sulfoxide
DTE	dithioerythritol
DTT	dithiothreitol
EDC	1-ethyl-3 (3-dimethylaminopropyl) carbodimide
EDTA	ethylenediaminetetraacetic acid
EGTA	1,2-bis (2-dicarboxymethylaminoethoxy)-ethane
g	gram
GAR-HRP	goat antirabbit horseradish peroxidase
g_{\max}	relative centrifugal field at maximum tube radius
hr	hour
KAF	kinase activating factor
kg	kilogram
MCE	2-mercaptoethanol

MES	(2 [N-morpholino]) ethanesulfonic acid
Mg	milligram
Min	minute
NEM	n-ethylmaleimide
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
Sec	second
TCA	trichloroacetic acid
Tris	Tris(hydroxymethyl)-aminomethane
v	volts
v/w	volume/weight
w/w	weight/weight
Z-arg-arg	benzyloxycarbonylarginylarginine 2-naphthylamide

I. INTRODUCTION

The postmortem aging of meat, or conditioning as it is sometimes called, results in increased meat tenderness and quality. Many factors such as age, sex, and nutritional state of the animal affect the overall acceptability of the meat. However, the increase in tenderness that is associated with conditioning is probably due primarily to the action of endogenous proteolytic enzymes. Recent studies have identified the cathepsins B, D, H, and L and a calcium activated protease (CAF) as being endogenous to muscle fibers. Because of their putative importance to protein turnover in vivo and to meat quality, much research is being conducted to better understand the action of these proteases on myofibrillar proteins and structure.

Increase in meat tenderness postmortem is assumed by many investigators to be the result of breaking, fragmenting, or at least weakening of the myofibrillar structure, possibly at or near the myofibrillar Z lines. Therefore, the action of a proteolytic enzyme that is effective in postmortem muscle may be to catalyze the hydrolysis of one or more proteins at a structurally important site(s) that results in weakening of the myofibril. Although a number of studies have been done to assess the relative effectiveness of the above mentioned enzymes in their ability to hydrolyze myofibrillar proteins, few studies have taken into account the actual chemical and physical environment which is present in postmortem muscle. The primary objective of this study was to better understand the role of CAF and cathepsin D in disassembly of myofibrils postmortem. The enzymes were examined with regard to their ability to

hydrolyze myofibrillar proteins and to cause changes in the structural integrity of myofibrils under conditions of pH and temperature mimicking those present during postmortem storage and the transition of muscle to meat.

II. LITERATURE REVIEW

A. Skeletal Muscle Structure, Composition, and Proteins

A brief review of skeletal muscle structure and composition is important to an understanding of the action of endogenous proteolytic enzymes on muscle structure and proteins. These enzymes and their effects on the structural integrity of myofibrils are thought to be of importance in the postmortem tenderization process. The following review is based in part on those of others (e.g., Huxley, 1972; Gould, 1973; Goll et al., 1983a,b, 1984; Huxley, 1983; Martonosi, 1983; Robson and Huiatt, 1983; Robson et al., 1984).

1. Skeletal muscle structure

Skeletal muscles and muscle cells (fibers) are held in anatomical register by a series of continuous connective tissue sheaths. The fairly thick sheath which surrounds the whole muscle is called the epimysium. That which anastomoses with the epimysium and then surrounds groups of muscle fibers (muscle bundles) is called the perimysium. Finally, a thin cover or sheath of connective tissue called the endomysium surrounds the individual muscle fiber and is in close association with the cell's membrane.

A skeletal muscle fiber is a long, unbranching cylindrical cell with tapering ends. The cell can extend completely from origin to insertion of the muscle, but rarely does. More commonly, it extends from one end of the muscle bundle to the midregion and terminates, or it originates and terminates in the belly of the muscle. The length

of a muscle fiber can range from about 1 mm to 34 cm in the most extreme cases; however, a more normal range is from 1 to 40 mm with an average length of 20 to 30 mm. The diameter of the muscle fiber also can vary widely with a range of about 10 to 100 μm . These differences in width are dependent upon a number of factors including the location along a cell's length (the ends taper), class of animal (fish > amphibians > reptiles > mammals > birds), exercise (increases with exercise), maturity (increases with age), and physiological type of fiber (red type fibers are usually smaller than white type fibers) (Huxley, 1972; Goll et al., 1984).

The cell membrane of a muscle fiber is often called the sarcolemma. It is approximately 100 nm thick and is composed of two basic parts. The innermost part is called the plasmalemma. It is 7.5 to 10 nm thick and consists of the typical trilaminar membrane structure. It is composed primarily of proteins, phospholipids, and cholesterol. The second part of the membrane is contiguous with the endomysium, but is distinct. It is composed of three morphological regions totaling approximately 70 to 90 nm in thickness and is collectively referred to as the basement membrane. It consists of an outer layer of fine non-collagenous fibrils, a middle layer of collagen fibers, and an inner somewhat amorphous layer. The sarcolemma functions in the active transport of metabolites and nutrients, and conducts electrical impulses which act as the signal for contraction (Huxley, 1972; Goll et al., 1984).

The composition of cellular constituents is uniquely specialized to the muscle fiber and its function. A skeletal muscle fiber is multinucleated, containing 100 to 200 nuclei per cell. This results because the mature cell arises from the fusion of many embryonic cells called myoblasts. The nuclei are ellipsoidal in shape and are located towards the periphery of the cell close to the sarcolemma. The mature cell does not divide mitotically. The muscle fiber is rich in mitochondria (sarcosomes), which are more numerous in red than in white fibers. It also contains the ribosomes needed to carry out the synthesis of proteins. Normal resting muscle contains many glycogen granules. These are the storage form of energy for glycolysis and are important in postmortem anaerobic metabolism. Lysosomes have been identified in muscle, but are very few in number. They may be associated with the sarcoplasmic reticular system, and increase during stress and disease (Bird, 1975; Bird et al., 1980). The cytoplasm (sarcoplasm) of a muscle cell contains many proteins including a large complement of glycolytic enzymes and myoglobin, which is unique to muscle cells and responsible for carrying and storing oxygen and for the characteristic color of red meat (Gould, 1973).

The sarcoplasmic reticulum and associated T system are membrane enclosed tubules that form an extensive network throughout the interior of the muscle cell (Franzini-Armstrong and Porter, 1962; Franzini-Armstrong, 1973). These two systems function in the initiation of contraction and relaxation. The T system is composed of T tubules that function to conduct nerve impulses from the sarcolemma into the center

of the cell. These tubules are oriented perpendicularly to the long axis of the cell and myofibrils. In mammalian skeletal muscle, these invaginations occur at the level of every A band/I band junction (two per sarcomere). The sarcoplasmic reticulum tubules or membranes (L system) run parallel to the long axis of the cell and myofibrils, and abut each side of the T tubule at each A-I junction. As the sarcoplasmic reticulum membranes approach the T tubule, they coalesce to form a large sac (lateral cisternae) adjacent to the T tubule. The sarcoplasmic reticulum functions by binding calcium ions to bring about relaxation (Martonosi, 1983).

Perhaps the most obvious and specialized structures of a skeletal muscle fiber are the myofibrils, which literally pack the inside of the muscle cell. Myofibrils are elongated protein threads, about 1 to 3 μm in diameter, with their long axis parallel to the long axis of the fiber. They extend from one end of the fiber to the other end. They also can branch at Z lines, which evidently is part of the growth process (Huxley, 1972). Myofibrils are the contractile elements of the muscle cell and comprise about 50% of total muscle protein (Huxley, 1983; Goll et al., 1984).

Myofibrils are responsible for giving skeletal muscle its characteristic striated appearance when viewed under the polarized light microscope (Huxley, 1972). Alternating patterns of light and dark regions arise from the arrangement and composition of filaments within the myofibrillar structure. The light region observed with the phase contrast light microscope is weakly birefringent or isotropic and is

termed the I band. Bisecting the I band is a thin dark line called the Z line or Z disk. The dark region of the myofibril observed when viewed with the phase contrast microscope is strongly birefringent or anisotropic and is termed the A band. The center of the A band in rest length myofibrils is bisected by a narrow, less dense zone or region called the H zone. The center of the A band also is bisected by a narrow dense line termed the M line, but this structure is difficult or impossible to see with the light microscope. The repeating unit from one Z line to the next Z line is called a sarcomere. The sarcomere length for normal resting muscle is 2.3 to 2.8 μm and for contracted muscle is between 1.8 and 2.1 μm . It has been shown in electron microscope studies that the myofibrils are composed of interdigitating arrays of thick and thin filaments (Huxley, 1972; Huxley, 1983). The I band region is composed of thin filaments, 6 to 8 nm in diameter and 1.0 μm long. They are attached at one end to the Z line. The other end extends into the A band. The A band region is composed of thick filaments, which are 14 to 16 nm in diameter and 1.5 μm long, plus the thin filaments that run up to the edge of the H zone. The sarcomere length changes during contraction/relaxation, but the lengths of the individual thick and thin filaments do not change (Huxley, 1972; Huxley, 1983).

Page (1968) has identified two dense lines, termed the N lines, in the I band. These lines are located on either side of and parallel to the Z line. The N_1 line (0.05 μm wide) is located approximately 0.1 to 0.2 μm from the Z line. Its position does not change with

sarcomere length. The N_2 line is slightly broader ($0.15 \mu\text{m}$ wide) and is located between the N_1 line and the edge of the A band. The position of the N_2 line varies as the sarcomere length changes. However, it maintains the same proportional distance from the Z and M lines (Franzini-Armstrong, 1970; Locker and Leet, 1976b; Wang, 1981).

2. Composition of muscle

The proximate composition of mature mammalian skeletal muscle is as follows: water, 55-78%; protein, 15-22%; lipid, 2-20%; carbohydrate, 1-2%; nucleic acid (DNA, 25-30 mg/100 g; RNA, 100 mg/100 g); and ash, 1-2% (Goll et al., 1984). The lipid content is the most variable constituent, with increases in its amount being offset by decreases in water content. Other nonprotein nitrogenous compounds are important in meat quality and some of these are listed as follows at their approximate concentrations in living muscle: ATP, 5-15 mM; ADP, 0.5 mM; phosphocreatine, 20 mM; creatine, 4-5 mM; carnosine, 350 mg/100 g; and anserine, 140 mg/100 g (Goll et al., 1984).

3. Proteins of muscle

The protein content of muscle often has been classified on the basis of its solubility in solutions of varying salt content. The sarcoplasmic (myogen) fraction can be defined roughly as those proteins that can be extracted in a short time (about one hour or less) from ground muscle by a buffer with ionic strength of 0.1 or less at neutral pH. This fraction comprises 30 to 35% of the total muscle protein and contains many of the enzymes associated with carbohydrate, lipid, and

amino acid metabolism plus myoglobin (Stromer et al., 1974; Goll et al., 1984). The myofibrillar fraction consists of those proteins extracted at higher ionic strength (above 0.3) and contains many of the proteins that make up the myofibril. It composes 52 to 56% of total muscle protein (Briskey, 1967). The third and final class of muscle proteins is composed of those proteins that are insoluble in neutral aqueous solutions and contains primarily the connective tissue proteins collagen and elastin. This fraction composes 10 to 15% of the total muscle protein and is called the stroma fraction (Briskey, 1967; Goll et al., 1970, 1984). For historical reasons, the classification of muscle proteins on the basis of their salt solubility has been included here. However, strict adherence to this scheme can lead to confusion because many of the myofibrillar proteins are soluble at low ionic strength after extraction and purification. They might better be classified on the basis of their function.

The myofibrillar protein fraction is composed of at least 12 to 14 proteins (Goll et al., 1984). The list may grow to as many as 20 or more if several additional, minor, poorly characterized proteins are shown to be bona fide myofibrillar components in skeletal muscle cells (Robson and Huiatt, 1983). A list of the more significant proteins and their respective approximate content as a percent of total myofibrillar protein is as follows: myosin, 45%; actin, 20%, tropomyosin, 5%; troponin, 5%; titin, 10%; nebulin, 5%; alpha-actinin, 2%; beta-actinin, 1%; C protein, 2%; M protein (165 K protein), 3%; myomesin (185 K protein), <1%; creatine kinase, <1%; desmin, <1%; and others

such as filamin and vinculin at less than 1% each (Grove and Eppenberger, 1983; Robson and Huiatt, 1983; Goll et al., 1984).

Myosin is one of the largest proteins in muscle and composes approximately 45% of the total myofibrillar protein (Robson and Huiatt, 1983; Yates and Greaser, 1983). It is of great importance in its biological function and its role in meat quality. It has a native molecular weight of about 520,000. The natural subunits of myosin consist of two heavy chains of approximately 200,000 to 223,000 daltons each and four light chains of approximately 16,000 to 21,000 daltons each (Stracher, 1969; Weeds and Lowey, 1971; Yates and Greaser, 1983). The molecule has a long, almost completely alpha-helical, rod-like tail and two globular heads, the sites for the enzymatic activity. The molecule is approximately 160 nm in length, 2 nm in diameter in the rod portion, and 6 nm in diameter in the head region (Goll et al., 1984). Myosin is the major constituent of the thick filament of myofibrils, has the ATPase enzymatic activity, and has the ability to bind actin during contraction. Myosin molecules have the ability to self-assemble into thick filaments in vitro. The tails of adjacent molecules aggregate to form the shaft of the thick filament, with the heads bent out to form the cross bridges. With respect to meat quality, myosin is directly related to water holding capacity, emulsion capacity, and overall texture of meat (Hamm, 1982; Lawrie, 1983).

Actin is the second most abundant myofibrillar protein and comprises 20% of the total myofibrillar protein (Greaser et al., 1981; Robson and Huiatt, 1983; Yates and Greaser, 1983). The molecule itself

is referred to as G actin and is composed of a single polypeptide chain of 42,000 daltons (Collins and Elzinga, 1975). In vivo, it forms the core or backbone of the thin filament by polymerization into long, double stranded, helical chains. The polymerized form is called F actin and contains one mole of ADP and one tightly bound calcium per G actin monomer. Polymerization of G actin to F actin in vitro can be accomplished by addition of 0.1 M KCl or 1 mM Mg^{2+} (Laki, 1971). F actin has the ability to bind myosin and to modify the Mg^{2+} -ATPase activity of myosin. In relation to meat quality, actin is very important because the amount of cross links between actin and myosin has pronounced effects on the tenderness, emulsion capacity, water binding capacity, and texture of the meat (Lawrie, 1983).

The tropomyosin molecule is located all along the thin filament and is part of the regulatory system of contraction. The molecule is composed of two subunits of approximately 33,000 daltons each (Cummins and Perry, 1973). These subunits are almost entirely alpha-helical and lie in register in a two-stranded, coiled-coil arrangement (Crick, 1953; Cohen and Szent-Gyorgyi, 1957; Mak et al., 1980).

Troponin and tropomyosin together are responsible for the regulation of contraction via calcium levels. Native troponin has a molecular weight of 69,000 and consists of three subunits. The largest of the three is troponin T, with a molecular weight of 30,500 (37,000 by SDS-PAGE). Troponin T binds to tropomyosin and to troponin C, but only weakly to troponin I and to F actin. Troponin I has a molecular weight of approximately 21,000 (24,000 by SDS-PAGE), binds strongly to

troponin C and to F actin, and weakly to troponin T and to tropomyosin. Troponin C has a molecular weight of approximately 18,500, binds strongly to troponins T and I, but not to actin, and has four binding sites for divalent cations. Two of the sites bind calcium reversibly during contraction/relaxation. One troponin molecule is bound to each tropomyosin molecule in the thin filament. One hypothesis for the control of contraction is called the steric blocking model (Huxley, 1983). In this model, the tropomyosin strand positions itself between actin in the thin filament and the myosin cross bridge when the calcium concentration is below about 10^{-8} M. As the calcium concentration rises in the cell following release from the sarcoplasmic reticulum, the calcium ions are bound by troponin C. Binding of this calcium induces a series of conformational changes in the thin filament and ultimately results in the tropomyosin strand being shifted out of the way, allowing myosin cross bridges to interact with actin in the thin filament (Greaser and Gergely, 1973; Huxley, 1983).

Alpha-actinin has a native molecular weight of 200,000 and is composed of two subunits of 100,000 daltons each (Robson et al., 1970, 1981; Robson and Zeece, 1973; Suzuki et al., 1976). Alpha-actinin is approximately 75% alpha-helical in its secondary structure (Suzuki et al., 1976). The protein is located in the Z line of the myofibril (Schollmeyer et al., 1976). Alpha-actinin, when added to reconstituted actomyosin suspensions in vitro, activates the Mg^{2+} -modified actomyosin ATPase and turbidity responses (Ebashi and Ebashi, 1964; Arakawa et al., 1970). It has been proposed that in vivo alpha-actinin has a structural

role in that it anchors thin filaments from opposing sarcomeres at the Z line (Yamaguchi et al., 1983).

Beta-actinin is a poorly understood myofibrillar protein that comprises less than 1% of the total myofibrillar protein. It has a molecular weight of approximately 70,000 and is composed of two subunits of 37,000 and 34,000 daltons (Maruyama et al., 1977). The protein seemingly is located at the free end of each thin filament (Maruyama and Kimura, 1981).

C protein is a constituent of the thick filament and has a molecular weight of 140,000 (Starr and Offer, 1978). It is localized as stripes or rings around the shaft of the thick filament. There are seven bands, approximately 43 nm apart, of C protein in each half of the thick filament (Craig and Offer, 1976). The molar ratio of C protein to myosin in the thick filament is about 1 to 3. The role of C protein is unclear, but it may give added strength to the thick filament (Starr and Offer, 1978). Interestingly, C protein also will bind to F actin (the only myofibrillar protein that will bind to both actin and myosin) and, thus, perhaps the protein has some function in modifying the interaction between thick and thin filaments (Starr and Offer, 1978; Moos et al., 1978; Moos, 1981).

The M line of thick filaments seemingly is composed of three proteins, namely, M protein (165 K protein), myomesin (185 K protein), and creatine kinase (Trinick and Lowey, 1977; Walliman et al., 1978; Strehler et al., 1980; Grove and Eppenberger, 1983). M protein consists of one subunit and has a molecular weight of about 165,000 (Trinick

and Lowey, 1977). Creatine kinase has a native molecular weight of about 84,000 and consists of two 42,000-dalton subunits (Walliman et al., 1978). Myomesin is a more recently discovered component of the M line structure and has a molecular weight of about 185,000 (Grove and Eppenberger, 1983). When longitudinal sections of muscle are examined by electron microscopy, the M line appears as 3 to 5 lines perpendicular to the long axis of the thick filaments. It has been proposed that the M line functions to hold the thick filaments in their proper three-dimensional register (Trinick and Lowey, 1977).

Titin is one of the more recently discovered myofibrillar proteins. It composes about 10% of the total myofibrillar protein. Titin has a molecular weight, as estimated by SDS-PAGE, of approximately 1,000,000 (Wang et al., 1979). Studies by Maruyama et al. (1981a) indicate that some of the high molecular weight components of the highly insoluble, elastic protein called connectin are identical to titin. Immunofluorescent localization of titin in myofibrils suggests that it is distributed throughout much of the sarcomere, with especially intense staining at the level of the A-I junction (Wang et al., 1979; Wang and Ramirez-Mitchell, 1983; LaSalle et al., 1983). It has been suggested that titin may compose a set of thin, longitudinally running, elastic filaments that extend from Z line to Z line within an intact sarcomere (Wang et al., 1979). Others earlier proposed the existence of a new set of elastic, longitudinally running filaments called gap filaments that link the cores of thick filaments of adjacent sarcomeres through the Z line (Locker and Leet, 1976a,b). It is unclear at present whether

gap filaments (or some type of longitudinally running filaments in the sarcomere) are composed of titin, but recent immunoelectron microscope results suggest this may be so (LaSalle et al., 1983; Robson and Huiatt, 1983; Robson et al., 1984). Titin's importance in meat quality is not known for certain; however, it has been shown that titin disappears coincident with postmortem aging (Lusby et al., 1983) during the time which meat becomes more tender. It also is known that titin is highly susceptible to proteolysis (Wang and Ramirez-Mitchell, 1983; Zeece et al., 1983).

Nebulin is another high molecular weight (about 500,000) protein that was discovered by Dr. Kuan Wang and his associates (Wang et al., 1979; Wang and Williamson, 1980). Nebulin has been localized at the N₂ line (Wang and Williamson, 1980; Ridpath et al., 1982), but the role of the protein in meat quality is not yet clear (Robson et al., 1984). It is known that nebulin is degraded during postmortem aging (Lusby et al., 1983; Robson and Huiatt, 1983). It comprises about 5% of the total myofibrillar protein and it is possible that nebulin may constitute part of a set of longitudinally running filaments in the sarcomere. If the latter is shown to be true, then nebulin may well have a significant role in the postmortem tenderization process.

Desmin is a protein having a molecular weight of approximately 55,000. It was first identified (Schollmeyer et al., 1976) and isolated from smooth muscle in a highly purified state (Huiatt et al., 1980). Subsequently, it was purified from skeletal muscle (O'Shea et al., 1981). Desmin has the ability to self-assemble into long, 10-nm diameter

filaments in vitro (Huiatt et al., 1980). Immunoelectron microscope localization studies suggest that desmin 10-nm filaments link adjacent myofibrils together at their Z line levels. Desmin may be quite important in meat quality because it disappears during the aging process (Robson et al., 1980, 1981, 1984; Kasang, 1983; Robson and Huiatt, 1983).

The stromal proteins make up approximately 10 to 15% of total muscle protein and are insoluble in neutral aqueous solutions (Stromer et al., 1974; Goll et al., 1977, 1984). The proteins collagen, elastin, and reticulin are the major constituents of this fraction. Collagen is by far the major protein of the three and is perhaps the most abundant single protein in mammals (Seifter and Gallup, 1966). Tropocollagen is the basic molecular unit of collagen. It is a rod-like molecule with a molecular weight of about 300,000, a length of 280 to 300 nm, and a diameter of 1.5 nm. It is composed of three polypeptide chains of about 100,000 daltons each that are arranged in a collagen triple helix. Collagen is a very unusual protein in its amino acid composition, with approximately one-third of its residues being hydroxyproline and proline. Tropocollagen is rather easily converted to a soluble form (gelatin) by heating. However, intermolecular cross-linking renders collagen molecules more resistant to denaturation by heating. It has been shown that the amount of intermolecular cross-linking increases with age of the animal. This increase adversely affects meat tenderness. There evidently is little change in collagen during postmortem

aging (Bailey and Sims, 1977; Goll et al., 1964, 1983a; Hill, 1966; Herring et al., 1967).

B. Effect of Sarcomere Length, Myofibril Fragmentation,
and Other Selected Postmortem Changes on Meat Quality

1. Sarcomere length

The factors responsible for determining meat quality are complex. With regard to meat tenderness, one of the primary determinants of meat palatability, it has been suggested by several investigators (Marsh and Leet, 1966; Davey and Gilbert, 1974; Marsh, 1977) that sarcomere length can be used as one of its indicators. It has been shown that only approximately 20% of the cross bridges on the thick filament are actively attached to thin filaments at any one time in vivo (Huxley and Brown, 1967). However, after the energy supply is depleted postmortem, up to 100% of the cross bridges can become firmly attached to the thin filaments and cause the muscle to become inextensible (Huxley, 1968; Goll et al., 1970). Up to a point, the potential number of cross bridges that can be formed postmortem between actin and myosin increases with decrease in sarcomere length. As a result, it often has been assumed that toughness is directly related to the degree of contraction that occurs just before ATP levels are exhausted postmortem (Marsh, 1977; Lawrie, 1980, 1983).

The average resting sarcomere length of vertebrate muscle is about 2.3 to 2.8 μm (Goll et al., 1984). Shortening by 20% only slightly affects tenderness; however, shortening of sarcomeres by 20% to 40% causes a four- to five-fold decrease in tenderness (Behnke et al., 1973;

Marsh and Crase, 1974). Extreme shortening to less than 60% of rest length results in increased tenderness, possibly reflecting damage to myofibril structure at the Z line (Marsh and Crase, 1974). Although sarcomere length has some relationship to meat tenderness, it is generally considered not to be a highly reliable predictor of tenderness (Olson et al., 1976).

2. Myofibril fragmentation

Several investigators have suggested that Z line degradation postmortem may be the cause for much of the increase in meat tenderness that occurs during aging (Stromer et al., 1967; Davey and Gilbert, 1969; Henderson et al., 1970; Penny, 1970). Loss of Z line integrity may result in many break points along the long myofibrils. It has been proposed that the Z line weakening also results in an increase in the amount of fragmentation and decrease in overall length of myofibrils that occurs when myofibrils are prepared from postmortem muscle. Parrish et al. (1973), for instance, observed that myofibrils prepared from tender muscle had shorter myofibril fragments. Olson et al. (1976) found a high correlation coefficient (-0.75) between myofibril fragmentation and Warner-Bratzler shear values for bovine longissimus muscle and suggested that degree of fragmentation was a better indicator of meat tenderness than was sarcomere length.

Further work from this laboratory, using several beef muscles including psoas, longissimus, and semitendinosus that had been stored at 2^oC or 25^oC and sampled at various times postmortem from one to thirteen days, demonstrated that: (1) myofibrils were fragmented

during postmortem aging, (2) fragmentation was muscle and storage condition dependent, and (3) the extent of fragmentation could be quantitatively measured and expressed as a "myofibril fragmentation index" (Olson et al., 1976). Their study showed that the greatest rate of fragmentation occurred from day one to day three at both storage temperatures (2°C and 25°C). Muscle stored at 25°C, however, had a higher myofibril fragmentation index score than muscle stored at 2°C (Olson et al., 1976). The quantitation of myofibril fragmentation index is performed by measuring the absorbance at 540 nm of a 0.5 mg per ml suspension of myofibrils. Olson and Parrish (1977) have reported relatively high correlation coefficients between myofibril fragmentation indexes and both Warner-Bratzler shear values and taste panel tenderness scores conducted on longissimus muscle samples aged for seven days. For instance, they reported correlation coefficients of 0.95 for veal and 0.73 for A maturity beef between myofibril fragmentation index scores and sensory panel tenderness scores. Thus, myofibril fragmentation seems to be a good predictor of tenderness compared to sarcomere length measurements. An exception to the high correlation of fragmentation index and tenderness has been observed (Olson and Parrish, 1977). The psoas muscle, which is very tender, does not have a high fragmentation index value. The reason for this exception is not clear.

3. Some postmortem changes in muscle

It is important in trying to understand the possible role of endogenous proteases in meat quality to be aware of some of the other changes that occur in muscle postmortem. Whether a given proteolytic

enzyme can be active probably is dependent on the environment in the muscle cell postmortem. The cessation of blood supply at death sets in motion a number of profound physical and chemical changes, collectively referred to as the "conversion of muscle to meat." As the flow of blood ceases, the cell's supply of oxygen and nutrients stops, as does its pathway for removal of metabolic by-products (Newbold, 1966; Lawrie, 1983). The most significant by-product is lactic acid, which accumulates in the muscle cell and reduces its pH. In normal resting muscle, glycogen reserves and ATP levels (10 to 15 mM) are high. The rapid depletion of available oxygen stores results in the cell no longer being able to maintain ATP levels by oxidative phosphorylation. Instead, the cell switches to anaerobic glycolysis and uses its glycogen reserves for the manufacture of ATP (Lawrie, 1980). As a result of this conversion, two moles of lactic acid are produced per glucose unit utilized. The cell also tries to keep ATP levels constant by rephosphorylating ADP from phosphocreatine via creatine kinase activity. Consequently, the concentration of phosphocreatine also falls postmortem. As lactic acid accumulates in the cell postmortem, the pH begins to decline. After glycogen and phosphocreatine reserves are exhausted, ATP levels drop rapidly, eventually reaching a level of approximately 20% or less of its initial concentration. The ultimate pH reached is inversely related to the amount of glycogen initially present, but often is about 5.5 (Lawrie, 1983). The rate at which glycolysis occurs postmortem is very temperature dependent. Bovine sternomandibularis

muscle can reach its ultimate pH in less than six hours at 37°C or in 24 hours at 2°C (Newbold, 1966).

Changes in pH and ATP levels have adverse effects on the sarcoplasmic reticulum and its ability to sequester calcium (Goll et al., 1970). The sarcoplasmic reticulum's adenosine triphosphatase enzyme has the ability to remove calcium from the sarcoplasm only so long as there is sufficient ATP and the temperature and pH remain high. Hay et al. (1973) showed that calcium uptake by the sarcoplasmic reticulum was strongly inhibited by low pH (5.6) or low temperature (2°C) and even more so by the combination of low pH and low temperature. Goll et al. (1974) have proposed that limited proteolysis of proteins of the sarcoplasmic reticulum also may account for some of the postmortem loss of this structure's calcium binding ability. They demonstrated that brief tryptic digestion of isolated sarcoplasmic reticulum membranes markedly reduced its calcium binding ability. As the intracellular ionic environment changes postmortem and as the sarcoplasmic reticulum's ability to sequester calcium is impaired, the intracellular calcium concentration is elevated from about 10^{-8} to 10^{-5} M. Some of this elevation in calcium levels postmortem also may result from leakage of calcium from mitochondria (Lawrie, 1980, 1983).

Considerable confusion has existed for many years among agricultural scientists over the definition of rigor mortis (Goll et al., 1970). It is now generally agreed that loss of extensibility is due to a decrease in ATP level postmortem and the formation of firm actin-myosin cross links (Goll et al., 1974). Defining rigor mortis only in

terms of muscle extensibility (Bate-Smith and Bendall, 1949) is insufficient, however, to explain the stiffening or hardening that occurs postmortem. Although loss of extensibility is a necessary condition for maintenance of the stiffening or rigidity developed postmortem, the primary cause of rigor stiffening is the result of postmortem muscle shortening (Goll, 1968; Goll et al., 1970). The attempted shortening by muscles on opposing sides of the same bone produces the stiffness or hardness similar to that observed in an isometric contraction (Goll, 1968). This "shortening-rigidity" concept of rigor mortis has become a useful way to approach and study postmortem changes in muscle (Busch, 1969; Goll et al., 1970).

One can measure the ability of a muscle strip to both develop isometric tension (development of rigor) postmortem and then to gradually lose the ability to maintain isometric tension (resolution of rigor) postmortem (Goll et al., 1970; Goll et al., 1974). This newer working definition of rigor mortis (shortening-rigidity) has enabled investigators to more easily relate structural and biochemical properties of the myofibril to gross events occurring in the muscle cell (Goll et al., 1970; Goll et al., 1974).

It now seems evident that the increase in calcium levels, which occurs in muscle cells postmortem, triggers the muscle's attempt to shorten. In a systematic study of the effect of temperature on isometric tension in bovine, porcine, and rabbit muscle, it was shown that postmortem isometric tension development and decline occurred in all muscles and at all temperatures studied (2° , 16° , 25° , and 37°C)

(Busch et al., 1972a). There was, however, a marked difference in the effect of differing temperatures on the degree of development of tension of bovine muscle strips. Bovine semitendinosus and psoas muscle strips developed maximal isometric tension at 2°C and minimal tension at 16°C. At 37°C, isometric tension was approximately half what it was at 2°C. The longissimus muscles of porcine and rabbit were similar to each other, and developed greatest tension at 37°C and minimal tension at 2° to 16°C (Busch et al., 1972a).

It has been proposed that the ability of bovine or ovine muscle to develop maximal isometric tension at low temperature is the cause for the phenomenon called "cold shortening," which results in very tough meat (Locker, 1960; Marsh and Leet, 1966). Contraction is initiated early postmortem in these muscles when calcium leaks from the sarcoplasmic reticulum and/or the mitochondria in response to lowered temperature. The muscle is in a highly contracted state that permits an increased number of actin-myosin interactions when ATP is depleted. The marked effects of cold shortening on tenderness are not completely reversed by aging, and a considerable loss in meat quality results (Hamm, 1982). The cold shortening effect can be avoided by waiting until the muscle pH has fallen to 6.0 to 6.5 before chilling, or by freezing very rapidly and holding the muscle at -1°C (Davey and Gilbert, 1976). During storage at -1°C, ATP is broken down and the concentration is reduced sufficiently so that a contraction does not occur upon thawing. Electrical stimulation also can be used to

effect rapid glycolysis, with the resultant lowering of pH and ATP concentration (Lawrie, 1980).

There is an eventual resolution of rigor mortis during postmortem storage provided rigor mortis is defined as a loss of ability of the muscle to maintain isometric tension. During this resolution of rigor, there is improvement in meat quality and tenderness. Although the muscle never becomes highly extensible again, there are several changes postmortem in the actin-myosin interaction. For instance, it has been observed that there is a partial lengthening of contracted sarcomeres in postmortem muscle that contains little or no ATP (Stromer et al., 1967; Takahashi et al., 1967). It also has been shown that the ATP concentration required to cause dissociation of post-rigor actomyosin is less than that required for dissociation of at-death actomyosin. Changes also have been observed in the Mg^{2+} -modified (Arakawa et al., 1976), Ca^{2+} -modified, and the Mg^{2+} plus EGTA-modified ATPase activities of myofibrils prepared from muscle stored postmortem (Goll and Robson, 1967).

Some distinct structural alterations have been observed in studies of postmortem muscle. Stromer et al. (1967) and Henderson et al. (1970) observed a progressive slow loss of Z line and M line structures in postmortem muscles. The disappearance or weakening of M line structure was slower than changes in the Z line structure. In contrast, the A and I band regions remained little changed in their ultrastructural appearances in muscle stored for up to 13 days at $16^{\circ}C$ (Stromer et al., 1974). These changes, when coupled with the lack of significant

change in the connective tissue fraction (Goll et al., 1970), suggest the loss of structural integrity of the myofibril is the cause for the resolution of rigor and the increase in meat quality.

In summary of significant changes in myofibrils postmortem, the actin-myosin interaction is altered, the M line and Z line structures are progressively lost or weakened, and there is an increase in the degree of fragmentation of isolated myofibrils. It is often assumed, albeit with little direct evidence, that proteolytic enzymes are responsible for these effects (Goll et al., 1983a). It also is generally assumed that these proteolytic enzymes are endogenous to the muscle cell because it seems unlikely that an enzyme extracellular to the muscle fiber could contribute to myofibrillar alterations and the postmortem tenderization process. It has been shown, for instance, that the sarcolemma is not porous to proteins and is rather resistant to attack by trypsin. Thin muscle strips that had been incubated in trypsin (0.05%, w/v) for 18 hours at 37°C showed no evidence of cellular penetration (Busch et al., 1972a). Proteases of extracellular origin, such as collagenases and elastases that presumably could degrade connective tissue, generally have been considered to be ineffective in postmortem muscle because no significant changes in the connective tissue fraction have been observed (Goll et al., 1970; Etherington, 1981).

Several studies have been done to determine the effect of post-mortem aging conditions on myofibrillar proteins (Locker, 1960; Davey and Gilbert, 1966; Parrish et al., 1973) and in general have shown no large

changes. By SDS-gel electrophoretic analysis, little or no change was observed in integrity of myosin heavy chains of myofibrils prepared from muscle stored at 0° to 4°C (Stromer et al., 1974; Yamamoto et al., 1979). Arakawa et al. (1976) did observe some myosin heavy chain degradation in myofibrils prepared from muscle stored at 40°C.

One of the more consistent changes that occurs in myofibrillar proteins during postmortem storage is the appearance of three closely spaced bands as observed by SDS-gel electrophoresis. These bands have approximate molecular weights of 34,000, 30,000, and 27,000 and have been observed by a number of investigators (e.g., MacBride and Parrish, 1977; Olson and Parrish, 1977; Penny, 1980; Parrish et al., 1981; Goll et al., 1983a). A 30,000-dalton band has been shown to be a CAF digestion product of troponin T (Olson et al., 1977). One or all of the bands appearing at about 30,000 dalton on gels of postmortem myofibrils may originate from troponin T degradation, but this has not been proven unequivocally (Goll et al., 1983a).

Cathepsins B, D, H, and L have been shown to be endogenous to the muscle fiber. These enzymes principally degrade myosin (Schwartz and Bird, 1977; Bird and Carter, 1980). Based upon observations of myofibrillar proteins prepared from muscle stored postmortem, it has been argued that the catheptic enzymes have little effect on resolution of rigor or tenderization (Goll et al., 1983a). These investigators point out that little or no degradation of myosin is observed in muscle held under normal postmortem conditions (Goll et al., 1983a).

Myofibrils treated with CAF closely resemble myofibrils made from muscle stored postmortem in several ways. First, both exhibit loss of Z lines, some subsequent loss of M line structure, but little effect on the A band structure (Dayton et al., 1975, 1976b; Olson et al., 1976). Second, both kinds of myofibrils show little or no change in integrity of actin or myosin polypeptides. CAF does not degrade these proteins (Dayton et al., 1975; Olson et al., 1977). Third, both kinds of myofibrils have peptides in the 30,000-dalton region when examined on SDS gels (Dayton et al., 1975; Olson et al., 1977). Fourth, both kinds of myofibrils show similar changes in the Mg^{2+} -modified and Ca^{2+} -modified ATPase activities (Suzuki and Goll, 1974). And fifth, SDS-gels of both kinds of myofibrils show degradation of the recently discovered proteins desmin and titin (Robson et al., 1980, 1981, 1984; Lusby et al., 1983; Robson and Huiatt, 1983; Zeece et al., 1983). These lines of evidence lend credence to the conclusion that CAF is the major enzyme involved in postmortem alterations in myofibrillar proteins and in tenderization (Goll et al., 1983a).

Nearly all studies done to demonstrate the effect of CAF on myofibrillar structure and proteins have been conducted in vitro under conditions that were at or near optimal for CAF activity. The effectiveness of CAF under conditions resembling more closely those found in the cell postmortem, during the transition from muscle to meat, is shown in the studies described in this dissertation.

C. Proteolytic Enzymes in Muscle

The identification and characterization of proteolytic enzymes in muscle is an area of very active research. Studies of protein turnover in muscle have provided a good deal of information on the basic mechanism of protein degradation and on some of the enzymes involved. Basal protein turnover is that which occurs under normal conditions in contrast to accelerated rates which occur under certain adverse conditions such as starvation and denervation atrophy. In basal protein turnover, a few general correlations have been proposed (Dean, 1980). The rate of turnover increases with an increase in protein subunit size. The lower a protein's pI, the greater is its rate of degradation. Proteins with substantial surface hydrophobicity are degraded more rapidly (Dean, 1980). Rates of basal protein turnover appear to be related to muscle fiber type; in general, cardiac muscle protein turnover is faster than in red type skeletal muscle which in turn is faster than in white type skeletal muscle (Millward, 1980).

Protein turnover involves the cooperative action of many enzymes to bring about the complete conversion of a protein to its constituent amino acids. There are several ways one can classify the enzymes. In muscle, the enzymes can be divided between two groups, lysosomal or nonlysosomal, with respect to their origin. The mechanism of cooperativity between enzymes involved in protein turnover is largely unknown. Much of the research currently in progress is directed at further characterization of known proteases and the search for others (Wildenthal and Crie, 1980).

Proteolytic enzymes also can be classified on the basis of their mode of attack. Exoproteases are those that act on a protein's amino or carboxy terminus by removing one or two amino acids at a time. Those exoproteases that act at the amino end are called aminopeptidases. Those that act at the carboxyl end are called carboxypeptidases. Endoproteases are those enzymes which cleave peptide bonds within the interior region of the polypeptide chain. They can be further classified according to the amino acid residue or cofactor present at their active site. Almost all endoproteases can be classified into four classes, namely, serine, thiol, carboxyl, and metallo enzymes (Barrett and Heath, 1977; Bird and Carter, 1980).

Serine proteases are enzymes that contain a serine residue at their active site and that characteristically have a pH optimum in the alkaline region (pH 7.5 to 9). They are inhibited by serine-reacting compounds such as diisopropyl phosphofluoridate (DFP), phenylmethylsulfonyl fluoride (PMSF), and other compounds of the halomethyl ketone type (Bird and Carter, 1980). They also are inhibited by small peptides such as chymostatin, leupeptin, and antipain that are produced by organisms of the actinomycetes species. Some serine type proteases are inhibited by soybean trypsin inhibitor (Barrett, 1977; Barrett and Heath, 1977; Bird and Carter, 1980).

Thiol proteases are enzymes that contain a sulfhydryl group at their active site and that characteristically have pH optimums within a wide range from pH 4 to 7.5. The sulfhydryl group must be in the reduced state for activity and, therefore, reducing agents such as MCE

or DTE are required for maximal activity. They are inhibited by reagents that react with sulfhydryl groups such as iodoacetic acid or n-ethylmaleimide (NEM). Thiol proteases are generally inhibited by the microbial peptides antipain and leupeptin (Barrett and Heath, 1977).

Carboxyl proteases are enzymes that contain a carboxyl group at their active site and that generally have a pH optimum in the acid region (pH 2 to 5). They can be inhibited by adjusting the pH to 7 to 7.5 or by addition of the microbial peptide pepstatin. Both thiol and carboxyl proteases can be inhibited irreversibly by diazoacetyl compounds (Barrett and Heath, 1977).

Metallo proteases are enzymes that require a metal ion for activity and that characteristically have pH optimums in the alkaline region (pH 7 to 9). As expected, they are inhibited by metal ion chelators such as EDTA. Many also have a requirement for a reducing agent such as MCE or DTT for full activity (Barrett and Heath, 1977).

There also is evidence that many of the proteases are regulated by endogenous inhibitors (Barrett, 1977; Bird and Carter, 1980; Goll et al., 1983a).

1. Proteases of lysosomal origin

Lysosomes and their accompanying complement of degradative enzymes have been demonstrated in all types of muscle cells (Bird, 1975). Their numbers increase during adverse conditions such as starvation or denervation atrophy. Their presence can be demonstrated by using a combination of histochemical or immunohistochemical and biochemical techniques. The histochemical procedures involve the use of dyes such

as acridine orange, which accumulates inside lysosomal vesicles, or the uptake of colloidal gold, which can be used for electron microscope investigations (Wildenthal and Crie, 1980). Recently, antibodies prepared to cathepsin D and coupled with peroxidase have been used in the immunohistochemical localization of lysosomes (Poole, 1977; Decker et al., 1980). Biochemical identification involves the use of differential centrifugation to demonstrate the presence of lysosomes. The procedure involves subcellular fractionation by centrifugation followed by demonstration of latency for marker enzymes such as cathepsin D and aryl sulfatase in order to confirm the presence of lysosomes (Wildenthal and Crie, 1980).

The lysosomal class of proteases is composed of many enzymes and is thought to contribute significantly to protein turnover in vivo (Dean, 1980). Some investigators feel that lysosomal proteases also are involved in the postmortem increase that occurs in meat quality (Dutson, 1983). This class of enzymes is composed of both exo and endo proteases that act cooperatively in protein degradation (Bird and Carter, 1980). Thus, lysosomal exopeptidases (exoproteases) may be of importance to meat quality. These include dipeptidyl-peptidases I-IV, glycyllucine dipeptidase, carboxypeptidase A, and carboxypeptidase B (Goll et al., 1983a). Recent studies have shown that both dipeptidyl-peptidase I and carboxypeptidase A may act synergistically with cathepsin D in the degradation of actin and myosin (Bird and Carter, 1980).

Lysosomal endoproteases and their actions on muscle proteins have been the subject of many investigations. However, many of these studies

have been flawed because of the use of "partially purified" enzyme preparations and the lack of proper controls (reviewed in Goll et al., 1983a). There are many proteolytic enzymes in a muscle homogenate or extract. Thus, there is the possibility of ascribing an effect to the enzyme of interest when it was produced in part or in whole by a contaminating protease. In the following, the term cathepsin will be used to refer to a lysosomally derived enzyme from animal cells, which is primarily an endoprotease, without restriction to an acid pH optimum (Barrett and Heath, 1977).

In older literature, cathepsin B was referred to as cathepsin B₁ to distinguish it from the exopeptidase with the same letter designation (Barrett, 1972). Recently, it has become agreed that the exopeptidase will be called carboxypeptidase B and the endoprotease will be referred to simply as cathepsin B (McDonald and Ellis, 1975). Cathepsin B is a thiol protease with a molecular weight of 25,000 and a pI in the region of about pH 5.0. Multiple forms of the enzyme have been observed by isoelectric focusing or by ion exchange chromatography (Barrett, 1977). The best synthetic substrates for cathepsin B are benzyl-DL-arginine 2-naphthylamide (BANA) (Barrett, 1976) and the more sensitive benzyloxycarbonylarginylarginine 2-naphthylamide (Z-arg-arg) (McDonald and Ellis, 1975). With most synthetic substrates, cathepsin B shows maximal activity at pH 6.0. However, much lower pH optima have been observed for some protein substrates. A reducing agent such as DTT or MCE is required for maximal activity, and EDTA also is included because some divalent cations (e.g., Mg²⁺) have an inhibitory effect on activity.

The thiol group at the enzyme's active site is inhibited by heavy metal ions (Hg) and their derivatives such as 4-chloromercuribenzoate (Barrett and Heath, 1977). Sulfhydryl reagents such as iodoacetic acid and NEM also will inhibit the enzyme. Egg white ovalbumin and the microbially derived peptide leupeptin (but not antipain) are potent inhibitors of its activity (Bird and Carter, 1980). Recently, an endogenous inhibitor of cathepsin B activity has been identified in rat muscle extracts. The effectiveness of this inhibitor decreases with time when the extract is incubated at pH 4.5 and 25°C (Bird and Schwartz, 1977). This endogenous inhibitor of cathepsin B was effective in the pH range of 4.5 to 7.8 and was heat stable at 60°C for 20 minutes. It also inhibited the enzyme against its synthetic substrates, BANA or Z arg-arg. The inhibitor had no effect on cathepsin D and also was found in skeletal and cardiac muscle of bovine, rabbit, and human muscle (Bird et al., 1978).

Cathepsin B selectively degrades the myofibrillar proteins myosin and actin. Purified native myosin is most rapidly cleaved at pH 5.2 and yields a 150,000-dalton fragment, which is resistant to further attack, and a number of fragments in the 50,000- to 10,000-dalton range. Purified skeletal muscle F actin is degraded by cathepsin B at an optimum pH of 5.0 and yields a major fragment of 35,000 daltons, which is resistant to further digestion (Schwartz and Bird, 1977).

Treatment of myofibrils with cathepsin B at pH 6.0 in the presence of EDTA has been shown to remove the Z line and M line structures (Noda et al., 1981). These investigators also reported the degradation of

troponin-T and tropomyosin by the enzyme at pH 6.0. Dutson (1983) reported that the enzyme exhibits a broad range of pH (4 to 6.5) in which it acts on myofibrillar proteins. Dutson (1983) has suggested that cathepsin B has a significant role in meat quality, but this view is not shared by all (Goll et al., 1983a).

Cathepsin D is perhaps the most thoroughly investigated of the lysosomal enzymes. It is a carboxyl protease and has a molecular weight of approximately 43,000 in mammalian muscle (Barrett and Heath, 1977). It has been reported that cathepsin D isolated from spleen is synthesized in a zymogen form (called cathepsinogen D) with a molecular weight of approximately 100,000 and that it is later converted to its active form (Lah and Turk, 1982). Cathepsin D isolated from bovine or human muscle has a molecular weight of about 42,000, but also shows two bands corresponding to proteins of about 28,000 and 14,000 daltons by SDS-PAGE (Barrett and Heath, 1977). However, the enzyme isolated from rabbit, pig, or rat muscle seemingly consists of a single 42,000-dalton chain (Barrett and Heath, 1977). It is uncertain at this time whether the 14,000 and 28,000 molecular weight components are naturally occurring or the result of autolysis. Cathepsin D has a pI of about 5 to 6 and contains multiple forms when examined by isoelectric focusing. Cathepsin D purified from rat skeletal muscle, for instance, contained three major forms on isoelectric focusing gels (Barrett and Heath, 1977).

Cathepsin D is a glycoprotein and contains residues of N-acetylglucosamine and mannose. The enzyme has a specificity for bonds involving residues with hydrophobic and preferably aromatic character (Barrett

and Heath, 1977). Denatured hemoglobin is very susceptible to hydrolysis by cathepsin D at pH 3.0 and is routinely used as a substrate for the determination of cathepsin D activity (Canonica and Bird, 1970).

Cathepsin D is inhibited by a variety of diazo compounds (e.g., diazoacetylnorleucine ester) and by a peptide of microbial origin called pepstatin. The ability of pepstatin to inhibit cathepsin D is pH dependent. The inhibition is maximal at pH 3.0 and decreases with increasing pH. Above pH 6.5, pepstatin is a very poor inhibitor because the enzyme undergoes a conformational change that reduces its ability to bind to the peptide (Barrett, 1977).

Cathepsins D and B have been localized in muscle cell lysosomes by the use of a cytochemical technique (Bird and Carter, 1980). The sub-cellular sites of proteolytic activity were observed by the deposition of an insoluble azo-dye metal chelate. Cathepsins B and D have been shown to hydrolyze CBZ-Ala-Arg-Arg 4-methoxy 2-naphthylamine and CBZ-Arg-Gly-Phe-Phe-Leu 4-methoxy 2-naphthylamine, respectively (Bird and Carter, 1980). The released 4-methoxy 2-naphthylamine is coupled with hexazotized pararosaniline, resulting in a dye product with a high degree of osmiophilia. This procedure permitted the demonstration of two types of lysosomes in muscle cells. The first type was found in the perinuclear region in the vicinity of the Golgi. The second type was found in the interior of the myofibril (Bird and Carter, 1980).

It has been shown that cathepsin D maximally degraded purified native myosin and actin at a pH of 4.0 (Schwartz and Bird, 1977). Myosin degraded by cathepsin D activity initially showed heavy chain

fragments in the 175,000- to 150,000-dalton region. These large fragments were then further degraded to many fragments of molecular weight less than 100,000 (Schwartz and Bird, 1977). Each of three isoelectric focusing isomers of cathepsin D produced the same reaction products when incubated with myosin and actin (Schwartz and Bird, 1977). These investigators also found that incubation of both cathepsin D and B showed no synergism. When each enzyme was incubated with the reaction products of the other, however, it was found that cathepsin D degraded a 150,000-dalton fragment obtained by digestion with cathepsin B. However, cathepsin B had no additional activity on the products resulting from cathepsin D digestion. It was shown that cathepsin D had a lower pH optimum (pH 4.0) with native myosin as substrate than did cathepsin B, plus a broad tail of activity up to about pH 6.5 (Schwartz and Bird, 1977).

Schwartz and Bird (1977) found that cathepsin D degraded F actin optimally at pH 4.0. Its rate of hydrolysis of actin was only about 10% of that observed with myosin. In general, it was concluded that cathepsin D was more effective in degrading myosin and actin than was cathepsin B (Schwartz and Bird, 1977). Other studies (Ogunro et al., 1979) have shown that cathepsin D isolated from cardiac muscle had similar activities toward myosin and actin as did the skeletal muscle enzyme except that the light chains of myosin also were degraded by the cardiac enzyme. Recently, it was shown that addition of cathepsin D to purified muscle proteins resulted in degradation of myosin heavy chains, tropomyosin, troponin T, troponin I, and alpha-actinin

(Matsumoto et al., 1983). It did not, however, degrade actin in their study. It also has been reported (Scott and Pearson, 1978) that cathepsin D degrades soluble collagen, or its cross linked peptides, to a limited degree.

Cathepsin H is a lysosomal protease that has both exo and endo-protease activities. The exoprotease is an aminopeptidase and releases the residues leucine or arginine (Okitani et al., 1980). The endo-protease is a thiol protease with a pH optimum in the range of 5.5 to 6.5. Cathepsin H has a pI of 7.1, a molecular weight of 28,000, and is very heat stable (Schwartz and Barrett, 1980). These investigators have shown that the enzyme loses only 10 to 20% of its activity when incubated at 50°C for 30 minutes. Cathepsin H is a glycoprotein (Schwartz and Barrett, 1980). The myofibrillar proteins myosin and actin are degraded by cathepsin H and it has been estimated that it is five times faster at degrading myosin than is cathepsin B (Bird and Carter, 1980). Cathepsin H is not inhibited by the peptide antipain and is only partially inhibited by leupeptin (Okitani et al., 1981).

Cathepsin L is a lysosomal endoprotease and belongs to the thiol class of enzymes. It has a molecular weight of 24,000 and a pI in the region of pH 5.8 to 6.1. Cathepsin L is very labile to autolysis, and has optimum activity in the range of pH 3.0 to 6.5 (Matsukura et al., 1981). Studies with purified cathepsin L have shown that it has very little activity on small synthetic substrates compared to cathepsins B and H. However, it is much more potent at degrading myofibrillar proteins (Bird and Carter, 1980). It has been estimated that cathepsin L

is ten times faster at degrading myosin and actin than is cathepsin B (Bird and Carter, 1980). Cathepsin L also has been shown to degrade collagen at a faster rate than does cathepsin N (Kirschke et al., 1982). Cathepsin L is only weakly inhibited by the microbial peptide leupeptin. However, a plasma protein has been identified which is an effective inhibitor of the enzyme (Pagano and Engler, 1982).

Cathepsin L degrades myosin heavy chains at pH 5.0 and initially produces a major fragment of 160,000 daltons (Matsukura et al., 1981). This fragment is subsequently degraded to 92,000-, 83,000-, and 60,000-dalton fragments. Cathepsin L digestion of actin initially produces fragments of 40,000 and 30,000 daltons with an additional band at 37,000 daltons appearing later (Matsukura et al., 1981). These investigators also have shown that tropomyosin is not appreciably degraded at pH 5.0; however, troponin T and troponin I are degraded after a short time. The myofibrillar protein alpha-actinin is not attacked by other known proteases endogenous to muscle cells. However, cathepsin L does degrade this protein. The rate of hydrolysis of alpha-actinin was approximately equal to that of actin and produced a major fragment of 80,000 daltons when the incubation was done at pH 5.0 (Matsukura et al., 1981). The pH optimum for the degradation of alpha-actinin was pH 3.0 to 3.5. At this low pH, cathepsin L produced many lower molecular weight fragments from alpha-actinin (Matsukura et al., 1981).

Cathepsin N is a thiol type endoprotease with a pH optimum in the region of 3.0 to 4.5. It has a molecular weight of 20,000 and a pI of 6.4. It degrades collagen with an acid pH optimum (Evans and Etherington,

1978). Cathepsin N has not been identified inside skeletal muscle cells (Goll et al., 1983a).

2. Proteases of nonlysosomal origin other than CAF

Muscle tissue contains a number of alkaline endoproteases that have been identified and characterized (Goll et al., 1983a). Most of these enzymes are of the serine type with the exception of one, myosin light chain proteinase which is a thiol enzyme (Bhan et al., 1978). These enzymes are generally very active against muscle proteins. Recent studies, however, indicate that they are not endogenous to the muscle cell, but rather originate in mast cells (Park et al., 1973; Woodbury et al., 1978; Goll et al., 1983a). Mast cells appear in significant numbers in the muscles of rat or mouse, sources often used for the preparation of alkaline proteases. It has been difficult to demonstrate the presence of alkaline protease activity in normal rabbit, pig, bovine, and human muscle samples (Bird and Carter, 1980). Additional evidence for the mast cell origin of these alkaline proteases has been provided by the use of the compound "48/80." This compound is a polymer of N-methylhomanisylamine and formaldehyde and causes degranulation of mast cells. After treatment of rat muscle homogenate with "48/80," alkaline protease activity is lost (Bird and Carter, 1980). Because alkaline proteases are located outside of muscle cells and because of their alkaline pH optimums, it is thought that they have little or no effect on meat quality (Goll et al., 1983a).

3. Identification and properties of the protease CAF

The most significant nonlysosomal protease in muscle is CAF (Goll et al., 1983a). It was first recognized as a factor which caused the activation of phosphorylase b kinase when muscle extracts were incubated with the factor in the presence of calcium (Krebs et al., 1964). The kinase activating factor (referred to as KAF) was later recognized as a protease and for being responsible for liberating ^{32}P peptides from labeled phosphorylase b kinase (Huston and Krebs, 1968). Other studies confirmed that KAF was a proteolytic enzyme and that it could be isolated from brain and myocardium as well as skeletal muscle tissue (Drummond and Duncan, 1966).

CAF was rediscovered when it was shown that very thin muscle strips incubated in the presence of 1 mM calcium at pH 7.1 lost their Z lines (Busch et al., 1972b). An enzyme, which had the ability to remove Z lines from myofibrils in the presence of calcium and MCE, subsequently was isolated from skeletal muscle and purified to a very homogenous state (Dayton et al., 1974, 1975, 1976a,b). The enzyme can degrade the myofibrillar proteins troponin T, troponin I, tropomyosin, and C protein, and also can release nondegraded alpha-actinin from myofibrils (Dayton et al., 1975). This enzyme was shown to be identical to the kinase activating factor (KAF) by Dayton and co-workers (1976a,b). There has been some confusion in the literature because calcium activated factor or protease has been named differently by different scientists, and the following terms are evidently used to refer to the same enzyme: (1) kinase activating factor (KAF) by Krebs et al. (1964),

(2) calcium activated factor (CAF) by Dayton et al. (1975), (3) calcium activated neutral protease (CANP) by Toyo-oka et al. (1979), and (4) calpain by Murachi et al. (1981).

Differential centrifugation studies demonstrated that CAF is either free in the muscle cell cytoplasm or adsorbed to myofibrils and not located within membrane enclosed vesicles such as lysosomes (Reville et al., 1976). Recent immunohistochemical studies have indicated that CAF is located inside the muscle cell at the level of the Z line (Ishiura et al., 1980; Dayton and Schollmeyer, 1981; Goll et al., 1983b) and adjacent to the cytoplasmic side of the cell membrane (Dayton and Schollmeyer, 1981). There is one report (Barth and Elce, 1981), however, that suggests some of the protease may be located outside the muscle cell.

CAF has been isolated from a number of nonmuscle sources including brain, kidney, and blood platelets, and from skeletal, cardiac, and smooth muscle (Phillips and Jakabova, 1977; Suzuki et al., 1979; Kishimoto et al., 1981). In most studies, it has been shown that the purified enzyme is composed of two subunits of 80,000 and 30,000 daltons (Goll et al., 1983b). The molecular weight of the skeletal muscle enzyme on gel exclusion chromatography is estimated to be 110,000 (Dayton et al., 1976b). However, several investigators have isolated an active enzyme supposedly containing only the 80,000-dalton component (Ishiura et al., 1978; Toyo-Oka et al., 1978; Azanza et al., 1979). Thus, the 80,000-dalton subunit evidently contains the active site of the enzyme. It now appears likely that all CAF enzymes contain two

subunits, and that reports indicating that CAF contains only one subunit have been conducted on autolyzed CAF (Goll et al., 1983b). CAF is a thiol type of proteolytic enzyme and also requires the divalent cation calcium for activation. Other divalent cations such as magnesium produce little or no activation, except for manganese which produces considerable activation (Edmunds et al., 1984). As expected, CAF is inhibited by divalent metal chelators such as EDTA or by compounds such as iodoacetic acid or NEM that react with the sulfhydryl group. When assayed with casein, CAF has a pH optimum in the region of 7.0 to 7.5 and a range of 6.5 to 8.0 (Dayton et al., 1976b).

The CAF enzyme isolated from pig skeletal muscle by Dayton et al. (1975, 1976a,b) requires millimolar concentrations of calcium for activation. Recent studies have indicated that there is another form of the enzyme in muscle that only requires micromolar concentrations of calcium for activation (Mellgren, 1980; Dayton et al., 1981; Spzacenko et al., 1981) and it is often referred to as low-Ca²⁺ CAF. Similarly, the millimolar calcium requiring form is now often referred to as high-Ca²⁺ CAF. These two CAF enzymes can be separated by ion exchange chromatography or by electrophoresis conducted under non-denaturing conditions (Goll et al., 1983b). During DEAE-cellulose chromatography at pH 7.5, low-Ca²⁺ CAF is eluted from the column with 105 to 145 mM KCl and high-Ca²⁺ CAF is eluted with 239 to 280 mM KCl, indicating that high-Ca²⁺ CAF has a higher net negative charge (Nagainis et al., 1983). Both forms of CAF elute from hydrophobic chromatography (e.g., phenyl Sepharose 4B) and gel permeation chromatography columns

identically (Goll et al., 1983b). During electrophoresis in non-denaturing conditions, low-Ca²⁺ CAF has a slower mobility than does high-Ca²⁺ CAF, also suggesting that low-Ca²⁺ CAF has a lower net negative charge. Both forms of the enzyme have two subunits of approximately 80,000 and 30,000 daltons and appear to be identical in their action on myofibrillar proteins (Goll et al., 1983b). The 80,000-dalton subunit of low-Ca²⁺ CAF cross-reacts with antibodies to high-Ca²⁺ CAF, and both forms of the enzyme are inhibited by endogenous CAF inhibitor (Goll et al., 1983b). The exact relationship between low-Ca²⁺ CAF and high-Ca²⁺ CAF remains unknown, but obviously they are extremely similar in many properties (Goll et al., 1983b). There is one report (Wheelock, 1982) that the 80,000-dalton subunits of these two forms of CAF have different peptide maps, suggesting the two forms may be products of different genes.

The activity profile for high-Ca²⁺ CAF shows it to be half maximally activated at 1.0 mM Ca²⁺ and to quickly rise to 100% activity as the Ca²⁺ concentration rises from 1.0 to 5.0 mM. The activity profile for low-Ca²⁺ CAF shows it to be half maximally activated at 10 μ M Ca²⁺ and to quickly rise to 100% activity in the 150 to 250 μ M range. It then remains at its maximum activity throughout the range of 250 μ M to 5.0 mM Ca²⁺ (Edmunds et al., 1984). Few tissues (e.g., gizzard smooth muscle, bovine brain, and kidney tissue) contain much of the low-Ca²⁺ form of CAF. Interestingly, bovine skeletal muscle is the highest source of low-Ca²⁺ CAF found so far (Goll et al., 1983b).

There are two additional forms of CAF that have been studied in vitro. Both high-Ca²⁺ CAF and low-Ca²⁺ CAF can undergo limited autolysis to form enzymes that require lower amounts of calcium for activation (Suzuki et al., 1981a,b; Goll et al., 1983b). Autolysis of high-Ca²⁺ CAF for two minutes at 0°C in 0.5 mM Ca²⁺ reduces the enzyme's requirement for Ca²⁺ for half maximal activity from 1 mM to 0.2 to 0.3 mM (values are for bovine skeletal muscle CAF; Edmunds et al., 1984). Whereas high-Ca²⁺ CAF has subunits of 80,000 and 28,000 daltons as determined by Laemmli gels, the autolyzed high-Ca²⁺ CAF has subunits of 78,000 (from the 80,000-dalton subunit) and 18,000 (from the 28,000-dalton subunit) daltons (Edmunds et al., 1984).

Autolysis of the naturally occurring low-Ca²⁺ CAF also occurs in vitro, which lowers the enzyme's requirement for calcium for half maximal activity from 5 μ M to 0.5 μ M. During this autolysis, the 80,000-dalton subunit is degraded to a 76,000-dalton component and the 28,000-dalton subunit is converted to an 18,000-dalton component (Edmunds et al., 1984). Because autolysis of high-Ca²⁺ CAF and low-Ca²⁺ CAF occurs during incubation assays in vitro, the products resulting from CAF digestion largely are from the autolyzed forms of the protease (Goll et al., 1983a,b).

In experiments designed to examine the effect of highly purified CAF (high-Ca²⁺ form) on individually purified myofibrillar proteins, the enzyme selectively degraded troponin T, troponin I, tropomyosin, and C-protein (Dayton et al., 1975). Troponin T (37,000 daltons by SDS-PAGE) is the most sensitive of these four proteins to degradation,

and initially was degraded to fragments of 34,000, 30,000, and 27,000 daltons. These were then subsequently degraded further to 14,000-dalton components. Purified tropomyosin also is quickly degraded with cleavages occurring in both alpha and beta subunits. The fragments have molecular weights of 17,000 and 16,000. Troponin I was the most slowly degraded of this group of four proteins and eventually was degraded to a fragment of about 14,000 daltons (Dayton et al., 1975; Olson et al., 1977). Skeletal muscle C protein is degraded from a subunit molecular weight of 135,000 to about 120,000 (Dayton et al., 1975).

When CAF has been incubated with skeletal muscle myosin, most reports have indicated that there is no effect on the heavy chain or on the light chains (Dayton et al., 1975, 1976b). An exception has been reported in which it was shown that the heavy chain of skeletal myosin is degraded to a 190,000-dalton species if light chain 2 is in the nonphosphorylated state (Pemrick et al., 1980). Additionally, it has been reported that the 20,000-dalton light chain of turkey gizzard smooth muscle myosin is degraded to a 17,000-dalton component (Goll et al., 1983b).

CAF has no effect on skeletal muscle actin, troponin C, and alpha-actinin (Dayton et al., 1975). It is interesting, however, that when CAF is incubated with purified myofibrils, alpha-actinin is released from the Z line in a nondegraded state (Dayton et al., 1975).

With regard to CAF's effect on other, more recently discovered myofibrillar/cytoskeletal proteins, CAF probably degrades filamin, a minor component at the periphery of the Z line, because gizzard filamin

(250,000-dalton subunits) is degraded to fragments of 240,000 and 9,500 daltons (Davies et al., 1978). The protein desmin, which evidently ties adjacent myofibrils together at their Z line levels, is rapidly degraded by CAF from a 55,000-dalton subunit to fragments of 32,000 and 18,000 daltons (O'Shea et al., 1979). The protein titin, which may be an integral part of a third set of longitudinal filaments in the sarcomere (LaSalle et al., 1983), is very susceptible to degradation by CAF even under conditions of low temperature and low pH (Zeece et al., 1983). The recently discovered protein nebulin, which is associated somehow with the N₂ lines of skeletal muscle, also is rapidly degraded by CAF (Maruyama et al., 1981a).

Not long after CAF was characterized (Dayton et al., 1975, 1976a,b), an inhibitor of this enzyme also was isolated from striated muscle (Okitani et al., 1976; Waxman, 1978; Waxman and Krebs, 1978; Otsuka and Goll, 1980). Its molecular weight as determined by gel exclusion chromatography in nondenaturing solvents was estimated to be 270,000 (Waxman and Krebs, 1978). The subunit molecular weights of bovine skeletal and cardiac muscle inhibitor have been estimated on SDS-PAGE to be about 125,000 and 100,000, respectively (Goll et al., 1983b; Shannon and Goll, 1984). Otsuka and Goll (1980) found that the inhibitor had no tryptophan and consequently a very low absorbance at 278 nm. Circular dichroism spectra indicate that it has little or no alpha helix and that it is largely in a random coil conformation. CAF inhibitor does not inhibit trypsin, chymotrypsin, subtilisin, thermolysin,

plasmin, thrombin, ficin, bromelain, or papain (Waxman and Krebs, 1978; Shannon and Goll, 1984).

Studies on its mechanism of action indicate that CAF inhibitor does not function by chelating available calcium (Okitani et al., 1976). The inhibitor will inhibit both high- and low-calcium requiring forms of CAF, as well as the two autolyzed forms of the enzyme (Shannon and Goll, 1984). There appears to be an excess amount of inhibitor present in skeletal muscle in comparison to the amount of CAF, and it has been estimated that one molecule of inhibitor will bind and inactivate 6 to 7 molecules of CAF (Goll et al., 1983b; Shannon and Goll, 1984). When CAF is present in amounts above what can be inhibited by CAF inhibitor, the inhibitor is rapidly degraded to smaller fragments by the CAF (Shannon and Goll, 1984). These fragments of CAF inhibitor, however, also have some inhibitory activity (Goll et al., 1983b). Recent studies using immunohistochemical methods have shown that the inhibitor is inside the muscle cell and located at the level of the Z line (Goll et al., 1983b). It is interesting that both CAF and CAF inhibitor are located at the Z line. A paradoxical situation is presented because CAF inhibitor requires calcium to bind CAF (Otsuka and Goll, 1980). At the same time, calcium also is required for the activation of CAF (Cottin et al., 1981; Goll et al., 1983b) so it would seem that CAF inhibitor would bind to CAF and inactivate it. Consequently, for CAF ever to be active in the presence of CAF inhibitor, the CAF/CAF inhibitor interaction must be regulated by some unknown mechanism.

Just how active CAF inhibitor is in muscle cells postmortem and how the postmortem environment affects the CAF/CAF inhibitor interaction are not known.

III. MATERIALS AND METHODS

All preparations were done at 0-4°C unless otherwise stated. Solutions were prepared using double-deionized, distilled water that had been redistilled in glass and stored in polyethylene containers. All reagents were analytical grade or better. Adjustment of pH was done at the temperature at which the buffer or solution was used.

A. Myofibril Preparation

A small portion of bovine longissimus muscle (approximately 100 g) was removed from market weight animals within 1 hr after exsanguination and transported in ice to the Food Technology Laboratory. The muscle was trimmed free of excess fat and connective tissue and passed through a pre-cooled meat grinder. Myofibrils were prepared from 50 g of ground muscle according to the method of Goll et al. (1974) with the following modifications: (1) each step in the myofibril washing procedure included a 10 sec homogenization in a 1 l Waring blender, and (2) washes done in 100 mM KCl also included 1 mM NaN₃.

B. Extraction and Purification of CAF

CAF was prepared from bovine cardiac muscle. Six to 8 hearts were removed from market weight animals within 1 hr after exsanguination at the Iowa State University Meat Laboratory and transported in ice to the Food Technology Laboratory. The hearts were trimmed free of connective tissue and fat. Only the left ventricle of each heart was used. The muscle was ground through a pre-chilled meat grinder and then homogenized in a 1 gallon Waring blender (three 30-sec bursts at a

setting of low, medium, and then high) in 2.5 volume of 4 mM EDTA, pH 7.2. The pH of the homogenate was adjusted to pH 6.5 by the addition of 1 M Tris. The homogenized suspension was then centrifuged at 14,000 $\times g_{\max}$ for 20 min. The supernatant was collected and its pH was adjusted to 4.9 by the slow addition of concentrated acetic acid while stirring. After standing at 0-4°C for 15 min, the suspension was centrifuged at 14,000 $\times g_{\max}$ for 20 min. The resulting pellets were collected and resuspended by vigorous stirring with a spatula in 200-300 ml of 0.1 M Tris-HCl, 10 mM EDTA, pH 8.0. An additional 200-300 ml of water was used for rinsing centrifuge tubes to insure complete collection of the pellets. The suspension was hand homogenized in a dounce homogenizer, the pH was adjusted to 7.0, and it was stirred gently overnight (16 hr). The next day, the suspension was centrifuged at 143,000 $\times g_{\max}$ for 1 hr to remove undissolved material. The volume of the supernatant was measured and solid ammonium sulfate was added to bring the concentration up to 40% saturation. After standing at 0-4°C for 15 min, the suspension was centrifuged at 14,000 $\times g_{\max}$ for 20 min. The supernatant was discarded and the pellets were dissolved in a solution consisting of 5 mM EDTA, 5 mM MCE, 1 mM KHCO₃ pH 7.5. The sample was then extensively dialyzed (6 changes) against 5 mM EDTA, 1 mM KHCO₃, pH 7.5. This sample is very similar to the "crude P₀₋₄₀ fraction" of Dayton et al. (1975) and was used for subsequent chromatographic purification of CAF after clarification at 143,000 $\times g_{\max}$ for 1 hr.

The sample, containing 3-4 g of protein, was applied to a column (5.0 cm x 45 cm) of Whatman DE-52 cellulose. This is a microgranular form of DEAE cellulose anion exchanger purchased from Sigma Chemical Company. The column was equilibrated with at least 10 column volumes of 20 mM Tris-HCl, 1 mM EDTA, 1 mM MCE, pH 7.5 (Buffer A), at a flow rate of 60 ml/hr. Elution was achieved with a linear gradient of KCl (0 to 400 mM) in Buffer A at the same flow rate. The presence of CAF in collected fractions was detected using a casein agarose plate method (described later). Fractions containing CAF activity were pooled.

The pooled fraction containing CAF activity from the DEAE-cellulose column was pumped directly onto a column (2.5 cm x 40 cm) of phenyl-Sepharose (Pharmacia Inc., Piscataway, N.J.) which previously had been equilibrated with 10 column volumes of 250 mM KCl in Buffer A. The flow rate during equilibration and elution was approximately 38 ml/hr. Elution was achieved by washing with a continuous gradient of KCl (250 mM to 0 mM) in Buffer A. Phenyl-Sepharose is a hydrophobic interactive type material to which proteins are more strongly attracted when present in solutions containing high concentrations of salt. The protein is eluted by lowering the salt concentration. The presence of CAF in collected fractions was identified using the casein agarose plate method. Fractions containing CAF activity were pooled.

The pooled fraction containing CAF from the phenyl-Sepharose column was pumped directly onto an affinity column of the peptide antipain covalently bound to Sepharose-4B. The column (1.0 cm x 10 cm) had been equilibrated with Buffer A containing no KCl at a flow rate

of approximately 25 ml/hr. After application of the sample, the column was washed with Buffer A until there was little or no absorbance at 278 nm. A solution of Buffer A containing 400 mM KCl was used to elute the CAF in a single step. The small number of collected fractions containing CAF activity was pooled and dialyzed against 5 mM EDTA, 1 mM KHCO_3 , pH 7.5. It was clarified by centrifugation at $143,000 \times g_{\text{max}}$ for 1 hr. This enzyme preparation, the purified CAF, was assayed using the casein tube method (described later) to determine its activity. The total amount of protein was determined by the Folin-Lowry method (Lowry et al., 1951). A specific activity was then calculated.

The total volume of the CAF enzyme solution was carefully measured and an amount of solid sucrose equal in gm to the number of ml was weighed out. The sucrose was carefully added to the enzyme solution by stirring slowly with a glass rod over a period of 1-2 hr. The final solution had a sucrose concentration of approximately 38% (w/v). The new volume was measured and used to calculate the protein concentration. One mg aliquots of the CAF enzyme were put into 2 ml vials and stored at -70°C until needed. CAF samples could be stored up to 1 year with little (less than 15%) loss of activity.

C. Preparation of Antipain-Sepharose 4B Affinity Matrix Used in the CAF Purification

Five gm of aminohexyl (AH) Sepharose 4B were allowed to swell overnight at 25°C in 1 l of 0.5 M NaCl. The next day the gel (20 ml volume) was washed 3 times with 200 ml (each time) of 0.5 M NaCl, followed by 3 times (200 ml each time) with water that had been

adjusted to pH 4.5 by the addition of 1 drop of 1 M HCl. All washes were done on a scintered glass funnel. The peptide antipain (5.0 mg) was dissolved in 2.0 ml of water and adjusted to pH 4.5 by the addition of 0.1 M HCl to prepare it for coupling. The dissolved peptide was then added to the gel suspension which then had a total volume of approximately 40 ml. Next, 200 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was added while gently swirling. The pH was monitored during the next hr and adjusted when necessary with 1 M HCl to keep the pH between 4.5 to 5.0. The suspension was then transferred to a large tube and stirred by "end over action" for 16 hr at 0-4^o C. Unreacted amino groups on the Sepharose were then blocked by the addition of 6 ml of 1 M acetic acid and incubation for an additional hr. The antipain-Sepharose was then washed with 1 l of 3 M KCl, 10 mM acetic acid to remove unreacted materials and reaction by-products. The coupled gel was then equilibrated for chromatography by washing with 1 l of 10 mM Tris-HCl, 1 mM EDTA, pH 7.5. After use in the purification of CAF, the gel was first washed with 500 ml of 1.0 M NaCl, then with 500 ml of 0.5 M NaCl (adjusted to pH 4.5), 1 mM NaN₃. The affinity material was stored at 0-4^o C for subsequent re-use.

D. Assays of CAF Activity

1. CAF activity against casein (tube assay)

CAF was assayed against casein (purified powder grade, Sigma) using the method described by Dayton et al. (1975) with the exception that imidazole-HCl at pH 7.5 was used in place of Tris-acetate. The calcium concentration was maintained at 5.0 mM in all cases unless

noted otherwise. The casein reaction mixture contained the following: 5 mg/ml casein, 100 mM imidazole-HCl, pH 7.5, 100 mM KCl, 5.0 mM MCE, 5.1 mM CaCl_2 , 0.1 mM EDTA, and 0.1 mM NaN_3 . Under these conditions, both low- Ca^{2+} and high- Ca^{2+} CAF activities were assayed. Low- Ca^{2+} CAF was measured specifically by lowering the calcium concentration to 0.2 mM.

All CAF casein assays (5 mM Ca^{2+} plus CAF) contained the following controls: (1) 5 mM EDTA-no enzyme, (2) 5 mM Ca^{+2} -no enzyme, and (3) 5 mM EDTA plus enzyme. The procedure for the assay was as follows: (1) 1.0 ml of a two-times concentrated reaction mixture was preincubated for 10 min at 25° C; (2) an equal volume of enzyme plus water was added; (3) the reaction was incubated for 30 min and then terminated by addition of 2.0 ml of 2.5% TCA; (4) the tubes were centrifuged at 1,000 x g_{max} for 10 min; and (5) the absorbance of the supernatants was measured in a spectrophotometer at 278 nm. The activity was calculated as the difference in absorbance between the calcium-plus enzyme tube and the calcium-no enzyme tube. The absorbances of the EDTA-no enzyme tube and of the EDTA-plus enzyme tube were usually very close (within 10%) to the absorbance of the calcium-no enzyme tube. Occasionally, however, a small amount of activity was detected in the EDTA-plus enzyme control tube with crude fractions of P_{0-40} , which suggested the presence of some non-CAF caseinolytic activity. The specific activity of the CAF enzyme was expressed as 0.1 absorbance units at 278 nm/mg of CAF enzyme fraction/30 min at 25° C.

2. CAF activity by the casein agarose plate method

In order to simplify assays of CAF activity conducted on the large number of fractions resulting from chromatography, a new "agarose plate method" was devised. In this method, the casein reaction mixture was incorporated into 1% agarose. The steps in the procedure were as follows: (1) the casein reaction mixture used for the tube assay (minus MCE) was heated to 80° C, cooled to 4° C, centrifuged at 10,000 x g_{\max} for 10 min, and then stored at 0-4° C; (2) the plates were prepared by adding 1.0 gm of LE agarose (a low endo-osmotic, low temperature gelling agarose from FMC Corporation, Rockland, Maine) to 100 ml of reaction mixture from step one and then heating in a boiling water bath up to 80° C in order to melt the agarose. Upon cooling to 50° C, the MCE was added to the mixture and the mixture was poured onto the hydrophilic side of gelbond (FMC Corporation) sheets. The mixture rapidly solidified and could be stored for 1 week at 0-4° C if protected from dehydration; (3) for the detection of CAF activity, 10 μ l aliquots of enzyme fractions were placed at regular intervals (approximately 1.5 cm) along the plate so that 75 to 100 samples could be tested simultaneously. A second sheet of gelbond marked with numbered positions was positioned underneath the transparent test sheet and used as a template; (4) the plate was incubated for 2 hr at 25° C in a covered container and then stained with a solution of 0.05% (w/v) amido black, 10% acetic acid for 15 min. The plate was washed with 50% methanol, 5% acetic acid for 15 min, and with several changes of 100% methanol to complete destaining and dehydration.

The plate was then allowed to air dry; (5) proteolytic activity appeared on the plate as clear circles against a blue background, and easily could be kept as a permanent record or photographed. As will be shown in the Results section, the method was a rather sensitive measure of CAF activity, detecting as little as 0.2 μg of CAF protein.

E. Treatment of Myofibrils with CAF

The myofibrils were prepared from bovine longissimus muscle as described earlier and assayed under the following conditions (called 5 mM Ca^{2+} plus enzyme): 2.0 mg/ml myofibrils, 100 mM KCl, 50 mM imidazole-HCl of desired pH, 0.1 mM EDTA, 5.1 mM CaCl_2 , 5 mM MCE. Imidazole was selected as the buffer because its pKa of 7.0 was in the range of the pH values tested, the concentration of 50 mM provided sufficient buffering capacity, and it did not alter the activity of CAF when compared to Tris buffered assays. The enzyme to substrate ratio was kept at 1:100 (w/w) unless noted differently. The pH values tested were 7.5, 7.0, 6.5, 6.0, and 5.5. At each pH, temperatures of 25 $^\circ$, 15 $^\circ$, and 5 $^\circ$ C were tested. The following controls were included: 5 mM EDTA-no enzyme (other reaction conditions the same), 5 mM Ca^{+2} -no enzyme (other reaction conditions the same), 5 mM EDTA-plus enzyme (other reaction conditions the same). The tubes were pre-incubated at the desired pH and temperature for 10 min. The reaction was started by the addition of enzyme or by an equal aliquot containing no enzyme in the controls without enzyme. The incubation was run for 2, 4, 8, 15, and 30 min and stopped by addition of enough concentrated EDTA to bring the final EDTA concentration to 10 mM. The suspension

was centrifuged at $50,000 \times g_{\max}$ for 15 min at the temperature used in the incubation. The activity of the enzyme was measured as the amount of protein present in the supernatant. For each time point tested, 0.1 ml of supernatant was diluted to 1.0 ml with water and assayed by the Folin-Lowry method (Lowry et al., 1951).

F. Extraction and Purification of Cathepsin D

1. Extraction of cathepsin D and preparation of crude enzyme

Four or 5 hearts were taken from 500 to 600 kg beef animals within 1 hr after exsanguination at Amends Packing Company (Des Moines, Iowa) and transported in ice to the Food Technology Laboratory. The muscle was carefully trimmed free of fat and connective tissue. Only the left ventricle of each heart was used for the preparation. The trimmed muscle was ground through a pre-chilled meat grinder and 2.5 kg of the ground tissue was homogenized in a 1 gallon capacity Waring blender (three 30-sec bursts at a setting of low, medium, and then high) in 2.5 volumes of 0.15 M NaCl, 1 mM EDTA, 2% n-butanol, pH 7.2. The pH of the suspension was adjusted to 6.5 with 1 M Tris and it was then centrifuged at $14,000 \times g_{\max}$ for 15 min. The supernatant was adjusted to pH 4.5 by addition of concentrated acetic acid and allowed to stand overnight (12-16 hr) at 25° C. Precipitated material was removed by clarification by centrifugation at $14,000 \times g_{\max}$ for 15 min at $0-4^{\circ}$ C. The supernatant was cooled further until it had reached 4° C in ice and then adjusted to 45% ammonium sulfate saturation by the addition of solid ammonium sulfate. After standing for 15 min, the suspension was centrifuged at $14,000 \times g_{\max}$ for 15 min. The volume of the

supernatant was measured and solid ammonium sulfate was added to bring the percent saturation to 65%. After standing for 30 min, the suspension was centrifuged at $14,000 \times g_{\max}$ for 15 min. The density of the aggregated material was less than that of the ammonium sulfate solution and was retrieved as a floating pellet or pad. The collected pellet was dissolved in 20 mM Na acetate, 1 mM EDTA, pH 5.0, homogenized by hand, and dialyzed versus several changes of 1 mM EDTA, 20 mM Na acetate pH 5.0 to remove all traces of n-butanol.

2. Purification of cathepsin D by chromatography

A two-step affinity chromatography procedure similar to that used by Afting and Becker (1981) was used for the purification of cathepsin D. The crude enzyme was dialyzed versus 0.03 M Na barbital, 0.6 M NaCl, pH 7.5. Several changes of buffer were used to insure that all EDTA was removed before chromatography on concanavalin A-Sepharose. The solution was removed from dialysis and centrifuged at $143,000 \times g_{\max}$ for 1 hr. The supernatant (300 to 400 ml) containing 2 to 3 g of protein was applied to a column (2.5 cm x 15 cm) of concanavalin A-Sepharose, which previously had been equilibrated in the sample buffer at a flow rate of 55 ml/hr. After application of the sample, the column was washed until little or no absorbance at 278 nm could be detected. The glycoprotein fraction containing cathepsin D was selectively eluted with a solution of 0.2 M alpha-methyl mannoside in 0.1 M NaH_2PO_4 , 0.6 M NaCl, pH 6.5. The fractions in this peak contained the cathepsin D activity and were pooled and then dialyzed versus 0.1 M Na citrate, 0.6 M NaCl, pH 3.0, for 6 to 8 hr at $0-4^\circ \text{C}$. This dialysis

step was done to lower the pH to 3.0, but was kept as brief as possible because substantial autolysis can occur at this low pH.

The concanavalin A-Sepharose 4B purified cathepsin D sample (15 to 25 ml) containing 100 to 150 mg of protein was applied to a column of pepstatin-Sepharose 4B (2.5 cm x 12 cm) which previously had been equilibrated with 0.1 M Na citrate, 0.6 M NaCl, pH 3.0. After sample application, the column was washed at a flow rate of 40 ml/hr until little or no absorbance at 278 nm could be detected in the effluent. The cathepsin D activity was eluted with a solution of 0.1 M NaHCO₃, 0.6 M NaCl, pH 8.0. The pooled fractions containing cathepsin D activity were dialyzed against 2 mM sodium phosphate, pH 6.5, and stored at 0-4° C.

3. Preparation of pepstatin-Sepharose 4B for cathepsin D chromatography

Approximately 30 g of aminoethyl(AH)-Sepharose 4B was suspended in 4 l of 0.5 M NaCl overnight. The next day the gel was washed on a scintered glass funnel with 3 l of 0.5 M NaCl that had been adjusted to pH 4.5 with 1 M HCl. The gel was then washed with water that had been adjusted to pH 4.5. After settling, the gel was collected in a total volume of 150 ml. Approximately 150 mg of pepstatin A (Sigma Chemical Company) was dissolved in 150 ml of 100% ethanol. First, the peptide solution was added to the gel suspension, then 1.2 g of EDC coupling reagent was added. This suspension was stirred gently with a glass rod for 1 hr, with the pH kept between 5.0 and 5.5 by addition of HCl. The gel was then mixed by "end over action" for 36 hr at 25°C. Excess amino groups on the Sepharose were blocked by addition of enough

concentrated acetic acid to bring the suspension to 1.0 M in acetic acid and incubation for 4 hr. To remove unreacted materials and reaction by-products, the pepstatin-Sepharose was washed by filtration with 1 l each of 50%, 25%, and 10% ethanol and then with 1 l of 0.1 M citrate, 0.5 M NaCl pH 3.0. Each wash step was followed by filtration on a scintered glass funnel.

G. Assays of Cathepsin D on Hemoglobin
and on Benzyl-DL-arginine 2-Naphthylamide (BANA)
and Benzylloxycarbonylarginylarginine 2-Naphthylamide (Z-arg-arg)

1. Hemoglobin assay

Because cathepsin D readily hydrolyzes denatured hemoglobin at pH 3.0 to 4.0 (Canonica and Bird, 1970), a hemoglobin assay was used as one of the measures for determining cathepsin D activity in selected fractions resulting from steps in the purification. An 8% (w/v) solution of bovine hemoglobin was dialyzed against water for 2 to 4 days and then centrifuged at $100,000 \times g_{\max}$ for 30 min. Steps in the assay were as follows: 0.25 ml of 8% hemoglobin plus 0.25 ml of 1.0 M Na formate, pH 3.8, were preincubated at 37° C for 10 min. Then 0.5 ml of enzyme was added and the reaction mixture was incubated for 1 hr at 37° C. The reaction was terminated by the addition of 3.0 ml of 5% TCA and the tubes were centrifuged for 10 min at $1,000 \times g_{\max}$. The amount of activity was determined by using the modified Folin-Lowry procedure for determining quantity of TCA soluble peptides in the supernatants as described by Barrett and Heath (1977) as follows: (1) A modified alkaline copper solution was made in two

parts. Solution A contained 1.0 g trisodium citrate dihydrate and 0.5 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per 100 ml. Solution B contained 16 g of NaOH and 50 g of NaCO_3 per 500 ml. (2) The modified alkaline copper solution was made by mixing 1.0 ml of solution A, 79 ml of water, and 20 ml of solution B. (3) The procedure for performing the Folin determination was to combine 0.5 ml of TCA supernatant, 0.5 ml of water, and 2.5 ml of modified alkaline copper solution, wait 15 min, add 0.25 ml of Folin solution (Sigma Chemical Company), and read the absorbance at 750 nm after 30 min. (4) The specific activity of cathepsin D was expressed as mg of tyrosine equivalent/mg of enzyme/60 min at 37° C. Confirmation that activity was due to cathepsin D rather than to another contaminating protease was done in two ways. First, controls containing 40 μl of 0.01 M pepstatin in the hemoglobin assay tubes were used. This demonstrated that hemoglobin hydrolyzing activity was inhibited by pepstatin, an effective inhibitor of cathepsin D. Second, the enzyme preparation also was assayed for the ability to hydrolyze BANA or Z-arg-arg (described below), which would indicate the presence of other catheptic enzymes such as cathepsins B, H, or L.

2. BANA and Z-arg-arg assays

The presence of proteolytic activity towards BANA or Z-arg-arg was tested in the following manner (Barrett and Heath, 1977). Substrate stock solutions were made by dissolving 40 mg of BANA (Sigma Chemical Company) or 13 mg of Z-arg-arg (Sigma Chemical Company)/ml in dimethylsulfoxide (DMSO). For the assay, 0.5 ml of enzyme and 1.5 ml of buffered "activator" solution containing 0.1 M NaH_2PO_4 , 1 mM EDTA,

pH 6.0, 1 mM cysteine were mixed and then preincubated at 40^o C. The reaction was initiated by addition of 50 μ l of one of the two substrate stock solutions. After 10 min, the reaction was stopped by addition of 2.0 ml of coupling reagent to each tube. The coupling reagent contained a diazonium salt called "fast garnet" which produced a colored product in the presence of free 2-naphthylamide (see Barrett and Heath, 1977, for complete composition of coupling reagent). The absorbances of each tube and standards containing 2-naphthylamide were read at 520 nm. The 2-naphthylamide and related naphthylamides are potent carcinogens; thus, the assays were handled with extreme caution.

H. Treatment of Myofibrils with Cathepsin D

The myofibrils were prepared from bovine longissimus muscle as described earlier and assayed under the following reaction conditions: 2.0 mg/ml myofibrils, 100 mM KCl, 50 mM imidazole-HCl of desired pH, 1 mM EDTA, plus enzyme. The enzyme to substrate ratio was kept at 1:100 (w/w). The pH values tested were 7.5, 6.5, and 5.5. At each of these pH values, temperatures of 37^o, 25^o, and 15^o C were tested. The following two controls were included: (1) myofibrils plus pepstatin-no enzyme and (2) myofibrils plus pepstatin plus enzyme. The tubes were preincubated for 10 min at the desired pH and temperature. The reactions were initiated by addition of enzyme or an equal volume of solution without enzyme (for myofibrils plus pepstatin-no enzyme control tube). The reactions were run for 30, 60, 90, and 120 min. The reactions were terminated by addition of 20 μ l of 0.01 M pepstatin. The terminated reaction tubes were centrifuged at 50,000 x g_{max} for

15 min at the temperature used in the reaction incubation. The proteolytic activity was determined by measuring the amount of protein in the supernatant by the Folin-Lowry method as described earlier in the CAF assays.

I. Fragmentation of Myofibrils Following Protease Digestions

Bovine skeletal myofibrils were prepared as described earlier and incubated with CAF or cathepsin D as described in the preceding respective sections except that the samples were not centrifuged at the end of the reaction incubation time. Instead, they were diluted with 1.0 ml of 100 mM KCl and subjected to a brief (10 sec) homogenization in a Virtis homogenizer at a setting of 15,000 rpm. The myofibrils then were examined with a phase contrast light microscope to determine their degree of structural integrity. The degree of fragmentation was estimated by counting number of sarcomeres per myofibril fragment in at least 10 fields.

J. Electrophoretic Methods

1. SDS-polyacrylamide electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) with slab gels measuring 110 mm x 100 mm x 1.1 mm. The slab gel consisted of a 5% stacking gel layered over a 10% separating gel. Myofibrils were prepared for electrophoresis after incubation as follows: (1) The myofibrils were separated into pellet and supernatant fractions by centrifugation as described previously; (2) 0.125 ml of tracking dye solution that contained 10% SDS, 50%

sucrose, 0.8% bromphenol blue, 0.1 M (2 [N-morpholino]) ethanesulfonic acid (MES), pH 6.5, and 50 μ l MCE was added to 0.5 ml of the myofibril supernatant and boiled for 3 min; (3) the pellets were carefully decanted of any remaining supernatant and 0.5 ml of water, 0.125 ml of tracking dye, and 50 μ l of MCE were added. The pellets were stirred vigorously with a glass rod to insure complete dispersal and then boiled for 3 min. Electrophoresis was performed at a setting of 10 mAmps for 4 hr. Staining was done overnight in a solution of 0.1% Coomassie blue R250 (Sigma Chemical Company), 10% acetic acid, 50% methanol. The gels were destained by diffusion with gentle shaking in a solution of 7.5% acetic acid, 5% methanol.

2. Electrophoresis in nondenaturing buffers

Electrophoresis in a nondenaturing buffer system was performed in slab or disc gels as described by Dayton et al. (1975). The slabs were 110 mm x 100 mm x 1.1 mm and the discs were 2 mm in diameter by 8.0 cm in length. The gel consisted of a 4% stacking gel, pH 5.5, layered over a 7.5 or 10% separating gel, pH 7.5. This electrophoresis system was used to examine the composition of purified CAF and cathepsin D under nondenaturing conditions. Samples were applied directly to the gel after incorporation of sucrose to 25% (w/v) final concentration and electrophoresed at 20 mAmps for 4 hr (slabs) or 2 mAmps per tube (disc) until the dye front was within 1 cm of the bottom of the gel. The gels (slab or disc) were then stained with 0.1% Coomassie blue as described above.

3. Two-dimensional gel electrophoresis

A two-dimensional gel electrophoresis system was used to examine the subunit composition of purified CAF and of cathepsin D. The first dimension consisted of electrophoresis under nondenaturing conditions (pH 7.5 as described above) on a 2 mm diameter disc gel, followed by incubation of the gel in 0.5% SDS, 0.1 M Tris-phosphate, pH 6.7, 1% MCE for 1 to 2 hr. At the end of the incubation period, the disc gel was laid horizontally on top of an SDS-containing slab gel (110 mm x 100 mm x 2.5 mm) prepared as described above and electrophoresed at 15 mAmps for 4 hr. Standards for electrophoresis in the second dimension were prepared by adding 1 to 2 mg of the desired proteins to 2 ml of SDS standard agar solution. The standard agar solution consisted of the following: 0.5 ml of stacking gel buffer, 0.2 g agarose, 0.25 ml MCE, 0.25 ml 20% SDS, standard proteins, and enough water to bring the total volume to 10 ml. The solution was boiled until the agarose melted and it was then pipetted into 2.0 mm diameter tubes. After the material had solidified, it was removed from the gel tubes, cut into 1 cm lengths, and placed on top of the slab gel used for the second dimension.

4. Immunoblotting

Electrophoresis of SDS-containing slab gels was performed as described earlier. Duplicate gels were always run. One was stained with Coomassie blue as described above and the other was used for blotting. The blotting procedure was carried out in a trans blot electrophoresis apparatus (Model 160) from Bio-Rad Corporation. Immediately after electrophoresis on the slab, the protein bands were

transferred to nitrocellulose paper by electrophoresis. They were transferred for 16 hr at a potential setting of 140 V and 0.6 amps, with a cooling coil used to dissipate heat. The transfer buffer contained 20% methanol, 0.15 M Tris-glycine, pH 8.5. At the end of the transfer time, the paper was removed and placed in a solution of 2% BSA in phosphate buffered saline (PBS), pH 7.0, for 2 hr. The blot was washed 3 times for 30 min each in PBS (no BSA), then incubated with antibody to the desired antigen. The antibodies were raised in New Zealand white rabbits by the methods of Richardson et al. (1981). The blot was again washed 3 times in PBS to remove excess antibody. Detection of antigen-antibody complexes was done by using horseradish peroxidase labeled goat-antirabbit antibody. The indirect method with GAR-HRP was detected by incubating 0.6% 4-chloro-1-naphthol in 0.01% H_2O_2 , which gives a dark blue reaction product.

IV. RESULTS

Several investigators (Olson et al., 1976; Goll et al., 1983a; Lawrie, 1983) have proposed that much of the postmortem increase in meat tenderness occurs because of weakening of the myofibril at the Z line. Although much is known about CAF and its ability to degrade myofibrillar proteins (Dayton et al., 1975; Goll et al., 1983a) and to cause the loss of Z and M line structures (Stromer et al., 1974) in vitro, little is known about its effectiveness under postmortem-like environments. The investigations described herein were done to evaluate the ability of purified CAF and purified cathepsin D to hydrolyze myofibrillar proteins and to cause structural alterations in myofibrils under in vitro conditions resembling those present in postmortem muscle.

The general protocol followed was to incubate myofibrils with purified CAF or cathepsin D under carefully controlled conditions and then to examine the enzyme's effect in several ways. First, the digested myofibrils were centrifuged at high centrifugal force so that only soluble proteins were left in the supernatant. The amount of protein remaining in the supernatant was measured by the Folin-Lowry method. Second, both the supernatant and pellet fractions were examined by SDS-PAGE. Third, the structural integrity of the myofibrils after incubation and brief homogenization (10 sec) was examined in the phase contrast microscope.

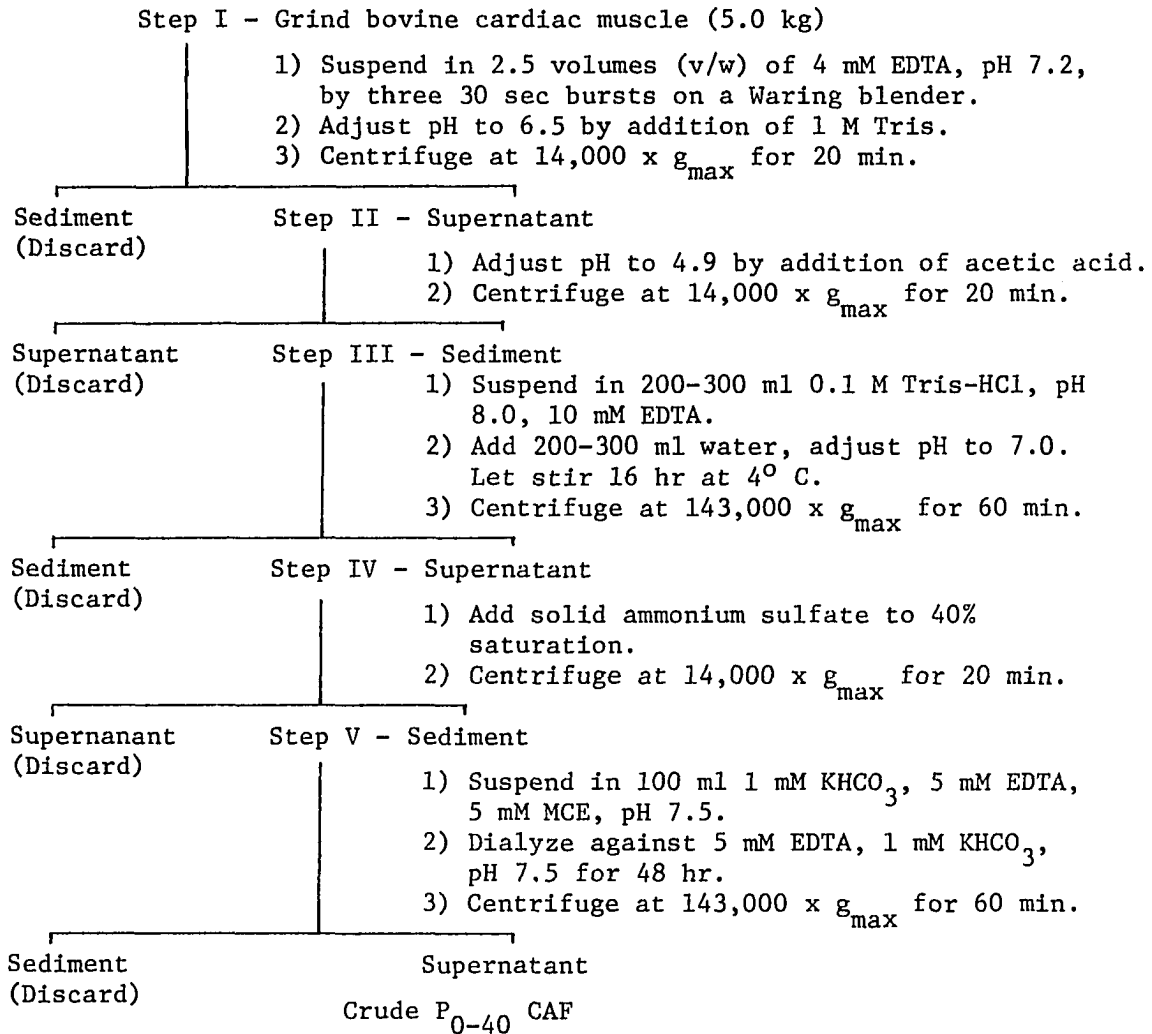
In a study of proteolytic enzyme action, it is important to be certain that the enzyme preparation is not contaminated with other

proteases. Therefore, a portion of this investigation concerned the isolation and purification of CAF and cathepsin D.

A. CAF: Purification and Properties

A "P₀₋₄₀ crude CAF" preparation was made by a slight modification of the method of Dayton et al. (1975) as outlined in Figure 1 and in the Materials and Methods from the ventricular portion of six to eight bovine hearts that were obtained within one hr of slaughter. The preparation of P₀₋₄₀ crude CAF typically contained 3 to 4 g of protein in approximately 125-150 ml of solution.

The P₀₋₄₀ crude CAF was equilibrated against 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM MCE (buffer A) and then loaded onto a 5.0 x 45 cm column of DEAE-cellulose. The elution profile is shown in Figure 2. The presence of CAF activity in the eluted fractions was detected by the spot assay method described in the Materials and Methods and shown in Figure 3. In this procedure, the casein substrate mixture for the CAF assay was incorporated into a thin agar matrix that was supported on a plastic sheet. To determine the presence of CAF activity, 10 μ l of each tube eluted from the column was spotted directly onto the agar plate. The plate was incubated for two hr at 25^o C, then fixed and stained as described in the Materials and Methods section. The presence of CAF activity was detected as clear or light blue circles against a dark blue background. The relative intensity of the "clearing" is indicated by the dashed line in Figure 2. In Figure 3, the plate was incubated with a sample of purified CAF in order to show its limits of sensitivity. As little as 0.2 μ g of purified CAF could be reliably

 Preparation of P₀₋₄₀ Crude CAF from Bovine Cardiac Muscle


Muscle tissue was obtained from the left ventricular portion of bovine hearts and processed as described in Materials and Methods. All solution volumes are based on the wet weight of ground muscle tissue used in Step I.

Figure 1. Flow sheet for the preparation of P₀₋₄₀ crude CAF from bovine cardiac muscle

Figure 2. Elution profile of the P₀₋₄₀ crude CAF fraction from a 5.0 x 45 cm DEAE-cellulose column

The column was loaded with 5,145 mg of P₀₋₄₀ crude CAF in 134 ml of solution. The column was eluted with a continuous gradient consisting of 1 each of 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM MCE, and of 400 mM KCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM MCE. Flow rate was 60 ml/hr and 12 ml fractions were collected. CAF activity was determined by the spot assay method using casein as a substrate. The relative intensity of the activity is shown by the dashed line. Tubes within the fraction represented by the vertical dashed lines were pooled. This fraction was designated DEAE-cellulose purified CAF and was used in the next purification step.

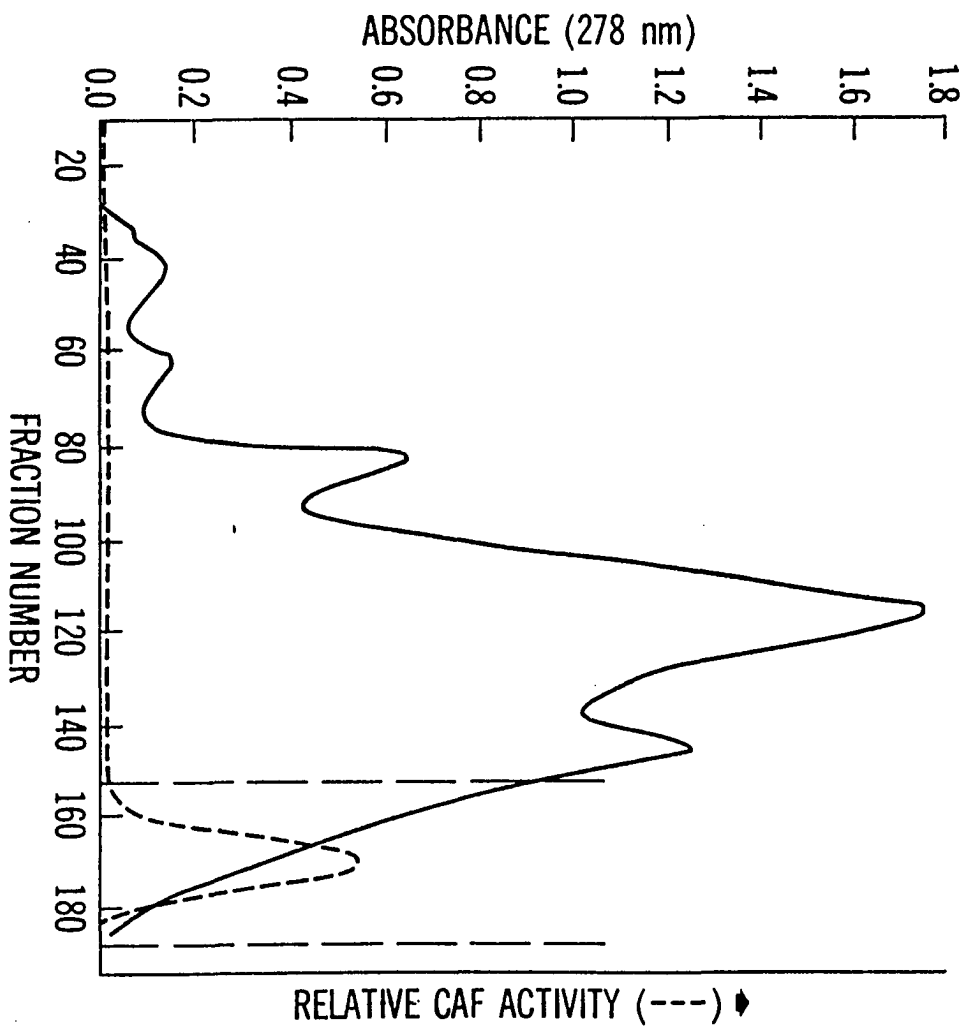


Figure 3. Detection of CAF activity by the spot assay method

Sensitivity for detection of CAF activity using casein as a substrate in an agarose matrix is demonstrated. Ten μl of purified CAF solution containing 10, 2.0, 1.0, 0.2, 0.1, and 0.05 μg protein, respectively, were spotted. The plate was incubated for 2 hr at 25^o C, stained with 0.05% amido black in 10% acetic acid, dehydrated in methanol, and air-dried. Additional details are given in the Materials and Methods section.



μg 10 2.0 1.0 0.2 0.1 0.05

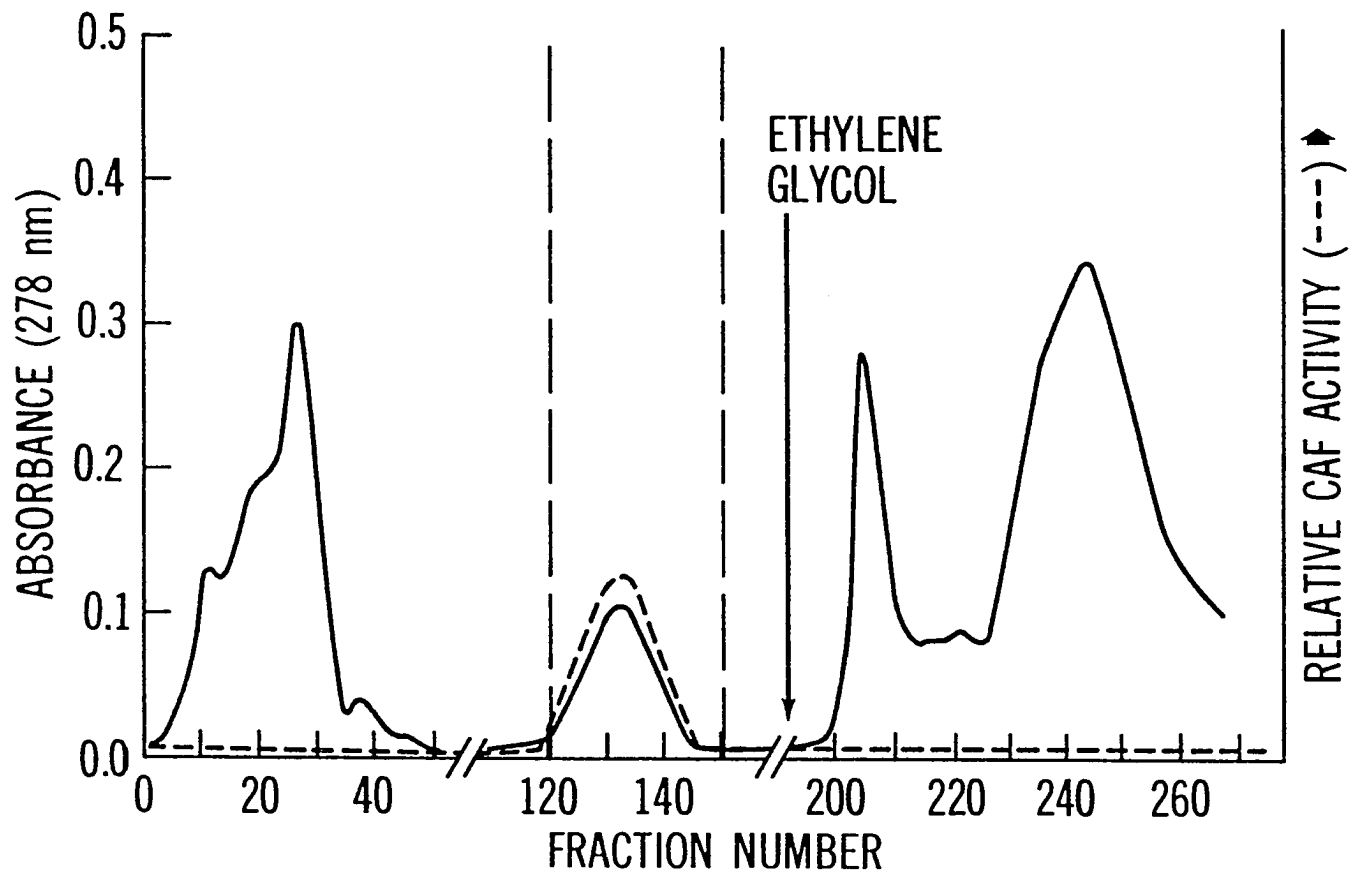
detected. The method rapidly and reliably allowed the identification of CAF in column fractions and helped to reduce the time required for purification. Tubes containing CAF activity indicated by the vertical dashed lines in Figure 2 were combined and called "DEAE-cellulose purified CAF."

DEAE-cellulose purified CAF was chromatographed on a phenyl-Sepharose column without previous concentration or desalting. This greatly reduced the time required to purify the enzyme. Phenyl-Sepharose is a hydrophobic column material and binds proteins more strongly under those conditions which increase hydrophobic interaction. Thus, DEAE-cellulose purified CAF, with its KCl content relatively high because of its late position in the elution profile, binds to the phenyl-Sepharose column. Elution from the phenyl-Sepharose column was accomplished using a continuous gradient from 250 mM KCl to 0 mM KCl in buffer A as shown in Figure 4. The CAF activity was determined using the spot assay method and is shown as a dashed line in Figure 4. Tightly bound proteins were removed from the column with a solution of 50% ethylene glycol in buffer A; however, no CAF activity was found in this portion of the elution profile. The column was then re-equilibrated in 250 mM KCl in buffer A, and reused at a later date. The tubes containing CAF activity, indicated by the vertical dashed lines in Figure 4, were combined and called "phenyl-Sepharose purified CAF."

Phenyl-Sepharose purified CAF was chromatographed on an antipain-Sepharose column without previous concentration. This also reduced the

Figure 4. Elution profile of the DEAE-cellulose purified CAF from a 2.5 x 40 cm phenyl-Sephrose column

A total of 458 mg of DEAE-cellulose purified CAF in 250 ml was loaded onto the column. The column was eluted with a continuous gradient consisting of 500 ml each of 250 mM KCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA and 20 mM Tris-HCl, pH 7.5, 1 mM EDTA. The arrow indicates start of elution with 50% ethylene glycol, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA. Flow rate was 42 ml/hr and 10.5 ml fractions were collected. CAF activity was determined by the spot assay method using casein as a substrate. The relative intensity of the activity is shown by the dashed line. Tubes within the fraction represented by the vertical dashed lines were pooled. This fraction was designated phenyl-Sephrose purified CAF and was used in the next purification step.



time required to purify the enzyme. Antipain is a peptide that acts as a competitive inhibitor of the enzyme. It was covalently attached to aminohexyl-Sepharose 4B as described in the Materials and Methods section. Phenyl-Sepharose purified CAF was directly loaded onto a small column (1.0 x 8.0 cm) of the antipain-Sepharose. The column was washed with buffer A as shown in Figure 5 until it was certain that no unbound material remained on the column. CAF was eluted from the column with a solution of 400 mM KCl in buffer A. The activity was detected using the spot assay method, and the tubes in the fractions indicated between the vertical dashed lines were combined and called "purified CAF."

The procedure outlined here for the purification of CAF from bovine cardiac muscle yielded 20 to 30 mg of highly purified enzyme and could be accomplished by one person in approximately 10 days. The enzyme's specific activity was typically in the range of 200 to 300 units/mg when assayed with casein as described in the Materials and Methods section. One unit of activity is defined as 0.1 absorbance unit at 278 nm in the TCA supernatant, after 30 min of incubation at 25° C. The preparation also was examined for low-Ca⁺² CAF activity with the casein substrate in the presence of 100 μM Ca⁺². The activity was less than 1% of the activity obtained in the presence of 5 mM Ca⁺².

Purified CAF was examined by several electrophoretic methods. Figure 6 shows PAGE results with purified CAF under both denaturing (SDS-PAGE) and nondenaturing conditions. When the enzyme was electrophoresed under nondenaturing conditions at pH 7.5 on a 7.5%

Figure 5. Elution profile of the phenyl-Sepharose purified CAF from a 1.0 x 8.0 cm antipain-Sepharose column

A total of 76 mg of phenyl-Sepharose purified CAF in 150 ml was loaded onto the column. The column was eluted with a continuous gradient consisting of 250 ml each of 20 mM Tris-HCl, pH 7.5, 1 mM EDTA and of 400 mM KCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA. Flow rate was 26 ml/hr and 6.5 ml fractions were collected. CAF activity was determined by the spot assay method using casein as substrate. The relative intensity of the activity is shown by the dashed line. Tubes within the fraction represented by the vertical dashed lines were combined and called purified CAF.

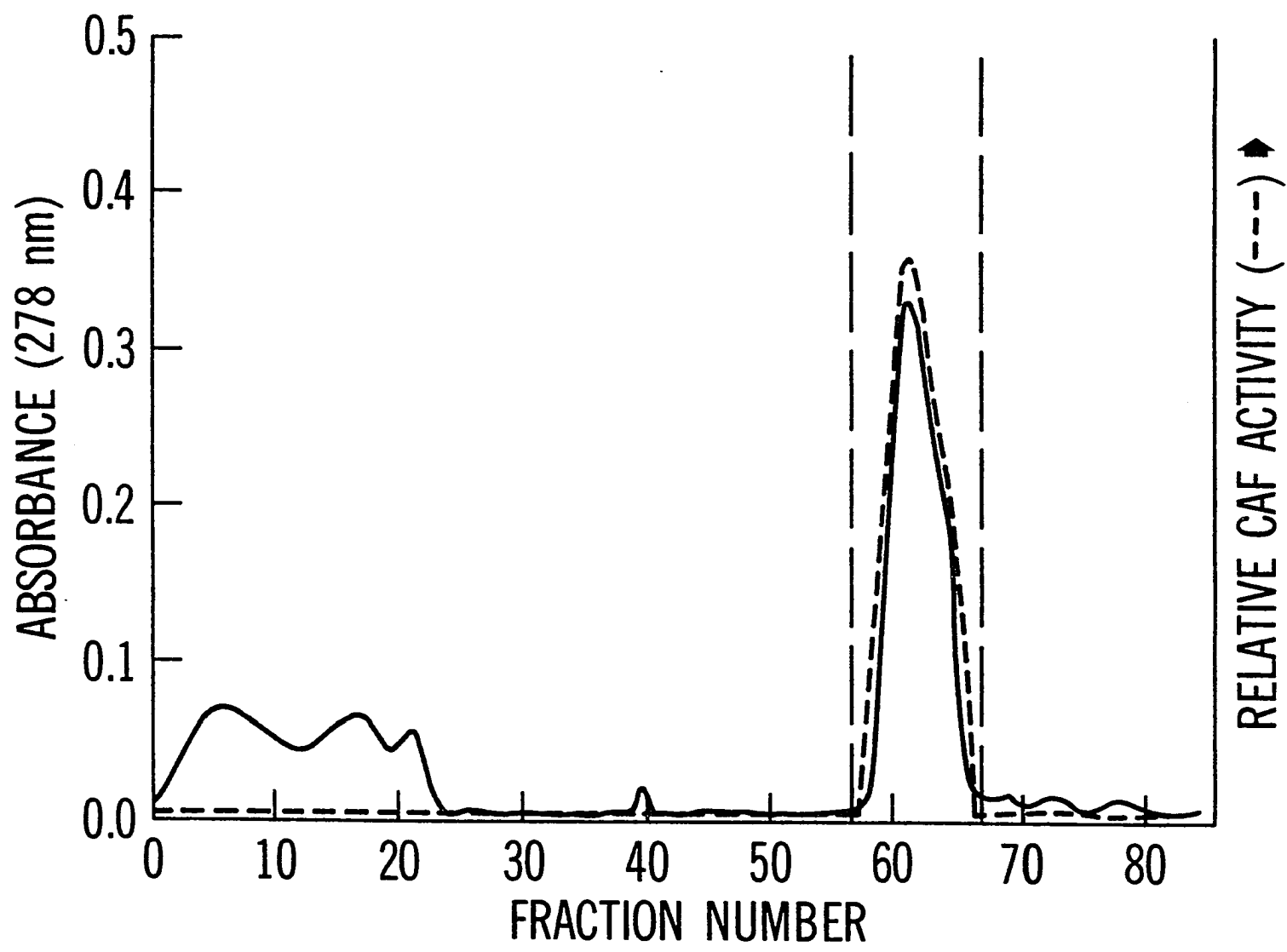
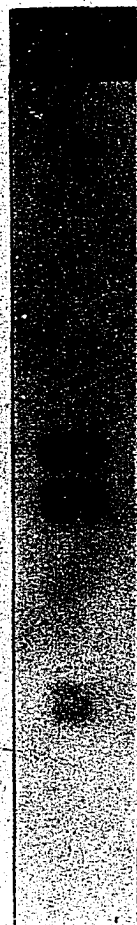


Figure 6. Polyacrylamide gel electrophoretic examination of purified CAF under nondenaturing and denaturing conditions

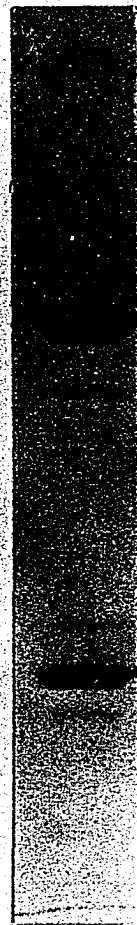
Purified CAF (20 μg) was electrophoresed under nondenaturing conditions at pH 7.5 on a seven and one-half percent acrylamide gel (shown on the left) as described in the Materials and Methods section. The CAF contained one major band, another band just below the major one, and two very minor bands close to the bottom of the gel. Purified CAF (28 μg) also was electrophoresed under denaturing conditions on a 10% acrylamide gel (shown on the right). The CAF contained two major bands corresponding to molecular weights of about 80,000 and 30,000.

**pH 7.5
ELECTROPHORESIS**



**SDS
ELECTROPHORESIS**

- 80,000



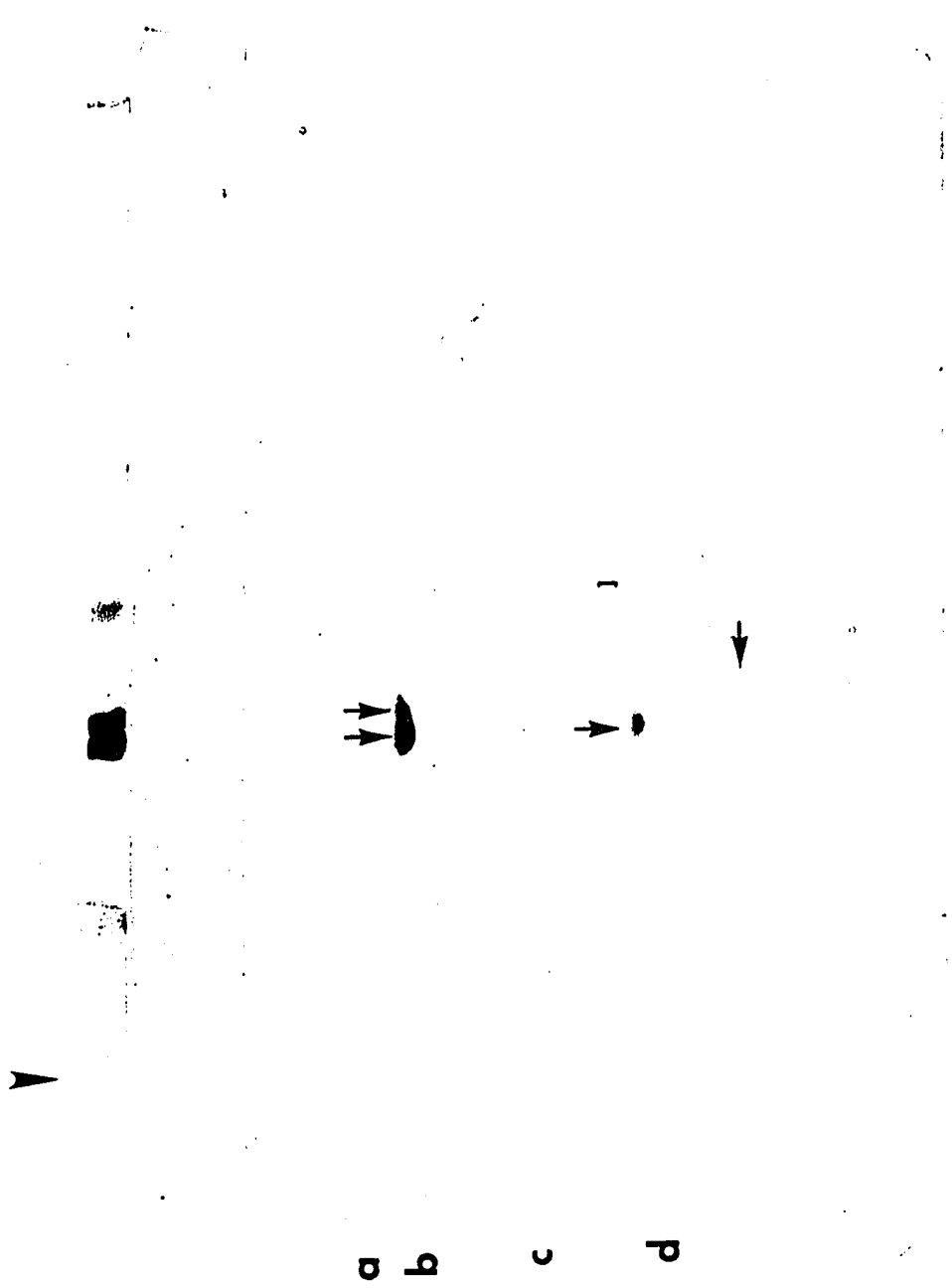
- 30,000

acrylamide gel, it consisted of one major band, with a lighter band migrating just below it, and some very faint bands near the bottom of the gel (left side of Figure 6). When purified CAF was electrophoresed under denaturing conditions in the presence of SDS on a 10% acrylamide gel, it showed two major bands with molecular weights of about 80,000 and 30,000 each (right side of Figure 6).

In order to ascertain the composition of the bands observed under nondenaturing electrophoretic conditions (gel on left in Figure 6), a two-dimensional PAGE system was used. Purified CAF first was electrophoresed under nondenaturing conditions at pH 7.5, on a 10% acrylamide disc gel. The disc gel then was equilibrated in SDS-containing buffer, placed on top of an SDS slab gel, and electrophoresed the second time as described in the Materials and Methods section. Figure 7 shows the results of the two-dimensional separation. The bands at the left of the gel correspond to molecular weight markers. It can be seen from this figure that the band corresponding to the major band observed under nondenaturing conditions migrated as two spots corresponding to subunits with molecular weights of about 80,000 and 30,000 each. The band, which migrated just below the major band on the nondenaturing gel, migrated as an 80,000-dalton species and as an approximately 18,000-dalton species (very faint spot indicated by the horizontal arrow in Figure 7). The 18,000-dalton species may be a degradation product of the 30,000-dalton CAF subunit (Goll et al., 1983b). The two closely spaced faint bands in the nondenaturing gel gave rise to

Figure 7. Two-dimensional PAGE analysis of purified CAF

Purified CAF (20 μg) was first electrophoresed on a 10% polyacrylamide disc gel under nondenaturing conditions at pH 7.5. The disc gel was then incubated for 1 hr in 1.0% SDS, 0.1% MCE, placed horizontally on a 10% polyacrylamide slab gel and subjected to SDS-PAGE as described in the Materials and Methods section. The large arrowhead indicates the position of the top of the disc gel. The bands at the left edge of the slab gel correspond to protein standards (approximately 2 μg each) as follows: a) alpha-actinin, 100,000 daltons; b) BSA, 68,000 daltons; c) ovalbumin, 43,000 daltons; d) chymotrypsinogen, 25,000 daltons. The four arrows and the bracket indicate spots on the slab gel derived from bands in the disc gel. The horizontal disc gel shown at the top is a stained duplicate of the disc gel used in the experiment. It has been positioned so that the stained bands are directly above the corresponding spots in the slab gel below.



two distinct spots corresponding to molecular weights of approximately 35,000. Their identity is unknown.

B. The Effect of Purified CAF on Myofibrils

1. Protein released from myofibrils by CAF treatment

CAF's ability to cause the release of soluble protein during incubation of myofibril suspensions was used as one measure of the enzyme's activity under varying conditions of pH and temperature. Figure 8 shows the release of soluble protein after incubation at 25° C for up to 30 min at pH 7.5, 6.5, or 5.5. The amount of protein in the supernatant was determined by the Folin-Lowry (Lowry et al., 1951) method. The curve at pH 7.5 shows the release of protein obtained under optimal pH conditions for CAF (Dayton et al., 1976a) and is reflected by the rapid release of protein into the supernatant. Decreasing the pH of the incubation to 6.5 decreased the rapid initial response, but after 30 min the total amount of protein released was fairly close to that observed at pH 7.5. Decreasing the pH of incubation to 5.5 also slowed the rapid initial response. At the end of 30 min of incubation, the total amount of protein released was slightly less than that observed at pH 6.5. In all samples, some protein was present in the supernatants of even the control (0-min) samples.

When the temperature of incubation was lowered to 15° C (Figure 9), differences were observed in the amount and rate of protein solubilized in comparison to results obtained at 25° C. The initial rate of release at pH 7.5 was lower than that obtained at pH 7.5 and 25° C, but substantial release still had occurred after 30 min of incubation.

Figure 8. Protein released from myofibrils after incubation with purified CAF at 25° C and pH 7.5, 6.5, or 5.5

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 7.5, 6.5, or 5.5, 0.1 mM EDTA, 5.1 mM CaCl₂, 5.0 mM MCE at 25° C were incubated with 40 μg of purified CAF. The reaction was stopped by addition of EDTA to a final concentration of 10 mM and each sample was then sedimented at 50,000 x g_{max} for 15 min at 25° C. Protein remaining in the supernatant was measured by the Folin-Lowry method. The data points are averages of two determinations done on a representative preparation.

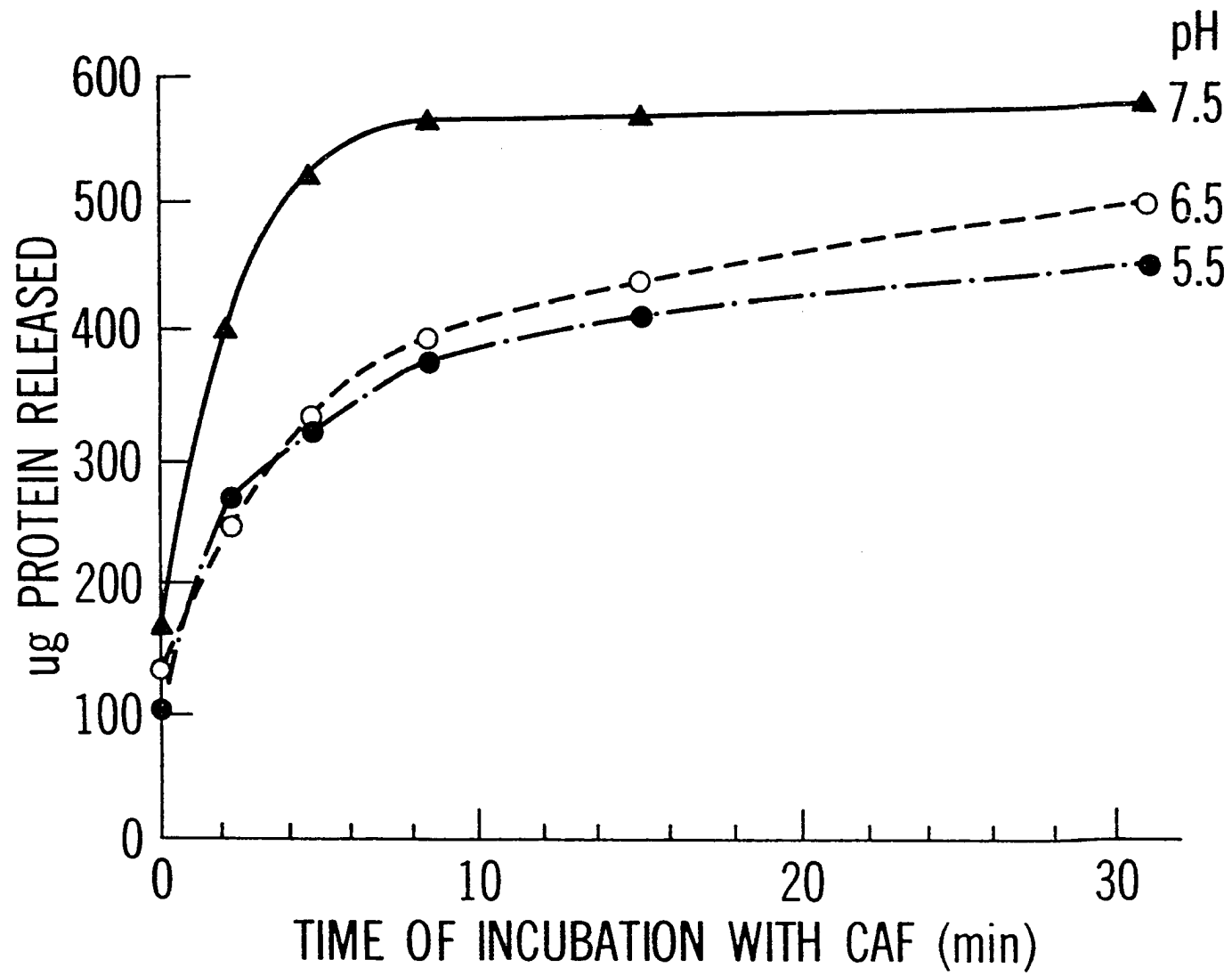
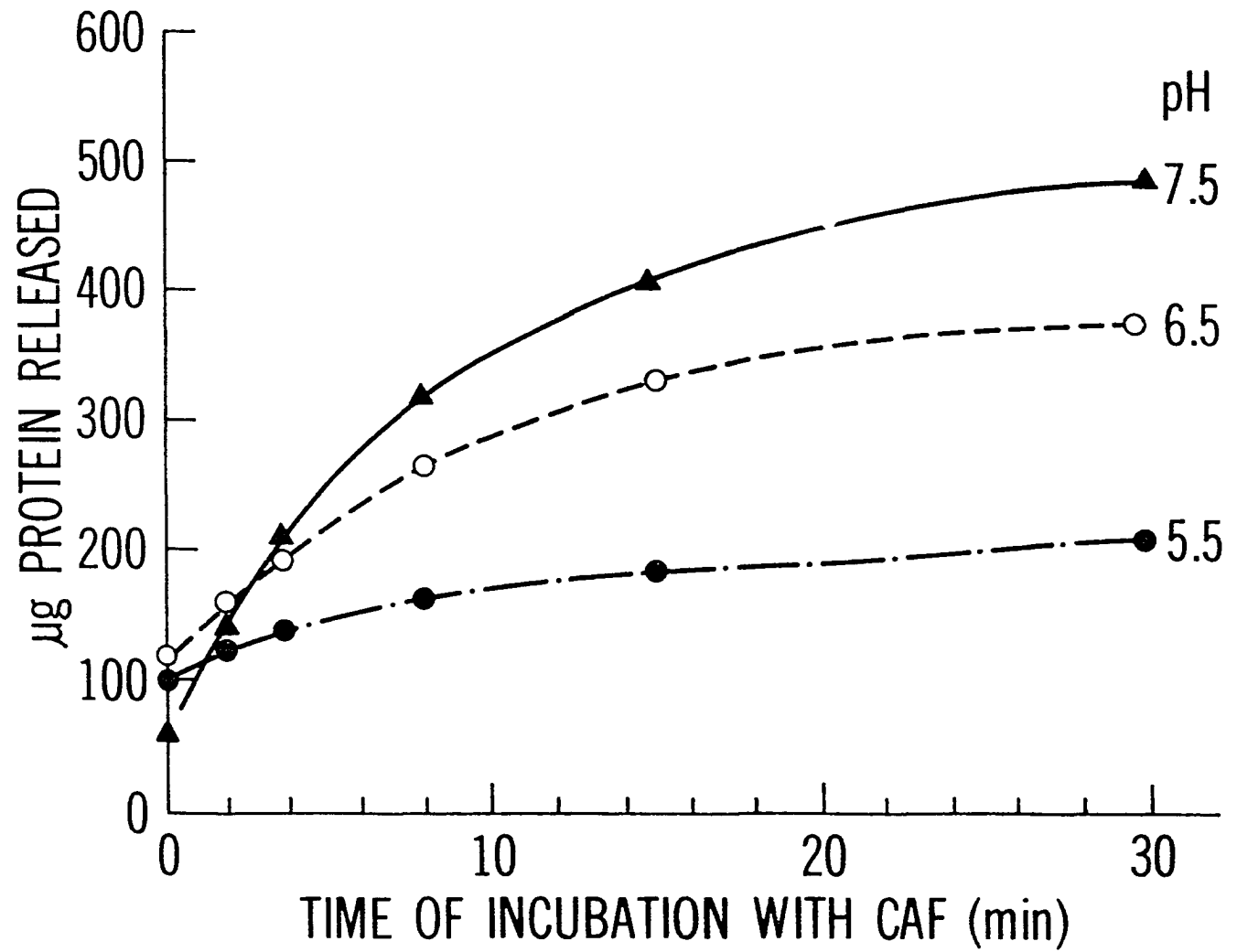


Figure 9. Protein released from myofibrils after incubation with purified CAF at 15° C and pH 7.5, 6.5, or 5.5

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 7.5, 6.5, or 5.5, 0.1 mM EDTA, 5.1 mM CaCl₂, 5.0 mM MCE at 15° C were incubated with 40 μg of purified CAF. The reaction was stopped by addition of EDTA to a final concentration of 10 mM and each sample was then sedimented at 50,000 x g_{max} for 15 min at 15° C. Protein remaining in the supernatant was measured by the Folin-Lowry method. The data points are averages of two determinations done on a representative preparation.



Lowering the pH of incubation to 6.5 at 15° C reduced both the initial rate and the final amount of protein released. When the pH of the incubation was lowered to 5.5 at 15° C, only little protein was released.

When the temperature of the incubation was further lowered to 5° C (Figure 10), there was a further decrease in both initial rates of the protein released and final amounts of protein solubilized. There still, however, was a substantial amount of protein released at the end of the 30 min incubation at pH 7.5. As expected, lowering the pH to 6.5 further reduced this amount. At pH 5.5, there was only very little CAF activity.

2. SDS-PAGE analysis of myofibril fractions after CAF treatment

CAF also was examined systematically with respect to its ability to hydrolyze myofibrillar proteins under varying conditions of pH and temperature by SDS-PAGE (Figures 11-25). In these experiments, myofibrils were incubated with CAF, centrifuged in order to separate sediment and supernatant fractions, and then examined by electrophoresis. Figures 11-15 show the results of SDS-PAGE analysis of supernatants and sediments resulting from CAF digestion of myofibrils at 25° C at selected pH values. The effect of incubation of myofibrils with CAF at 25° C and pH 7.5 is shown in Figure 11. Lanes a through f contain supernatant fractions corresponding to 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes g through l contain sediment fractions corresponding to similar incubation times. Examination of the supernatant fractions, starting with 2 min of incubation, shows that a number

Figure 10. Protein released from myofibrils after incubation with purified CAF at 5° C and pH 7.5, 6.5, or 5.5

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 7.5, 6.5, or 5.5, 0.1 mM EDTA, 5.1 mM CaCl₂, 5.0 mM MCE at 5° C were incubated with 40 g of purified CAF. The reaction was stopped by addition of EDTA to a final concentration of 10 mM and each sample was then sedimented at 50,000 x g_{max} for 15 min at 5° C. Protein remaining in the supernatant was measured by the Folin-Lowry method. The data points are averages of two determinations done on a representative preparation.

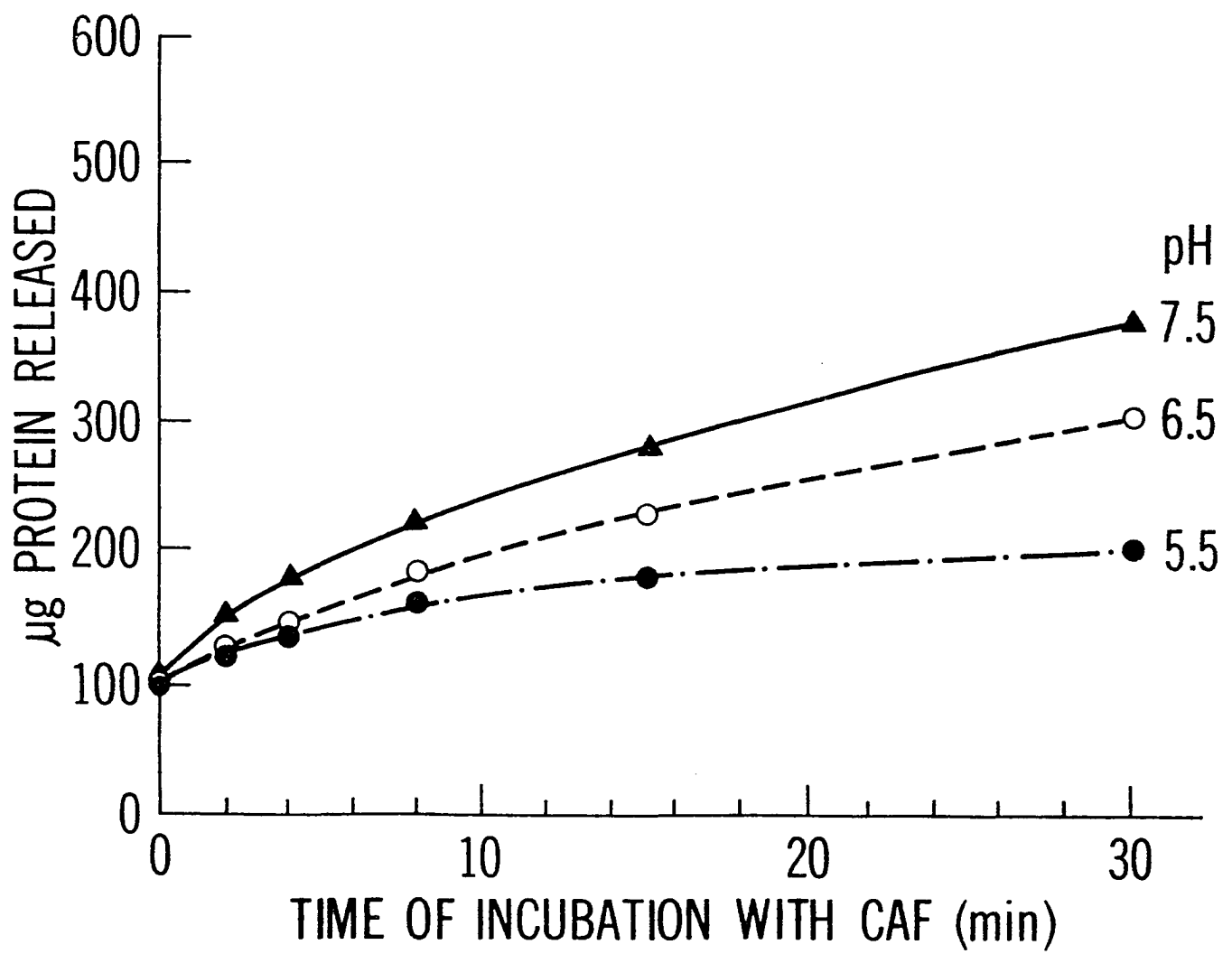
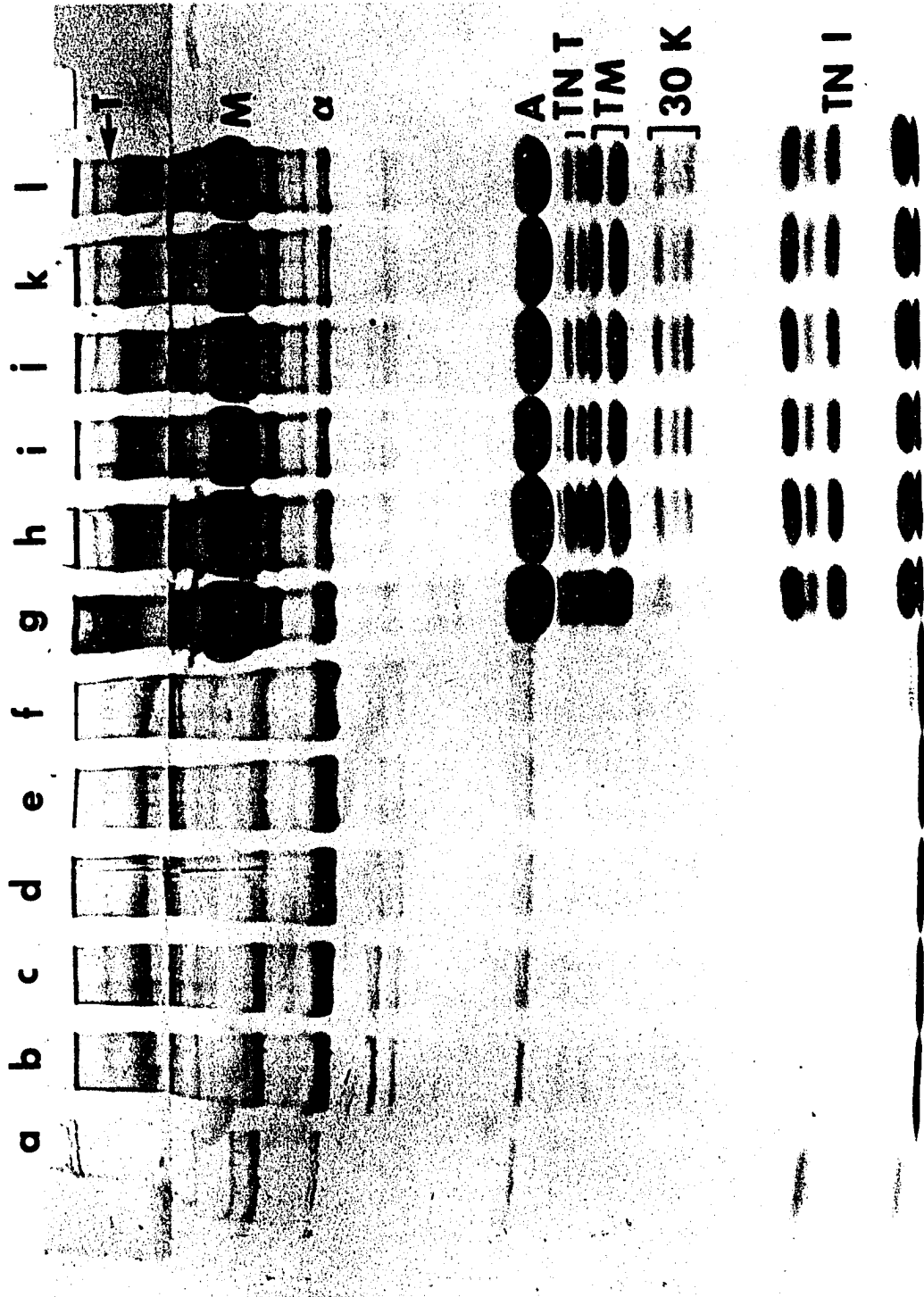


Figure 11. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 25° C and pH 7.5

CAF assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 7.5, 0.1 mM EDTA, 5.1 mM CaCl₂, 5 mM MCE, were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 25° C. Lanes a through f were loaded with 50 μl of supernatant fractions corresponding to 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes g through l were loaded with 48 μg of sediment fractions from 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes a and g (0 min) correspond to EDTA control supernatant and sediment fractions, respectively (see Materials and Methods). Labels at the right edge of the gel are as follows: T, titin; M, myosin heavy chains; α, alpha-actinin; A, actin; TN T, a small bracket indicates troponin T isomers; TM, a bracket indicates beta and alpha tropomyosin subunits; 30 K, a bracket indicates the position of a trio of bands of approximately 30,000 daltons, if present; TN I, troponin I.

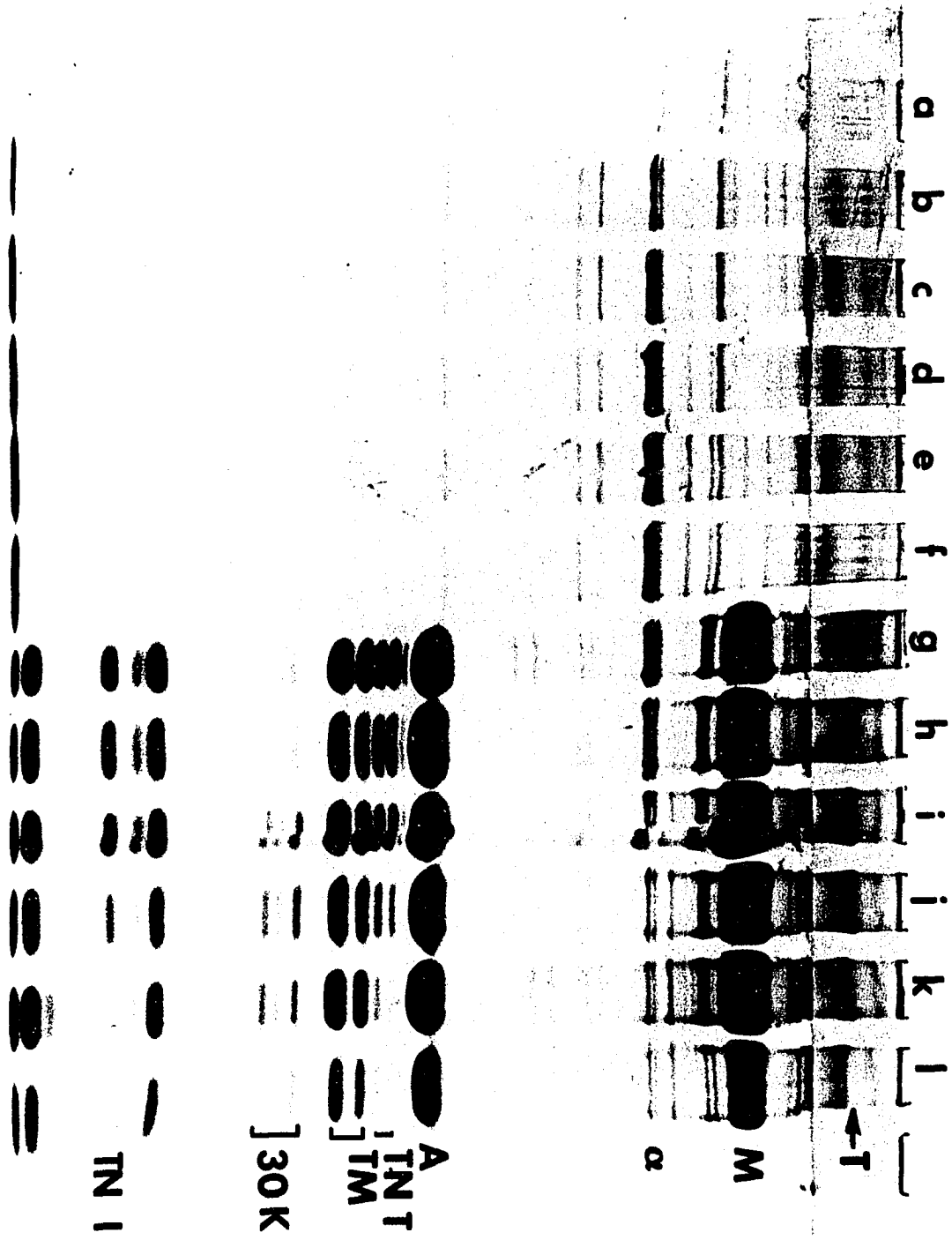


of proteins were solubilized from the myofibril by CAF. There were small amounts of several unidentified proteins with molecular weights greater than myosin heavy chains (about 200,000) and one just below it which were rapidly released. The major protein that migrated just ahead of the myosin heavy chains may have been M-protein (about 170,000 daltons), but this was not shown conclusively. This band appeared in the supernatants of essentially all samples (Figures 11-25), including the controls. The major constituent released appeared to be intact alpha-actinin. In the sediment fractions, there was a decrease in the intensity of the titin band with increased time of incubation. Putative titin breakdown products, which migrated progressively faster than titin (c.f. lanes h to l with g), also appeared. The identity of the small amount of protein that migrated slightly slower than titin in these samples is unknown. The alpha-actinin band decreased in amount during the incubation. Troponin T isomers were degraded substantially by 4 min of incubation time. Tropomyosin subunits were only slightly decreased in amount after 30 min. A trio of bands at about 30,000 daltons appeared in the sediment after only 2 min of incubation. It has been proposed that these three bands are breakdown products of troponin T (Dayton et al., 1975; Olson et al., 1976; Goll et al., 1983a). Troponin I was slowly but progressively degraded with increased time of incubation.

The effect of incubation of myofibrils with CAF at 25° C and pH 7.0 is shown in Figure 12. The results were qualitatively rather similar to those obtained at pH 7.5 (Figure 11). Again, there was a

Figure 12. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 25° C and pH 7.0

CAF assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 7.0, 0.1 mM EDTA, 5.1 mM CaCl₂, 5 mM MCE, were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 25° C. Lanes a through f were loaded with 50 μl of supernatant fractions corresponding to 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes g through l were loaded with 48 μg of sediment fractions from 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes a and g (0 min) correspond to EDTA control supernatant and sediment fractions, respectively (see Materials and Methods). Labels at the right edge of the gel are as follows: T, titin; M, myosin heavy chains; α, alpha-actinin; A, actin; TN T, a small bracket indicates troponin T isomers; TM, a bracket indicates beta and alpha tropomyosin subunits; 30 K, a bracket indicates the position of a trio of bands of approximately 30,000 daltons, if present; TN I, troponin I.

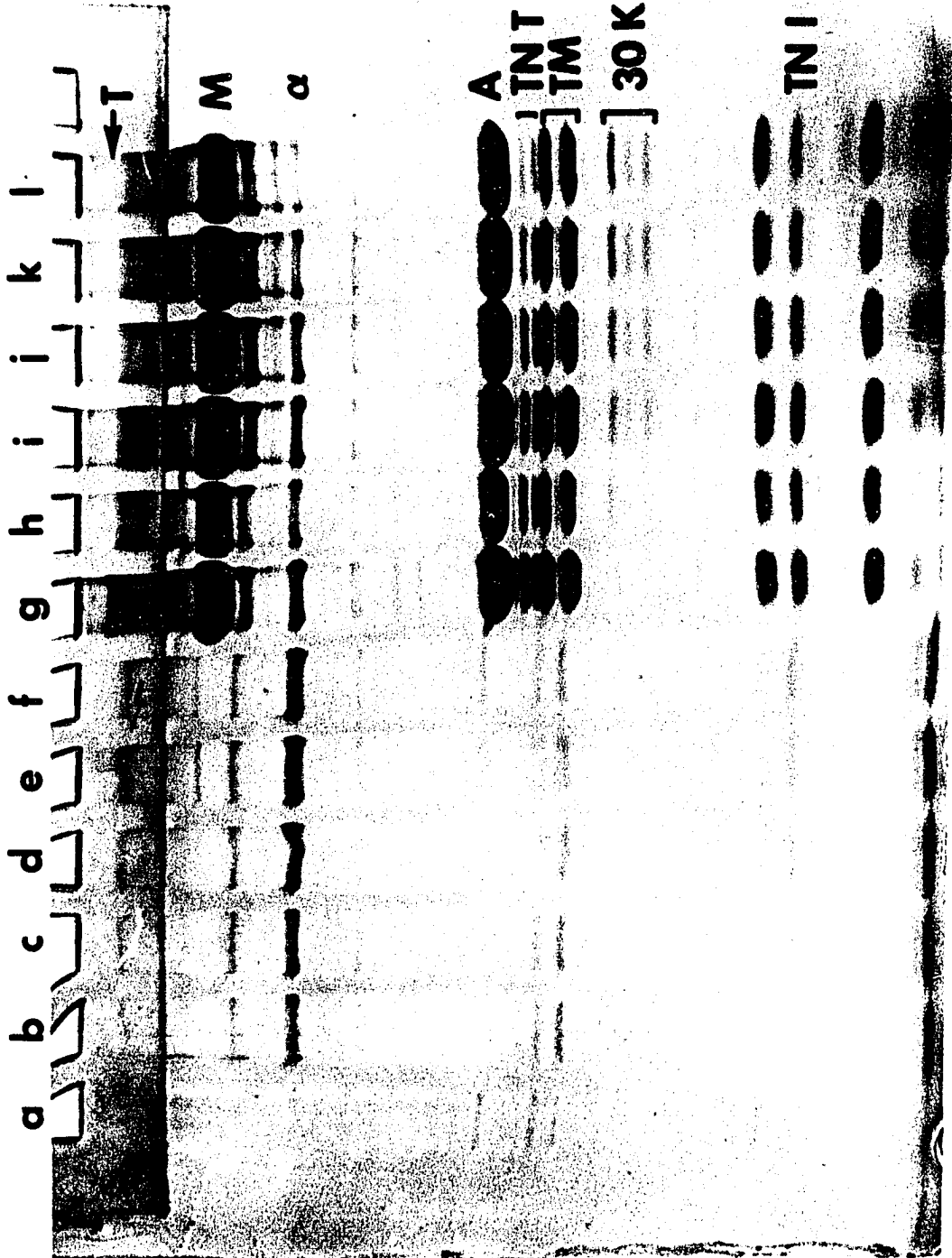


significant release of protein after only two min of incubation (lane b, Figure 12), alpha-actinin was the major constituent released, and there were a number of unidentified proteins with molecular weights greater than myosin heavy chains and one just below myosin heavy chains (lanes b-f, Figure 12). In lanes g to l (sediments), it was evident that the titin band decreased in intensity and that it apparently was degraded to smaller fragments that migrated just ahead of the position of intact titin with increase in incubation time. The alpha-actinin band in the sediments decreased in amount with increased time of incubation at the same time that it appeared in the supernatant fractions. The troponin T isomers initially were degraded slightly more slowly than at pH 7.5, but for unknown reasons they were seemingly degraded more completely than at pH 7.5 by the end of 30 min of incubation. Similarly, the tropomyosin bands were degraded initially more slowly, but more completely at the end of 30 min. The trio of bands at 30,000 daltons appeared at about 4 to 8 min of incubation. Troponin I appeared to be more completely degraded after 15 min of incubation at pH 7.0 than after 30 min at pH 7.5.

The effect of incubating myofibrils with CAF at 25° C and pH 6.5 is shown in Figure 13. The lanes (a-f) of the supernatants show that somewhat less protein was released from the myofibrils than in the experiments done at higher pH. The lanes run with the supernatants contain some unidentified protein, which appears after 4 to 8 min of incubation, migrating with a molecular weight greater than myosin heavy chains. Alpha-actinin appears to be the major constituent in the

Figure 13. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 25° C and pH 6.5

CAF assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 6.5, 0.1 mM EDTA, 5.1 mM CaCl₂, 5 mM MCE, were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 25° C. Lanes a through f were loaded with 50 μl of supernatant fractions corresponding to 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes g through l were loaded with 48 μg of sediment fractions from 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes a and g (0 min) correspond to EDTA control supernatant and sediment fractions, respectively (see Materials and Methods). Labels at the right edge of the gel are as follows: T, titin; M, myosin heavy chains; α, alpha-actinin; A, actin; TN T, a small bracket indicates troponin T isomers, TM, a bracket indicates beta and alpha tropomyosin subunits; 30 K, a bracket indicates the position of a trio of bands of approximately 30,000 daltons, if present; TN I, troponin I.



solubilized protein. In the lanes (g-1) run with the sediments, the titin band decreases in intensity and migrates as putative breakdown products with increased incubation time. Troponin T and tropomyosin appear to be more slowly degraded than at pH 7.0. The 30,000-dalton trio appeared after 8 to 15 min of incubation. Troponin I was only partly degraded after 30 min of incubation.

Figure 14 contains the electrophoretic results obtained on supernatants and sediments from myofibrils incubated with CAF at 25° C and pH 6.0. The lanes (b-f) with the supernatants show that a significant amount of protein was still solubilized during the digestion at pH 6.0 compared to the control (lane a). A considerable proportion of the released protein was unidentified high molecular weight material. The major single constituent of protein released was alpha-actinin. There was also some solubilization of intact actin, tropomyosin, troponin T, troponin I, etc. during the incubation at this pH. However, the intensity of these bands did not seem to change much with increased incubation time, which suggests that lowered pH alone may have been responsible for this result. The lanes (g-1) run with the sediments showed a decrease in amount of intact titin with increase in incubation time. The intensity of the alpha-actinin band decreased in the lanes run with sediments at the same time as it appeared in the lanes run with the supernatants. Troponin T was degraded, but at a slower rate than at the higher pH values. The trio of bands in the 30,000-dalton region again appeared after 4 to 8 min of incubation. Troponin I had been substantially degraded after 15 min of incubation.

Figure 14. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 25° C and pH 6.0

CAF assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 6.0, 0.1 mM EDTA, 5.1 mM CaCl₂, 5 mM MCE, were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 25° C. Lanes a through f were loaded with 50 μl of supernatant fractions corresponding to 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes g through l were loaded with 48 μg of sediment fractions from 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes a and g (0 min) correspond to EDTA control supernatant and sediment fractions, respectively (see Materials and Methods). Labels at the right edge of the gel are as follows: T, titin; M, myosin heavy chains; α, alpha-actinin; A, actin; TN T, a small bracket indicates troponin T isomers; TM, a bracket indicates beta and alpha tropomyosin subunits; 30 K, a bracket indicates the position of a trio of bands of approximately 30,000 daltons, if present; TN I, troponin I.

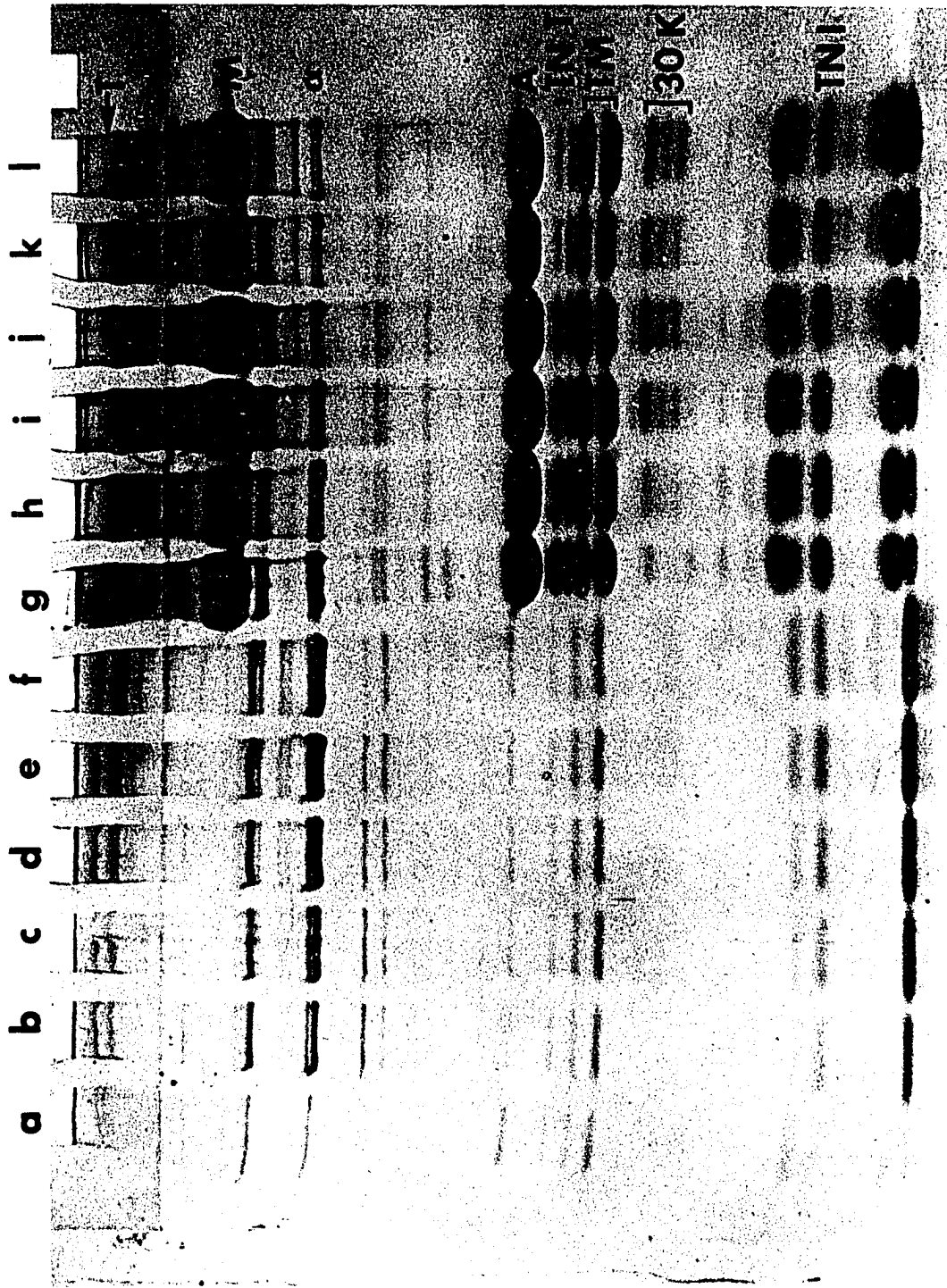
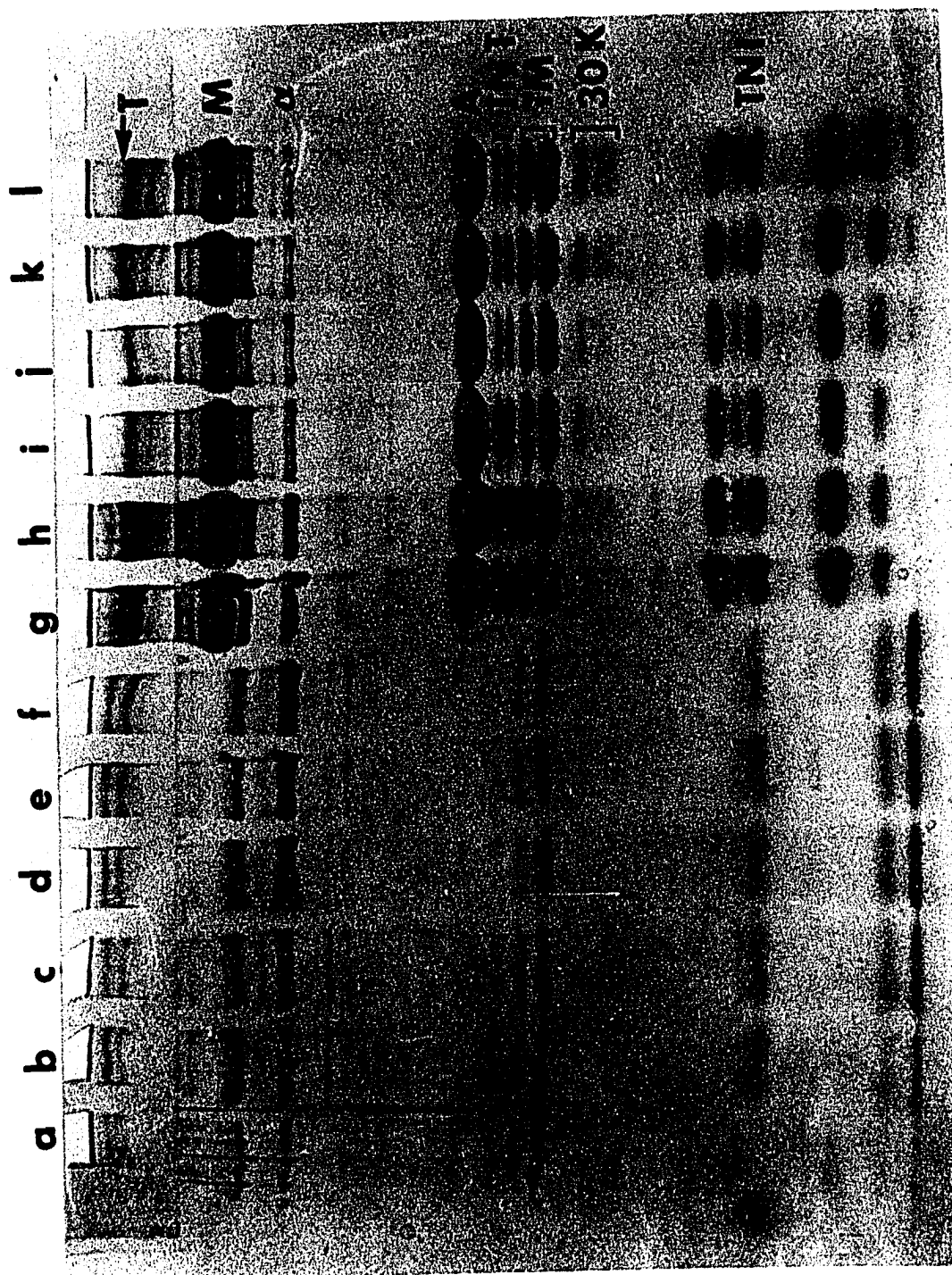


Figure 15 contains the electrophoretic results of supernatants and sediments from myofibrils incubated with CAF at 25° C and pH 5.5. The lanes (a-f) run with the supernatants show that a significant amount of myofibrillar protein was released during digestion with CAF and that it increased in amount with increase in incubation time. A significant portion of the released protein was unidentified high molecular weight material. The major constituent of the solubilized protein in the supernatants was alpha-actinin. As was evident in the digestion done at pH 6.0 (Figure 14), some proteins were released intact into the supernatant of even control samples (lane a) at this low pH. Some of the protein in the supernatants migrated with the dye front, as it also did in the supernatants of samples digested at the higher pH values. There was an increase in this material with increase in incubation time. The nature of this material was unknown, but it presumably was small molecular weight breakdown products of myofibrillar proteins. The lanes (g-l) run with the sediments show that the titin band decreased somewhat in intensity with incubation time and that putative titin breakdown products, just below the position of intact titin, appeared (c.f. lane l with g). The alpha-actinin band decreased in intensity in the sediments at the same time that it appeared in the supernatants. The troponin T bands still decreased in amount with increased incubation time. The trio of bands at about 30,000 daltons appeared somewhat later during the digestion at pH 5.5 than at higher pH values. Troponin I was slightly degraded.

Figure 15. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 25° C and pH 5.5

CAF assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 5.5, 0.1 mM EDTA, 5.1 mM CaCl₂, 5 mM MCE, were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 25° C. Lanes a through f were loaded with 50 μl of supernatant fractions corresponding to 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes g through l were loaded with 48 μg of sediment fractions from 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes a and g (0 min) correspond to EDTA control supernatant and sediment fractions, respectively (see Materials and Methods). Labels at the right edge of the gel are as follows: T, titin; M, myosin heavy chains; α, alpha-actinin; A, actin; TN T, a small bracket indicates troponin T isomers; TM, a bracket indicates beta and alpha tropomyosin subunits; 30 K, a bracket indicates the position of a trio of bands of approximately 30,000 daltons, if present; TN I, troponin I.



Figures 16 to 20 show the results of SDS-PAGE analysis of supernatants and sediments resulting from CAF digestion of myofibrils at 15° C and selected pH values. Figure 16 shows the results for the digestion done at pH 7.5. The lanes (a-f) with the supernatants exhibited several unidentified high molecular weight proteins and alpha-actinin. The lanes (g-l) of the sediments show that titin was degraded and that the amount of alpha-actinin was decreased as the digestion proceeded. Troponin T was partly degraded and the trio of bands at about 30,000 daltons appeared after 2 to 4 min of incubation. Tropomyosin and troponin I showed only a small amount of proteolysis during the 30-min incubation. In general, much more alpha-actinin remained in the sediments of the samples treated at 15° C (Figure 16) than when treated at 25° C (Figure 11).

The effect of incubation of myofibrils with CAF at 15° C and pH 7.0 is shown in Figure 17. The lanes (a-f) run with supernatants show that a significant amount of protein was solubilized and released into the supernatant during the digestion. A significant proportion of the released material was unidentified high molecular weight protein. The major single constituent of the solubilized protein was alpha-actinin. The lanes (g-l) run with the sediments show that titin was rapidly degraded, that alpha-actinin was decreased, and that troponin T was significantly degraded by about 15 min of incubation. Tropomyosin and troponin I were slightly degraded and the trio of bands at about 30,000 daltons appeared after about 8 to 15 min of incubation.

Figure 16. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 15° C and pH 7.5

CAF assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 7.5, 0.1 mM EDTA, 5.1 mM CaCl₂, 5 mM MCE, were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 15° C. Lanes a through f were loaded with 50 μl of supernatant fractions corresponding to 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes g through l were loaded with 48 μg of sediment fractions from 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes a and g (0 min) correspond to EDTA control supernatant and sediment fractions, respectively (see Materials and Methods). Labels at the right edge of the gel are as follows: T, titin; M, myosin heavy chains; α, alpha-actinin; A, actin; TN T, a small bracket indicates troponin T isomers; TM, a bracket indicates beta and alpha tropomyosin subunits; 30 K, a bracket indicates the position of a trio of bands of approximately 30,000 daltons, if present; TN I, troponin I.

a b c d e f g h i j k l

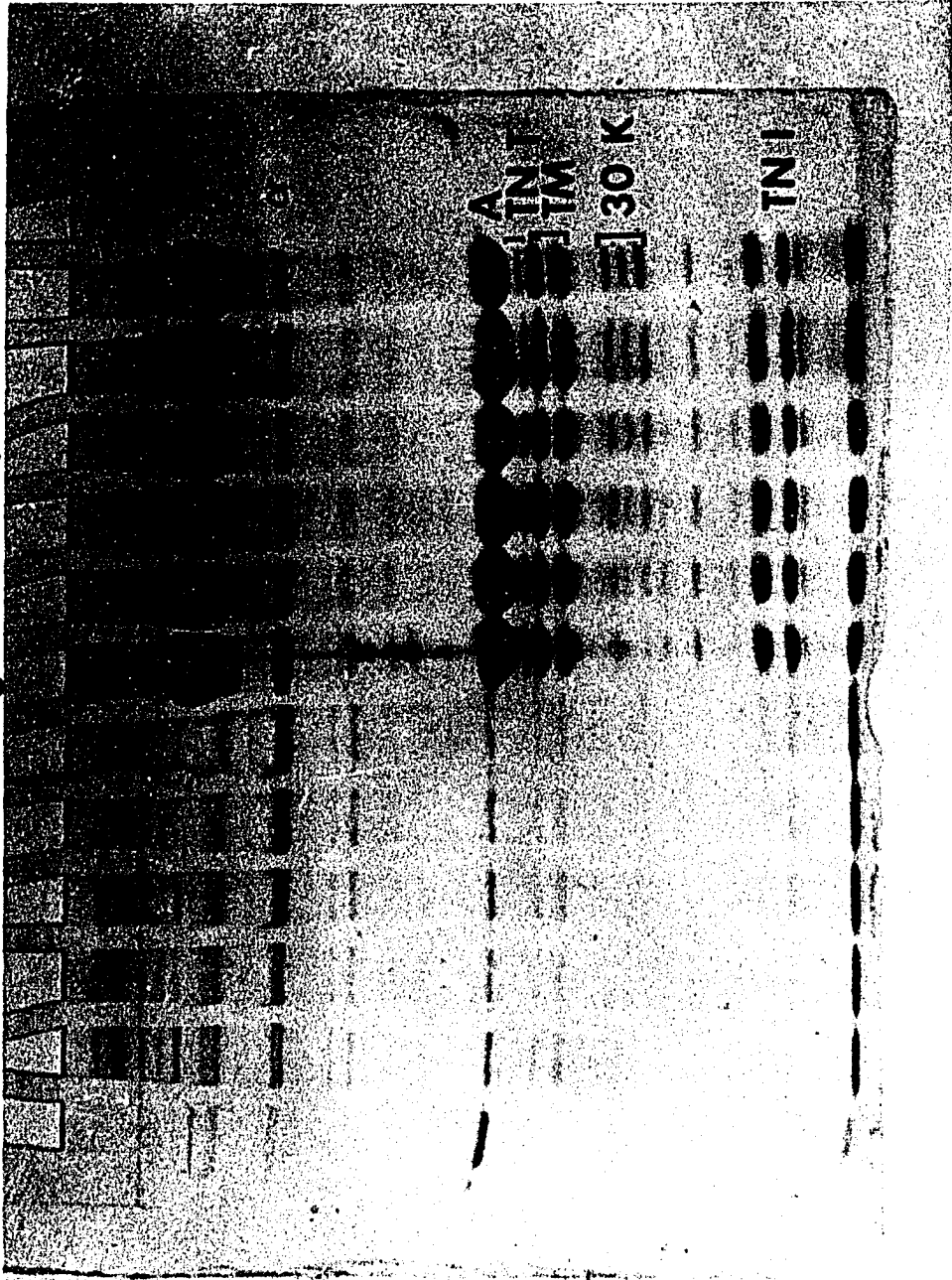


Figure 17. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 15° C and pH 7.0

CAF assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 7.0, 0.1 mM EDTA, 5.1 mM CaCl₂, 5 mM MCE, were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 15° C. Lanes a through f were loaded with 50 μl of supernatant fractions corresponding to 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes g through l were loaded with 48 μg of sediment fractions from 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes a and g (0 min) correspond to EDTA control supernatant and sediment fractions, respectively (see Materials and Methods). Labels at the right edge of the gel are as follows: T, titin; M, myosin heavy chains; α, alpha-actinin; A, actin; TN T, a small bracket indicates troponin T isomers; TM, a bracket indicates beta and alpha tropomyosin subunits; 30 K, a bracket indicates the position of a trio of bands of approximately 30,000 daltons, if present; TN I, troponin I.

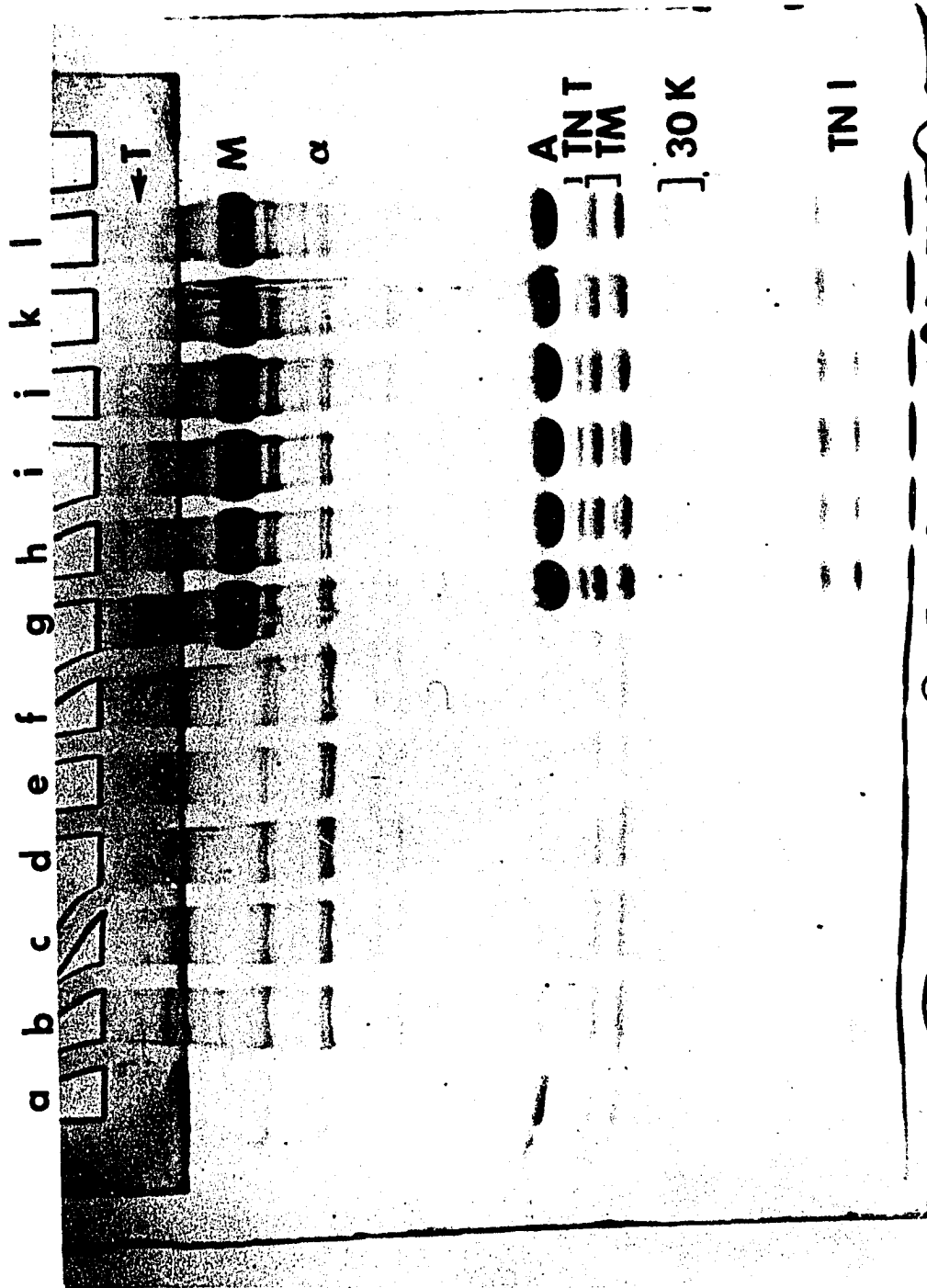


Figure 18 shows the electrophoretic results obtained on supernatants and sediments from myofibrils incubated with CAF at 15° C and pH 6.5. The lanes (a-f) of the supernatants show that somewhat less protein was solubilized than at the higher pH values. The unidentified high molecular weight proteins appeared after about 8 to 15 min of incubation. The major single constituent in the released protein fraction was alpha-actinin. The lanes (g-l) of the sediments show that titin was degraded to lower molecular weight species and that the alpha-actinin band decreased in amount with increase in time of digestion. Troponin T was only partly degraded during the 30-min incubation and tropomyosin and troponin I were degraded only slightly. The trio of bands at about 30,000 daltons appeared after about 8 to 15 min of incubation.

Figure 19 contains the electrophoretic results obtained on supernatants and sediments from myofibrils incubated with CAF at 15° C and pH 6.0. The lanes (a-f) run with the supernatants show that only a small amount of protein was solubilized by CAF. There was a small amount of unidentified high molecular weight protein solubilized. The unidentified species with a molecular weight of about 170,000 was prominent. It did not, however, increase in amount with increase in incubation time. The major single constituent released into the supernatant was alpha-actinin. The lanes (g-l) run with the sediments show that the intact titin band was again decreased in intensity and that it was apparently degraded to lower molecular weight fragments during the 30-min digestion. The alpha-actinin band was reduced in

Figure 18. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 15° C and pH 6.5

CAF assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 6.5, 0.1 mM EDTA, 5.1 mM CaCl₂, 5 mM MCE, were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 15° C. Lanes a through f were loaded with 50 μl of supernatant fractions corresponding to 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes g through l were loaded with 48 μg of sediment fractions from 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes a and g (0 min) correspond to EDTA control supernatant and sediment fractions, respectively (see Materials and Methods). Labels at the right edge of the gel are as follows: T, titin; M, myosin heavy chains; α, alpha-actinin; A, actin; TN T, a small bracket indicates troponin T isomers; TM, a bracket indicates beta and alpha tropomyosin subunits; 30 K, a bracket indicates the position of a trio of bands of approximately 30,000 daltons, if present; TN I, troponin I.

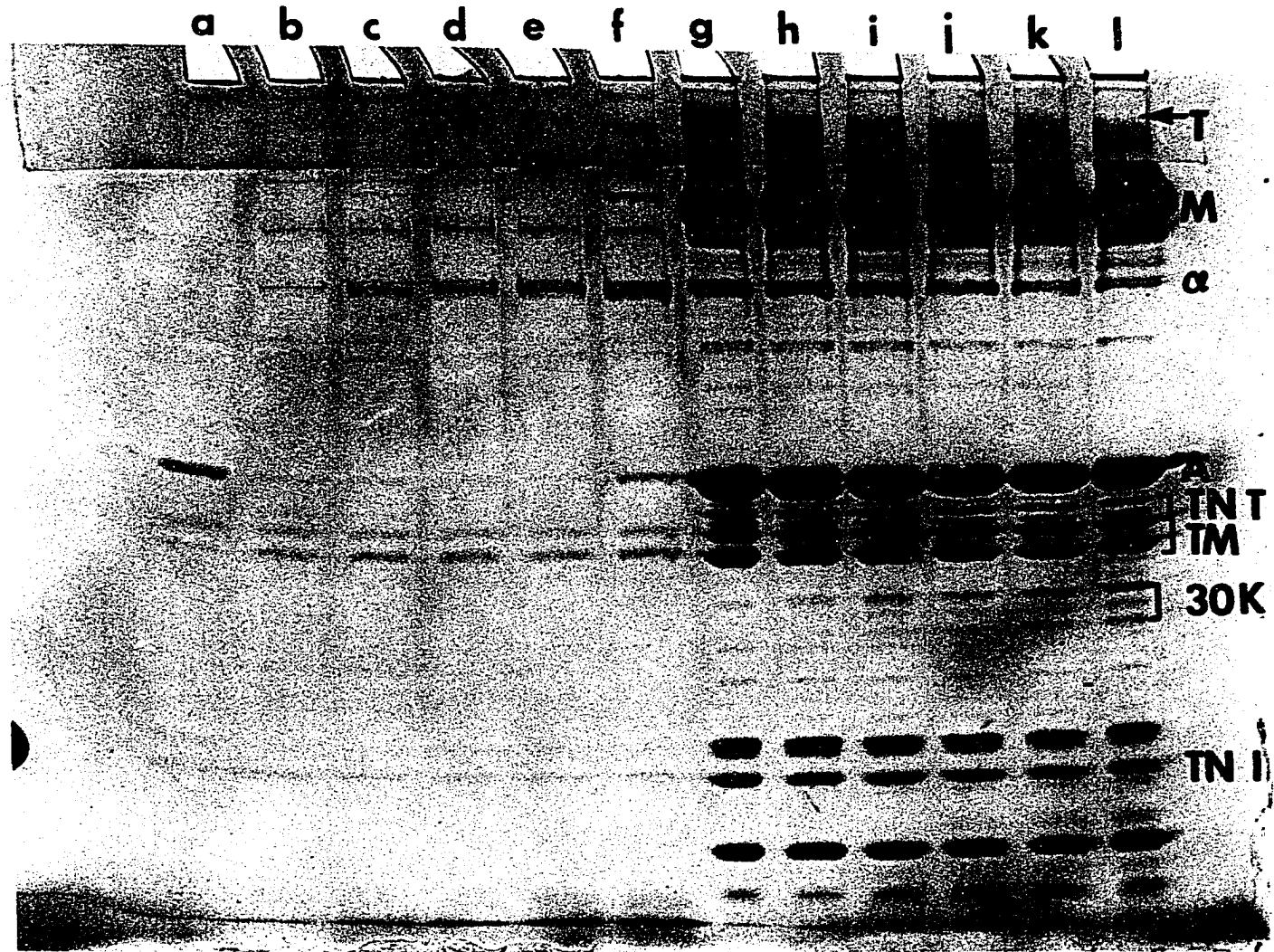
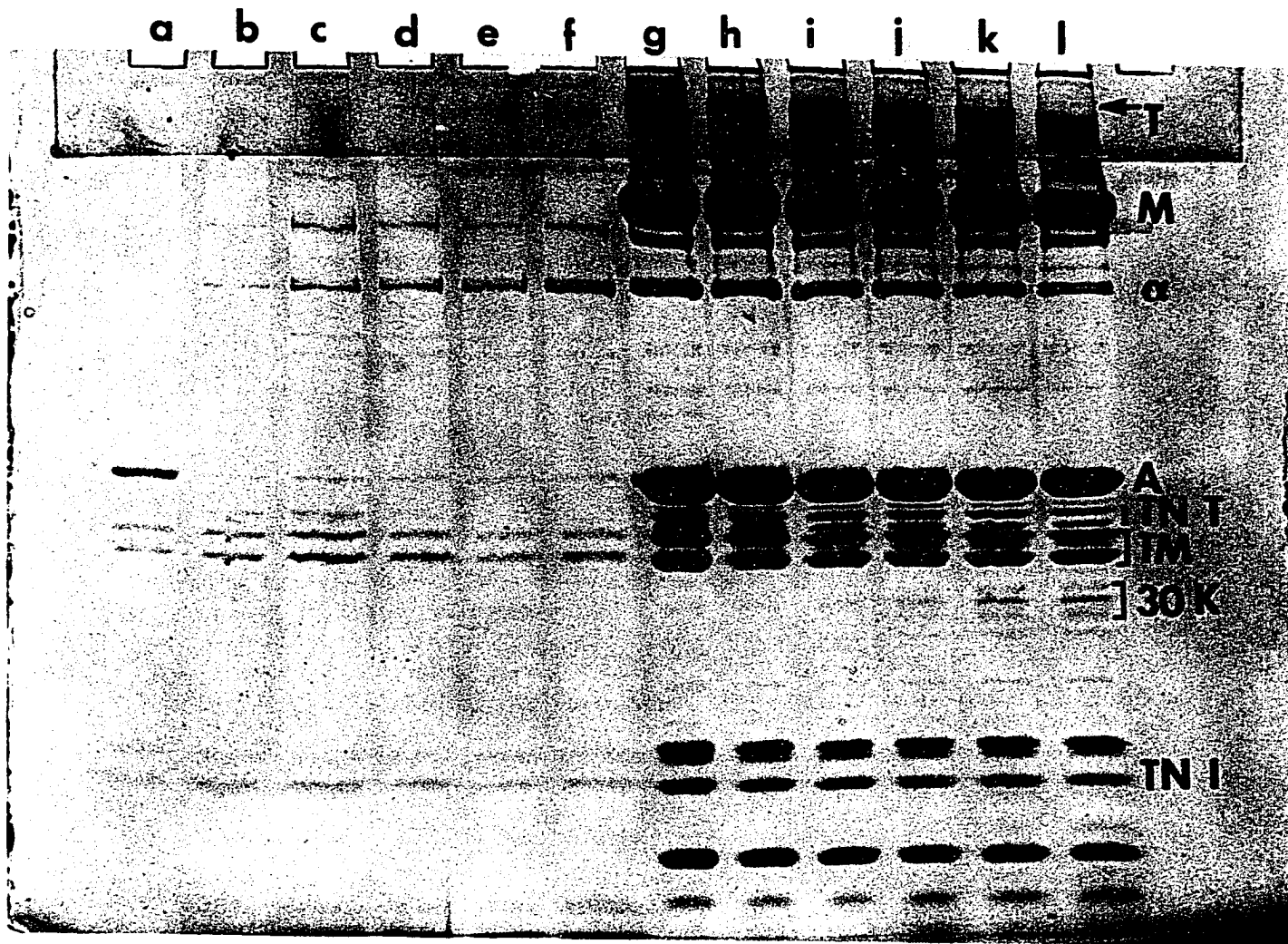


Figure 19. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 15° C and pH 6.0

CAF assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 6.0, 0.1 mM EDTA, 5.1 mM CaCl₂, 5 mM MCE, were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 15° C. Lanes a through f were loaded with 50 μl of supernatant fractions corresponding to 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes g through l were loaded with 48 μg of sediment fractions from 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes a and g (0 min) correspond to EDTA control supernatant and sediment fractions, respectively (see Materials and Methods). Labels at the right edge of the gel are as follows: T, titin; M, myosin heavy chains; α, alpha-actinin; A, actin; TN T, a small bracket indicates troponin T isomers; TM, a bracket indicates beta and alpha tropomyosin subunits; 30 K, a bracket indicates the position of a trio of bands of approximately 30,000 daltons, if present; TN I, troponin I.

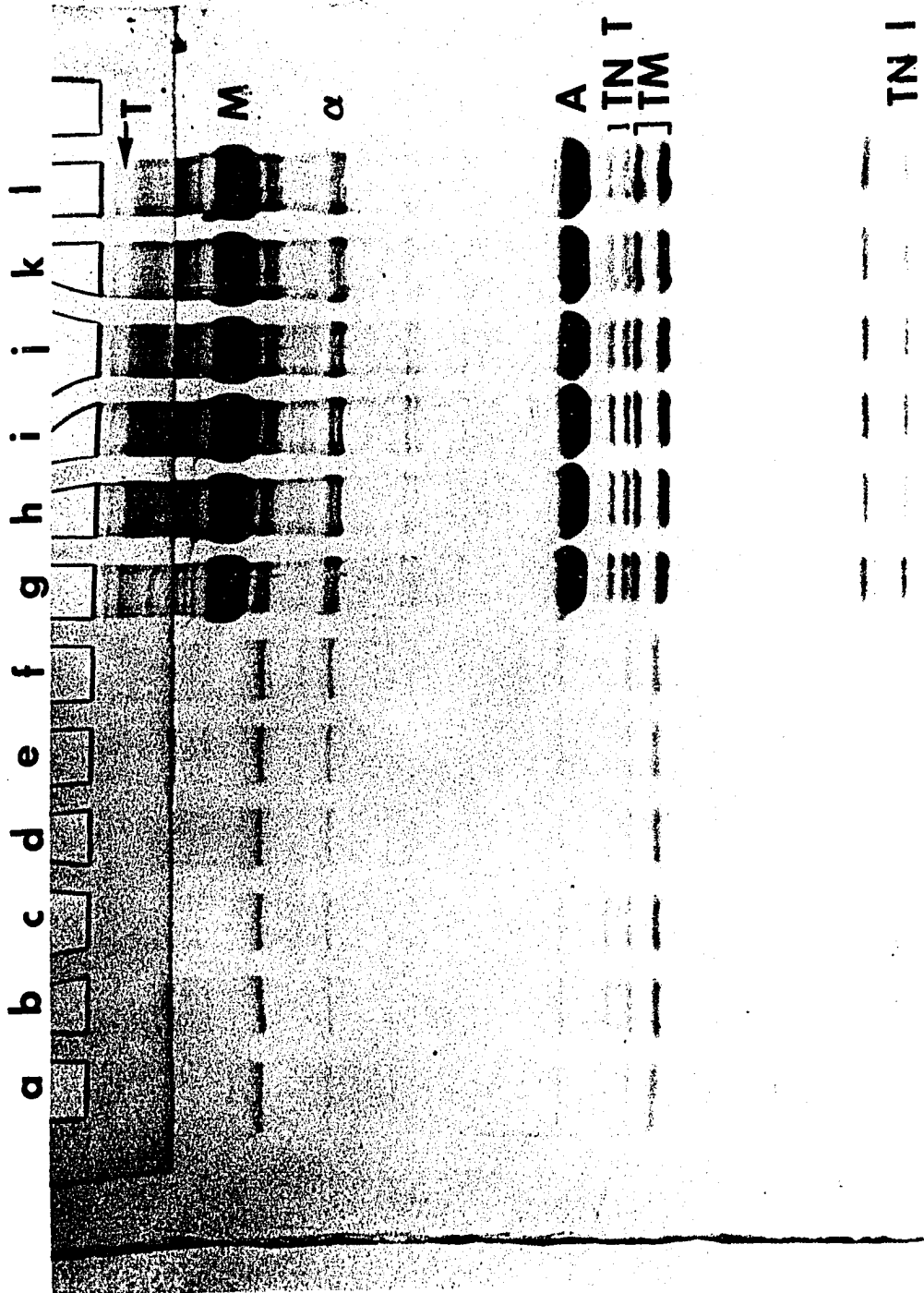


intensity, but not to the degree it was in the digestion done at higher pH values. The troponin T was only partly degraded after 30 min of incubation. The subunits of tropomyosin and the troponin I were only slightly degraded. The three bands at about 30,000 daltons appeared in small quantity near the end of the incubation. As in the case of the incubations done at 25° C and the lower pH values (Figures 14 and 15), a considerable number of the bands in the supernatant samples (including the control) migrated at the same rate as proteins in the sediment, which suggests that lowered pH alone was causing release of some intact proteins (e.g., actin, tropomyosin).

Figure 20 shows the electrophoretic results obtained on supernatants and sediments from myofibrils incubated with CAF at 15° C and pH 5.5. The lanes (b-f) of the supernatant show little protein was solubilized compared to the control supernatant (lane a). Alpha-actinin was released to a very small degree. The unidentified species (possibly M-protein) at about 170,000 daltons was a significant component in all of the supernatant samples, including the control (lane a). The lanes (g-l) of the sediments showed that there was some degradation of the titin band during 30 min of incubation. There was only a small decrease in amount of the alpha-actinin band. Troponin T was only slightly degraded by the end of 30 min of incubation. There was little evidence of bands appearing in the 30,000-dalton region during the digestion. Tropomyosin and troponin I were digested very little.

Figure 20. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 15° C and pH 5.5

CAF assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 5.5, 0.1 mM EDTA, 5.1 mM CaCl₂, 5 mM MCE, were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 15° C. Lanes a through f were loaded with 50 μl of supernatant fractions corresponding to 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes g through l were loaded with 48 μg of sediment fractions from 0, 2, 4, 8, 15, and 30 min incubation, respectively. Lanes a and g (0 min) correspond to EDTA control supernatant and sediment fractions, respectively (see Materials and Methods). Labels at the right edge of the gel are as follows: T, titin; M, myosin heavy chains; α, alpha-actinin; A, actin; TN T, a small bracket indicates toponin T isomers; TM, a bracket indicates beta and alpha tropomyosin subunits; 30 K, a bracket indicates the position of a trio of bands of approximately 30,000 daltons, if present; TN I, troponin I.



Figures 21 to 25 show the results of SDS-PAGE analysis of supernatants and sediments resulting from CAF digestion of myofibrils at 5° C at selected pH values. Figure 21 shows the results for the digestion done at pH 7.5. The lanes (a-f) run with the supernatant samples show that only a small amount of protein was solubilized. There were some unidentified high molecular weight proteins and alpha-actinin. The latter protein was quite evident after 8 to 15 min of incubation. The lanes (g-l) of the sediments show that titin was degraded during the 30 min of incubation. As expected, there was a small decrease in the amount of the alpha-actinin band with increased incubation time. A small decrease in troponins T and I occurred as well. The trio of degradation bands at about 30,000 daltons was present in minor amounts after 15 to 30 min of incubation.

The effect of incubation of CAF with myofibrils at 5° C and pH 7.0 is shown in Figure 22. The lanes (a-f) of the supernatants again show that only a small amount of protein was solubilized by CAF digestion. It consisted, in part, of unidentified high molecular weight material and alpha-actinin, which were most easily seen after 8 to 15 min of incubation. The lanes (g-l) of the sediments show that titin was degraded to smaller fragments during the 30 min of incubation. There was a slight decrease in the intensity of the tropomyosin, the troponin T, and troponin I bands after about 30 min of incubation. The trio of bands at about 30,000 daltons appeared, but only near the end of the digestion and in very small amounts.

Figure 21. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 5° C and pH 7.5

CAF assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 7.5, 0.1 mM EDTA, 5.1 mM CaCl₂, 5 mM MCE, were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 5° C. Lanes a through f were loaded with 50 μl of supernatant fractions corresponding to 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes g through l were loaded with 48 μg of sediment fractions from 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes a and g (0 min) correspond to EDTA control supernatant and sediment fractions, respectively (see Materials and Methods). Labels at the right edge of the gel are as follows: T, titin; M, myosin heavy chains; α, alpha-actinin; A, actin; TN T, a small bracket indicates troponin T isomers; TM, a bracket indicates beta and alpha tropomyosin subunits; 30 K, a bracket indicates the position of a trio of bands of approximately 30,000 daltons, if present; TN I, troponin I.

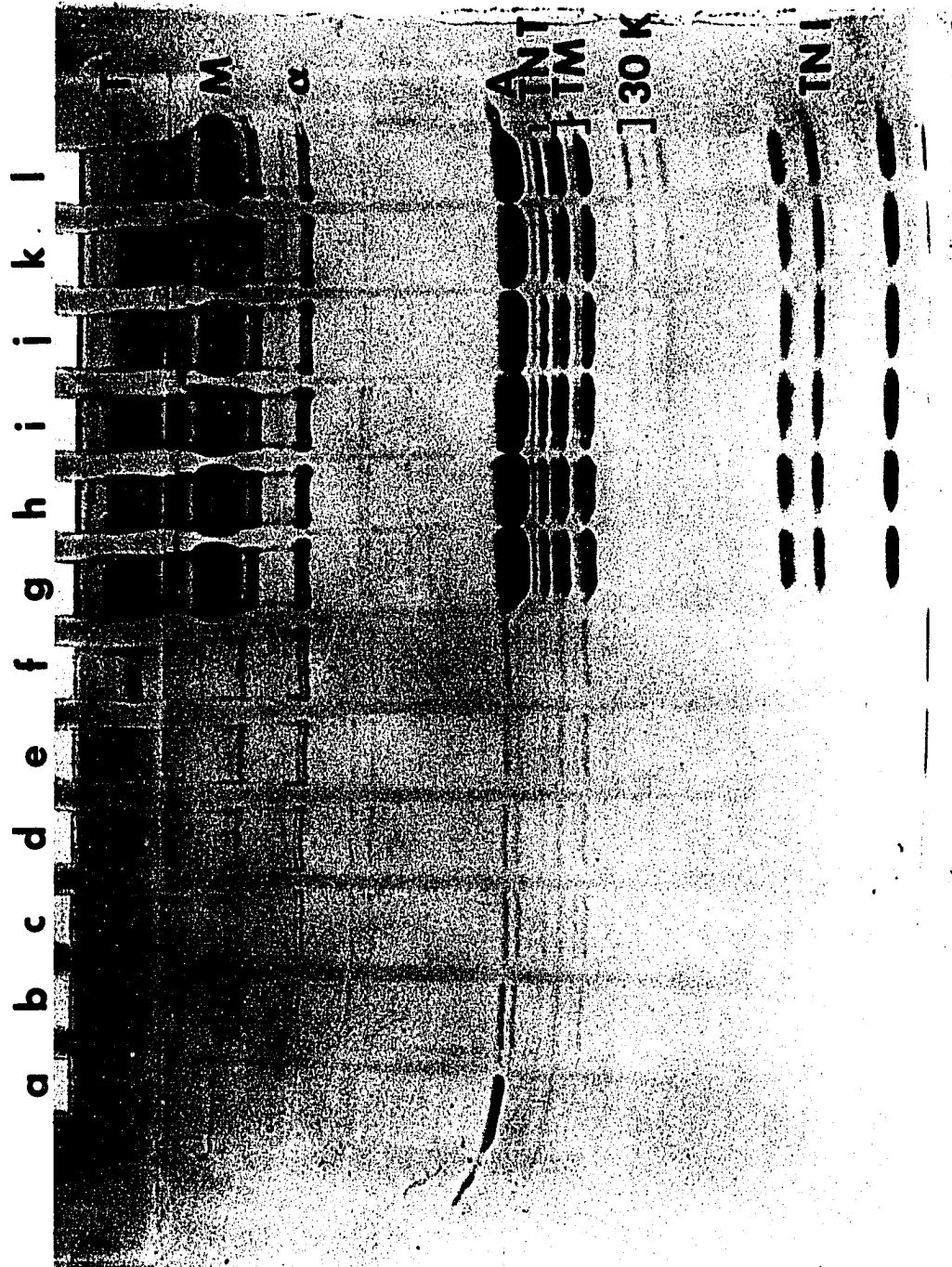


Figure 22. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 5° C and pH 7.0

CAF assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 7.0, 0.1 mM EDTA, 5.1 mM CaCl₂, 5 mM MCE, were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 5° C. Lanes a through f were loaded with 50 μl of supernatant fractions corresponding to 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes g through l were loaded with 48 μg of sediment fractions from 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes a and g (0 min) correspond to EDTA control supernatant and sediment fractions, respectively (see Materials and Methods). Labels at the right edge of the gel are as follows: T, titin; M, myosin heavy chains; α, alpha-actinin; A, actin; TN T, a small bracket indicates troponin T isomers; TM, a bracket indicates beta and alpha tropomyosin subunits; 30 K, a bracket indicates the position of a trio of bands of approximately 30,000 daltons, if present; TN I, troponin I.

Figure 23 shows the electrophoretic results obtained on supernatants and sediments from myofibrils incubated with CAF at 5° C and pH 6.5. The lanes (b-f) of the supernatants show that only a slight amount of protein was solubilized compared to the control supernatant (lane a). The proteins that were released included alpha-actinin and the unidentified high molecular weight material. The lanes (g-l) of the sediments show that titin was degraded to lower molecular weight fragments during the incubation. The alpha-actinin and troponin T bands were slightly reduced in amount after 30 min of incubation. There was no significant amount of bands in the 30,000-dalton region of the gel, even after 30 min of incubation.

Figure 24 contains the electrophoretic results obtained on supernatants and sediments from myofibrils incubated with CAF at 5° C and pH 6.0. The lanes (a-f) with the supernatant samples show that almost no protein was solubilized by the CAF treatment. A small amount of alpha-actinin could be seen after 8 to 15 min of incubation. The lanes with the sediments show that titin was slowly degraded to lower molecular weight fragments. Other changes were very small.

Figure 25 shows the electrophoretic results obtained on the supernatants (lanes a-f) and sediments (lanes g-l) from myofibrils incubated at 5° C and pH 5.5. The supernatant lanes show that little protein was solubilized by the CAF treatment when lanes b to f are compared with lane a (0-min control). A very slight amount of alpha-actinin was released after about 8 to 15 min of incubation. Most of the proteins in the supernatants (lanes b-f) co-migrated with bands in the control

Figure 23. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 5° C and pH 6.5

CAF assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 6.5, 0.1 mM EDTA, 5.1 mM CaCl₂, 5 mM MCE, were incubated with 40 µg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 5° C. Lanes a through f were loaded with 50 µl of supernatant fractions corresponding to 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes g through l were loaded with 48 µg of sediment fractions from 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes a and g (0 min) correspond to EDTA control supernatant and sediment fractions, respectively (see Materials and Methods). Labels at the right edge of the gel are as follows: T, titin; M, myosin heavy chains; α, alpha-actinin; A, actin; TN T, a small bracket indicates troponin T isomers; TM, a bracket indicates beta and alpha tropomyosin subunits; TN I, troponin I.

a b c d e f g h i j k l

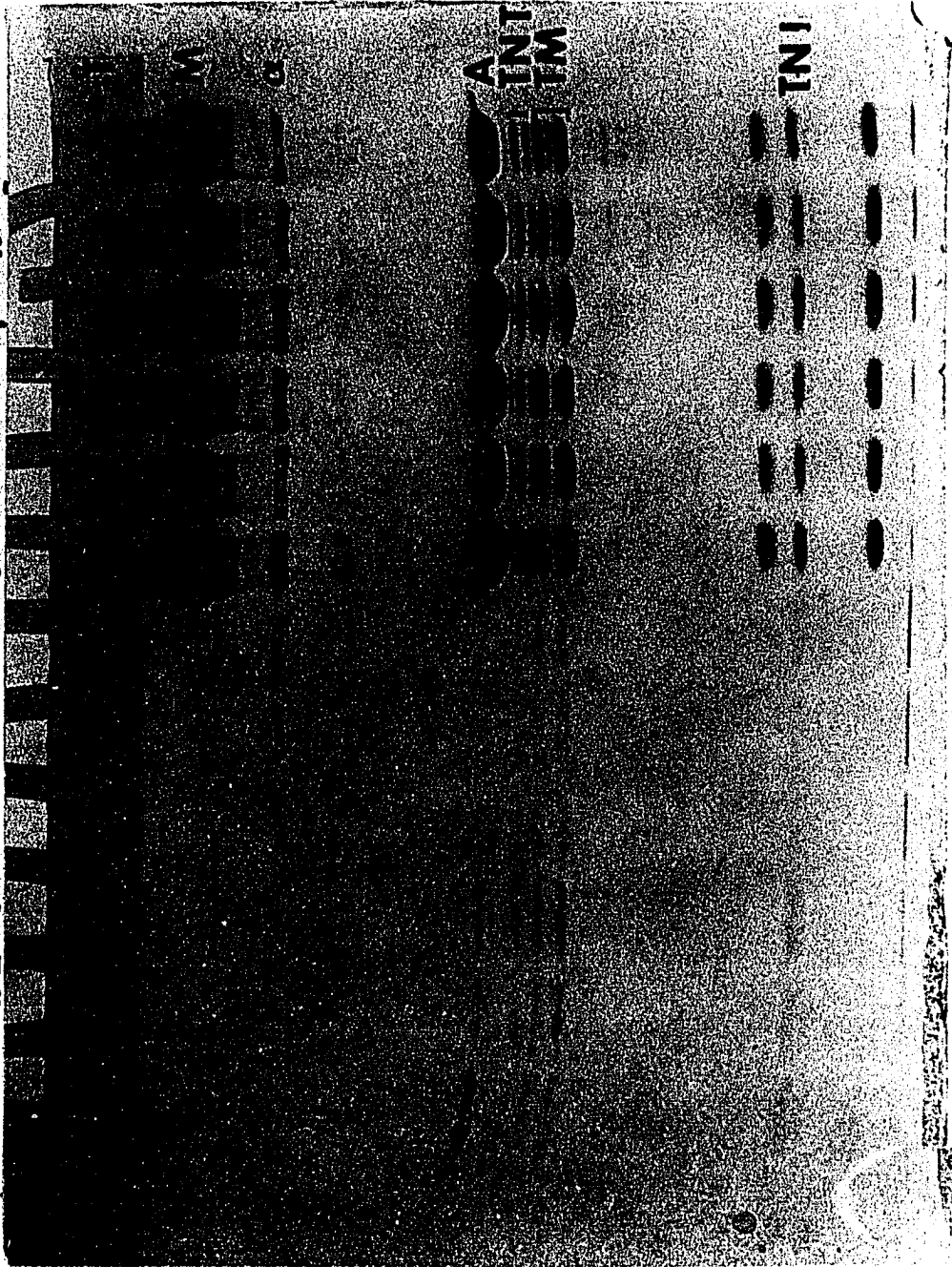


Figure 24. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 5° C and pH 6.0

CAF assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 6.0, 0.1 mM EDTA, 5.1 mM CaCl₂, 5 mM MCE, were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM and each sample was then centrifuged at 50,000 x ϵ_{max} for 15 min at 5° C. Lanes a through f were loaded with 50 μl of supernatant fractions corresponding to 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes g through l were loaded with 48 μg of sediment fractions from 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes a and g (0 min) correspond to EDTA control supernatant and sediment fractions, respectively (see Materials and Methods). Labels at the right edge of the gel are as follows: T, titin; M, myosin heavy chains; α , alpha-actinin; A, actin; TN T, a small bracket indicates troponin T isomers; TM, a bracket indicates beta and alpha tropomyosin subunits; TN I, troponin I.

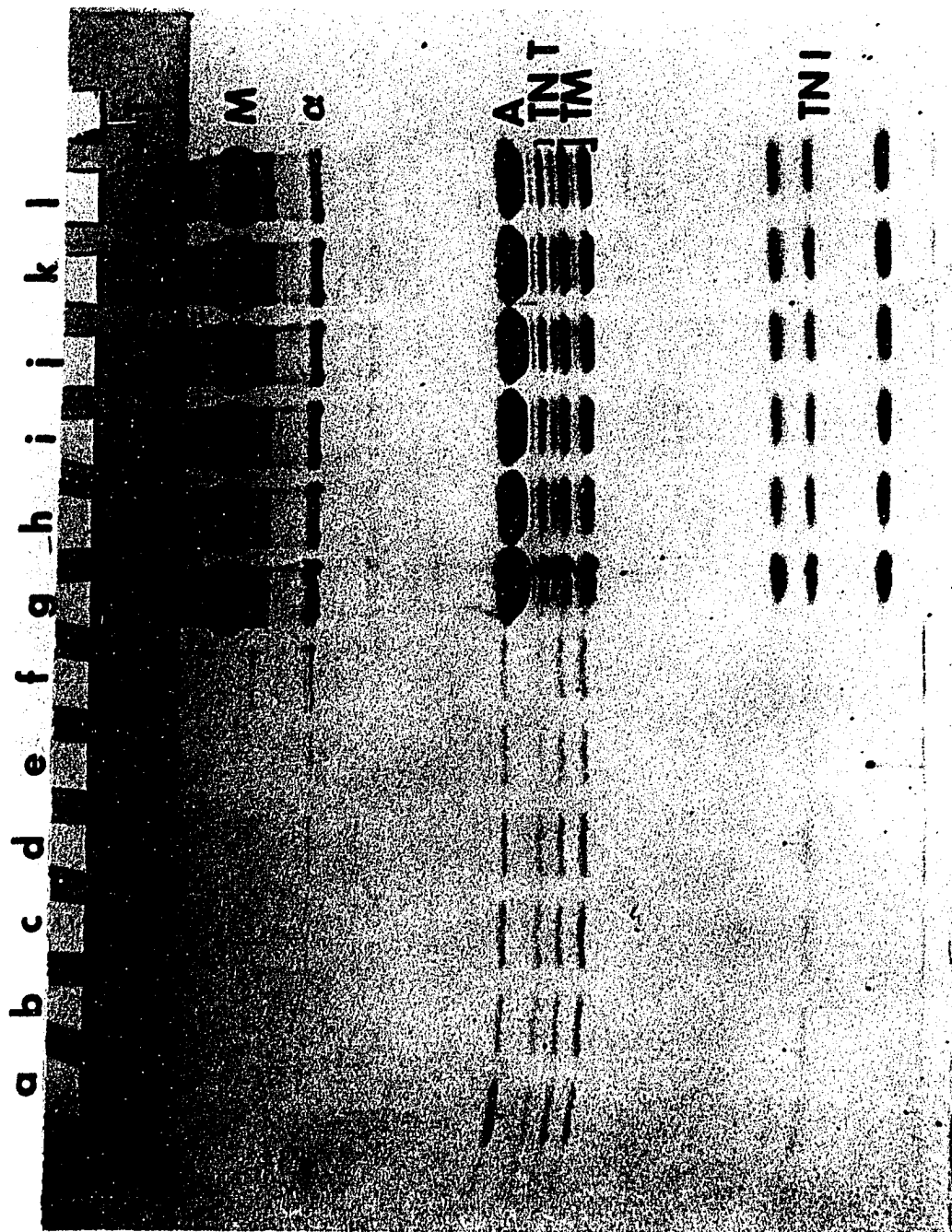
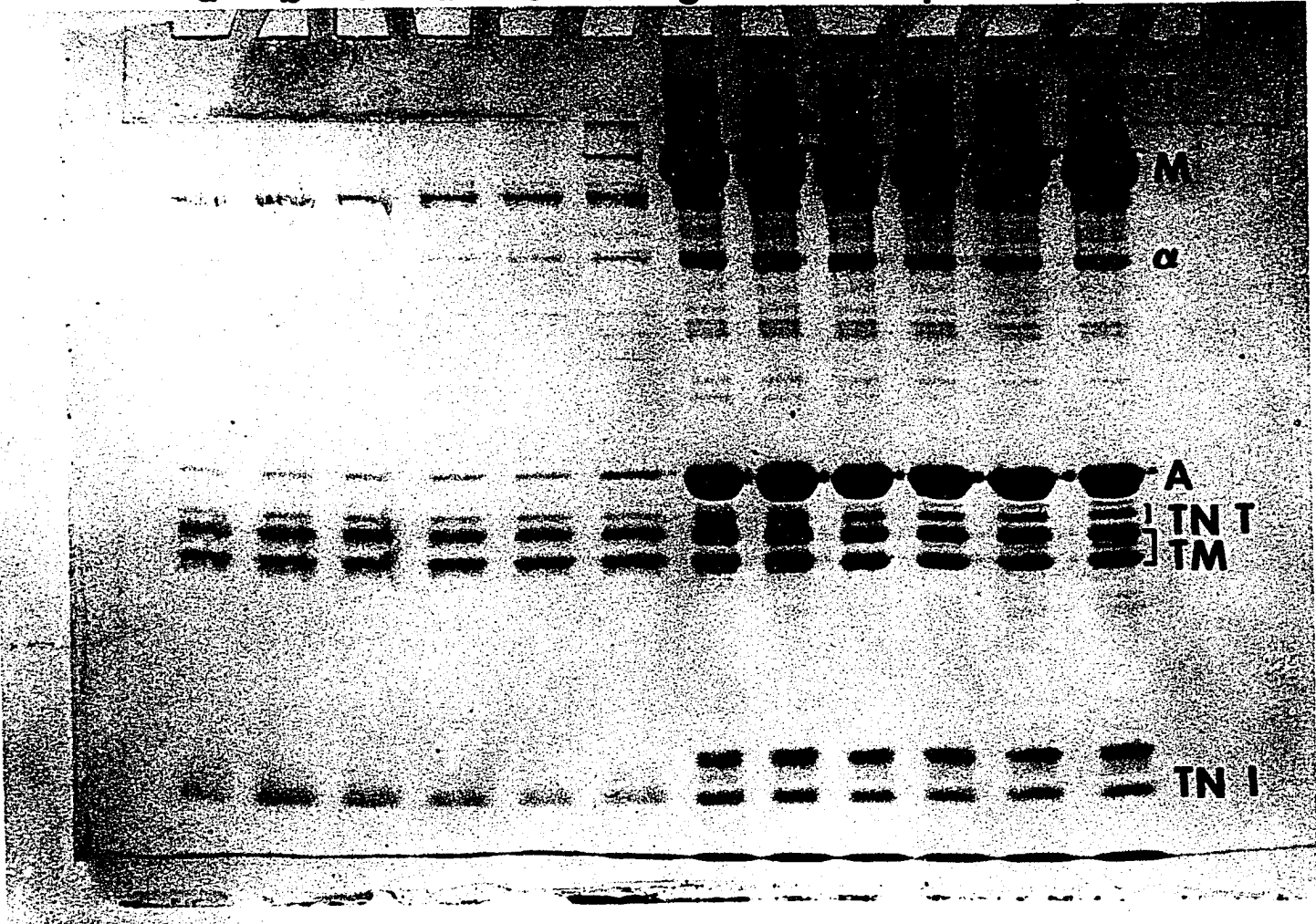


Figure 25. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified CAF at 5° C and pH 5.5

CAF assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 5.5, 0.1 mM EDTA, 5.1 mM CaCl₂, 5 mM MCE, were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 5° C. Lanes a through f were loaded with 50 μl of supernatant fractions corresponding to 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes g through l were loaded with 48 μg of sediment fractions from 0, 2, 4, 8, 15, and 30 min of incubation, respectively. Lanes a and g (0 min) correspond to EDTA control supernatant and sediment fractions, respectively (see Materials and Methods). Labels at the right edge of the gel are as follows: T, titin; M, myosin heavy chains; α, alpha-actinin; A, actin; TN T, a small bracket indicates troponin T isomers; TM, a bracket indicates beta and alpha tropomyosin subunits; TN I, troponin I.

a b c d e f g h i i k l



supernatant, suggesting that intact proteins were being released by the low pH treatment itself. The lanes (g-1) of the sediments show a slight change in the position of the titin band, suggesting it was still being slowly degraded under these conditions. Decreases in amounts of alpha-actinin, troponin T, and troponin I were all small.

3. Titin immunoblot of myofibril fractions after CAF treatment

The previous electrophoretic results indicated that titin was one of the proteins that was very susceptible to CAF-induced degradation. A preliminary study using an immunoblot technique was done in an attempt to localize the protein and/or proteolytic fragments in electrophoretic separations of myofibril fractions after CAF treatment. Figure 26 shows an immunoblot of a slab gel run with supernatants and sediments from myofibrils that had been incubated with CAF at 25^o C and pH 7.5. Figure 11 contains the conventionally stained electrophoretic gel of similar myofibril fractions incubated with CAF under identical conditions. In the immunoblot method, the samples were electrophoretically separated as shown in Figure 11 and then transferred to nitrocellulose paper as described in the Materials and Methods section. The blot was incubated with polyclonal antibodies to titin. In the supernatant lanes (b through f) in Figure 26, many bands are recognized by the titin antibody. No proteins were recognized by the antibody in the control supernatant (lane a). In lanes b through f, which contain supernatant fractions from myofibrils treated with CAF for increasing incubation times, the number of bands recognized by the titin antibody increases with increase in incubation time. And the population of bands shifts

Figure 26. Identification of titin and/or titin fragments by an immunoblot procedure

Supernatants and sediments from myofibrils that had been treated with CAF at 25° C and pH 7.5 were subjected to SDS-PAGE on a 10% acrylamide slab gel, containing a 5% stacking gel (refer to Figure 11 for a stained gel with similar samples). The immunoblot was prepared as described in the Materials and Methods section. The dark bands in the blot have been recognized by polyclonal antibodies to titin. Lanes a through f in the slab gel were loaded with 50 μ l of supernatant fractions corresponding to 0, 2, 4, 8, 15, and 30 min incubations, respectively. Lanes g through l were loaded with 48 μ g of corresponding sediment fractions from 0, 2, 4, 8, 15, and 30 min incubations, respectively. The letter T at the right edge of the gel indicates the position of titin in the control sediment (lane g).

a . b c d e f g h i j k l

← T



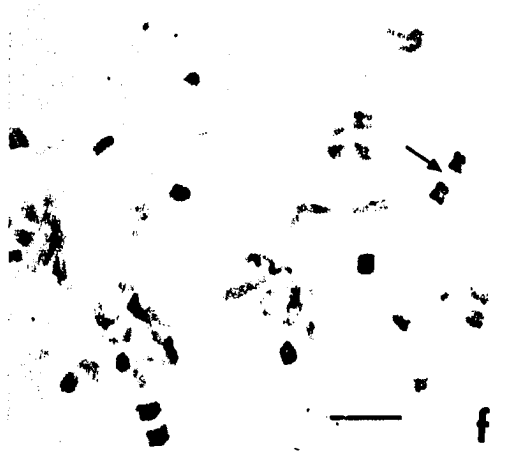
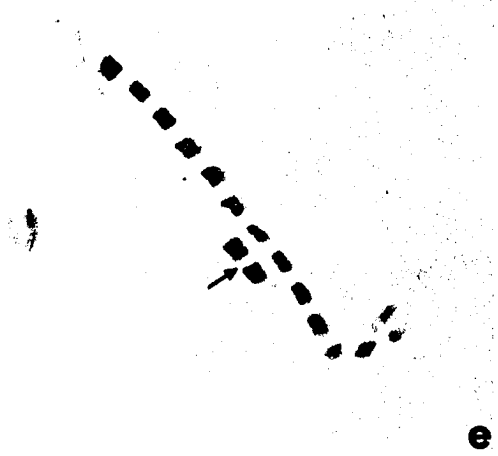
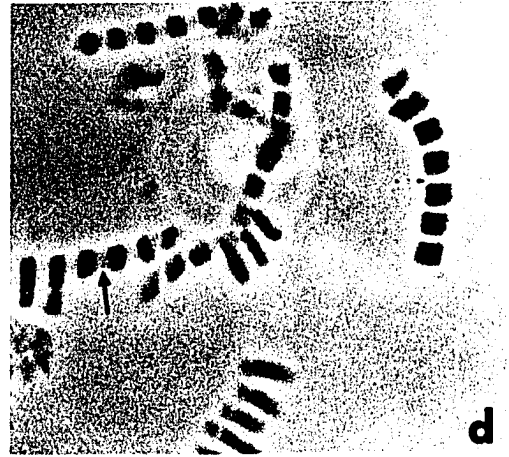
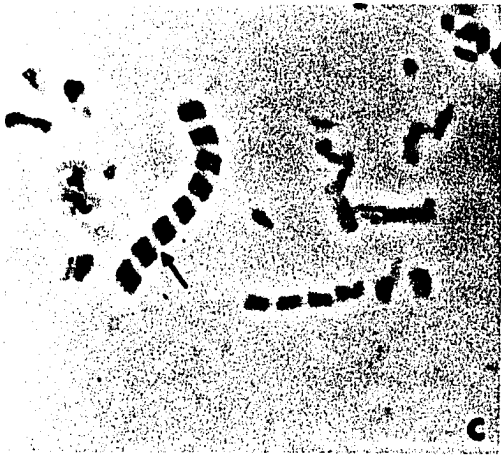
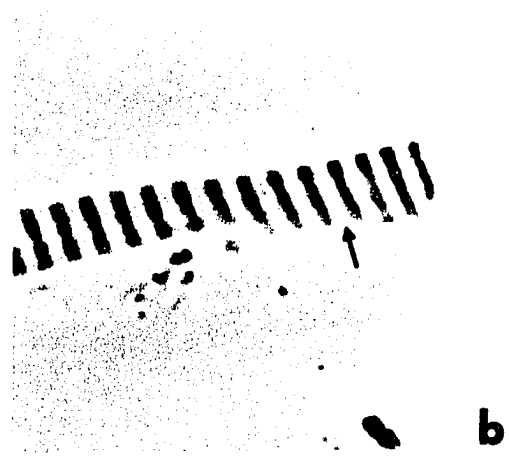
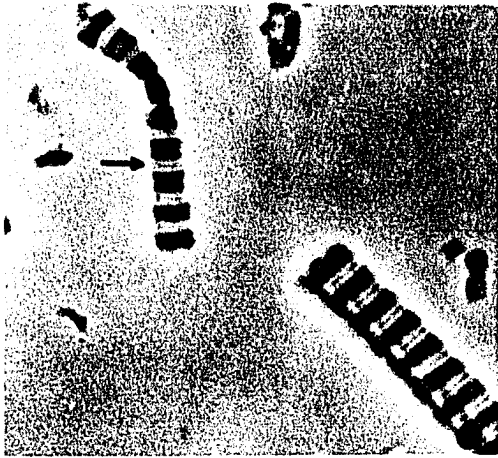
from a few heavily labeled bands present in samples from the short incubations to a greater number of bands of lower molecular weight present in samples from longer incubations (c.f., lane b with lanes e and f). This pattern is typical of what might be expected in a proteolytic digestion as larger polypeptides are degraded to smaller ones. Lanes g through l contain the sediment fractions of myofibrils after corresponding incubation times with CAF. Lane g shows the position of titin in the control (0-time). This band decreases in intensity and migrates (slightly faster mobility) slightly faster with increasing incubation time. The nature of the two or three other bands labeled in the lanes with the sediments is unknown.

4. Fragmentation of myofibrils following CAF digestion

CAF also was examined systematically with respect to its effectiveness in altering the structural integrity of the myofibril under varying conditions of pH and temperature. Following incubation with CAF under selected conditions of time, temperature, and pH, the myofibrils were subjected to a brief homogenization (10 sec) and then examined in the phase contrast microscope to ascertain the degree of fragmentation caused by CAF. Figure 27 contains representative phase contrast micrographs of myofibrils that had incubated with CAF for selected periods of time at 25° C and pH 7.0 and then homogenized. Figure 27a shows the appearance of the 0-min (EDTA control) myofibrils, which tend to be fairly long and exhibit intact Z lines. The position of a Z line or the position from which the Z line has been removed is indicated in every figure by an arrow. Figure 27b through f show the

Figure 27. Phase contrast micrographs of myofibrils after incubation with purified CAF at 25° C and pH 7.0 and a brief homogenization

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 7.0, 0.1 mM EDTA, 5.1 mM CaCl₂, 5.0 mM MCE, at 25° C were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM. One ml of this suspension was diluted with 1.0 ml of 100 mM KCl, and then homogenized for 10 sec at 15,000 rpm as described in the Materials and Methods section. Micrographs a through f represent 0 (EDTA control), 2, 4, 8, 15, and 30 min incubations, respectively. The arrow in each indicates the position of a Z line or the position from which the Z line has been removed. The bar in micrograph f represents 5 μm. Magnification was X2000 in all figures.



appearance of myofibrils that have been incubated with CAF for 2, 4, 8, 15, and 30 min, respectively. The Z lines of the myofibrils disappear quickly (Figure 27b). By the end of 30 min of incubation with CAF, the myofibrils appeared highly fragmented (Figure 27f). Many fragments containing less than three or four sarcomeres were evident. Perhaps because of the presence of some myofibril "clumps," which may have partly resisted CAF's action, some variability in appearance of myofibrils appeared in all samples viewed in this study. For example, some myofibrils could be found with intact Z lines when most of the myofibrils exhibited no Z lines and were highly fragmented.

Figure 28 contains phase contrast micrographs of myofibrils incubated with CAF for selected periods of time at 25^o C and pH 6.0 and then homogenized. Figure 28a shows the appearance of the 0-min (EDTA control) sample in which the myofibril fragments tend to be long with intact Z-line structures. Figure 28b through f show the appearance of myofibrils after increasing incubation times. The Z lines generally had disappeared after only a short time (2-4 min) of incubation (Figure 28b and c). By the end of the 30 min of incubation (Figure 28f), there was a marked increase in degree of fragmentation. The myofibrils also occasionally exhibited a rather distorted or stretched appearance following CAF treatment and homogenization such as that shown in Figure 28d (note the myofibril on the left).

Figure 29 contains phase contrast micrographs of myofibrils incubated with CAF for selected periods of time at 15^o C and pH 7.5 and then homogenized. Figure 29a contains the 0-min (EDTA control)

Figure 28. Phase contrast micrographs of myofibrils after incubation with purified CAF at 25° C and pH 6.0 and a brief homogenization

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 6.0, 0.1 mM EDTA, 5.1 mM CaCl₂, 5.0 mM MCE, at 25° C were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM. One ml of this suspension was diluted with 1.0 ml of 100 mM KCl, and then homogenized for 10 sec at 15,000 rpm as described in the Materials and Methods section. Micrographs a through f represent 0 (EDTA control), 2, 4, 8, 15, and 30 min incubations, respectively. The arrow in each indicates the position of a Z line or the position from which the Z line has been removed. The bar in micrograph f represents 5 μm. Magnification was X2000 in all figures.

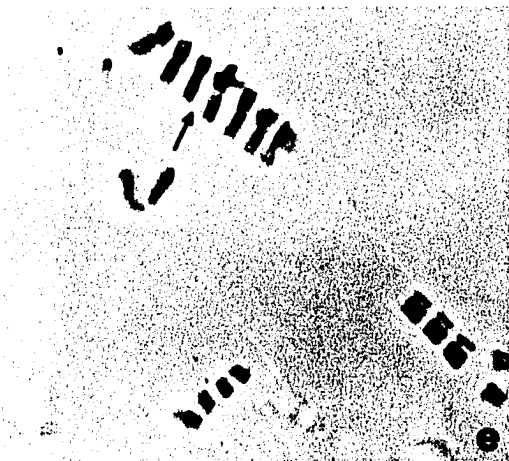
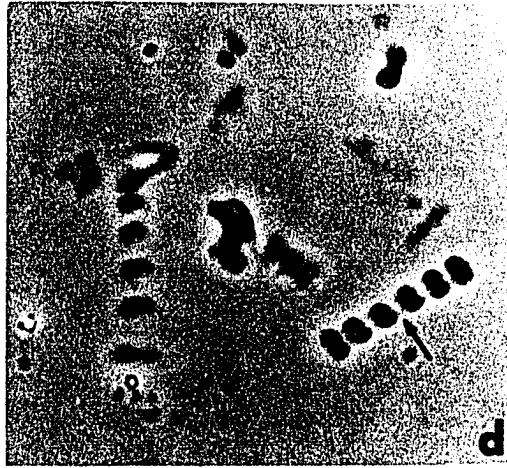
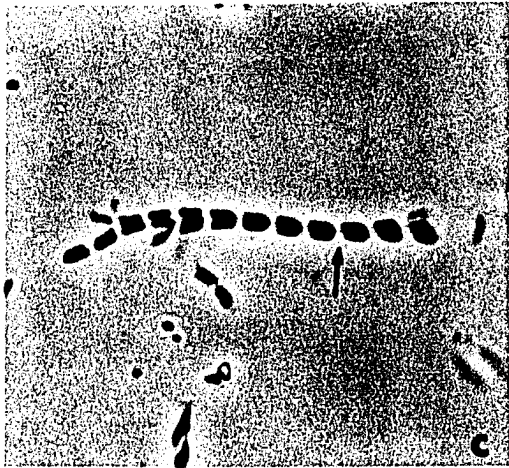
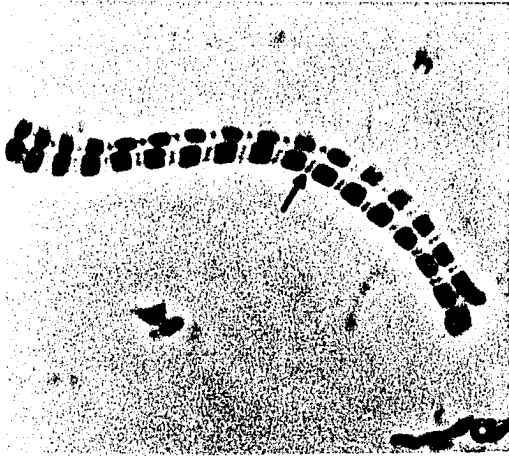
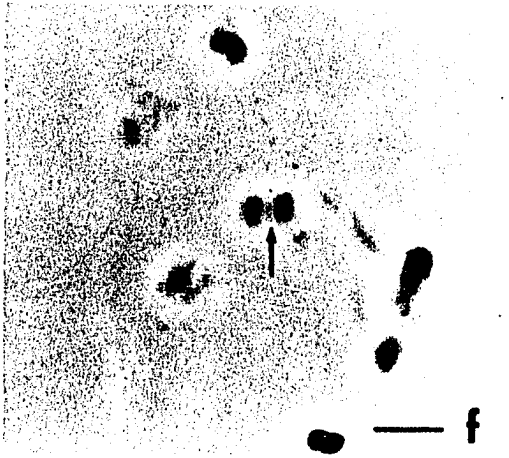
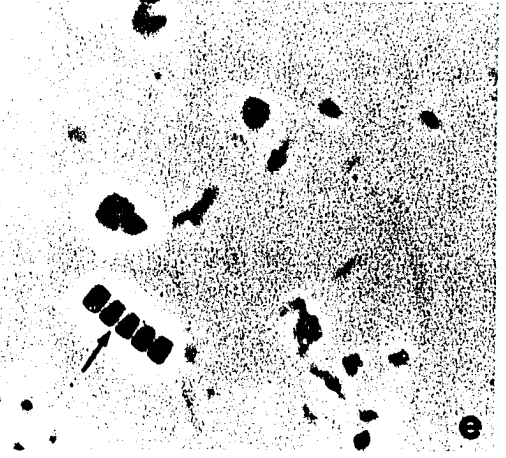
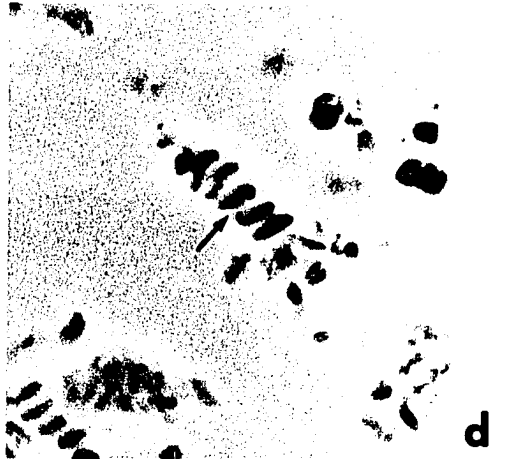


Figure 29. Phase contrast micrographs of myofibrils after incubation with purified CAF at 15° C and pH 7.5 and a brief homogenization

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 7.5, 0.1 mM EDTA, 5.1 mM CaCl₂, 5.0 mM MCE, at 15° C were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM. One ml of this suspension was diluted with 1.0 ml of 100 mM KCl, and then homogenized for 10 sec at 15,000 rpm as described in the Materials and Methods section. Micrographs a through f represent 0 (EDTA control), 2, 4, 8, 15, and 30 min incubations, respectively. The arrow in each indicates the position of a Z line or the position from which the Z line has been removed. The bar in micrograph f represents 5 μm. Magnification was X2000 in all figures.



sample and shows long myofibrils with intact Z lines. Figure 29b through f shows that the Z-line material was degraded during the incubation and that there was a marked increase in amount of fragmentation by the end of the 30-min incubation. There was, however, a tendency for myofibrils to retain more Z-line structure when compared to myofibrils that had been digested at 25° C at similar pH values. When the incubation was conducted at pH 6.5 and 15° C (Figure 30), the phase contrast micrographs show that the loss of Z-line material from the myofibril required slightly longer incubation times (4- to 8-min incubations) than it had at pH 7.5. By the end of the 30-min incubation, there was still a significant increase in degree of fragmentation. When the incubations were conducted at pH 5.5 and 15° C (Figure 31), the phase contrast micrographs show that loss of Z-line material required much longer incubation time (15 to 30 min) before most Z lines were removed. And the degree of fragmentation at the end of 30 min was lower (Figure 31f) than had occurred at 25° C at a similar pH value.

Figure 32 contains phase contrast micrographs of myofibrils incubated with CAF for selected periods of time at pH 7.5 and 5° C and then homogenized. Figure 32a contains the 0-min (EDTA control) and shows long myofibrils with intact Z lines. Figure 32b through f show that the Z-line material was lost during the incubation and that there was still a significant increase in the degree of fragmentation (less sarcomeres/myofibril) at the end of the incubation conducted at 5° C. Figure 32e contains an example of a myofibril with a distorted

Figure 30. Phase contrast micrographs of myofibrils after incubation with purified CAF at 15° C and pH 6.5 and a brief homogenization

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 6.5, 0.1 mM EDTA, 5.1 mM CaCl₂, 5.0 mM MCE, at 15° C were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM. One ml of this suspension was diluted with 1.0 ml of 100 mM KCl, and then homogenized for 10 sec at 15,000 rpm as described in the Materials and Methods section. Micrographs a through f represent 0 (EDTA control), 2, 4, 8, 15, and 30 min incubations, respectively. The arrow in each indicates the position of a Z line or the position from which the Z line has been removed. The bar in micrograph f represents 5 μm. Magnification was X2000 in all figures.

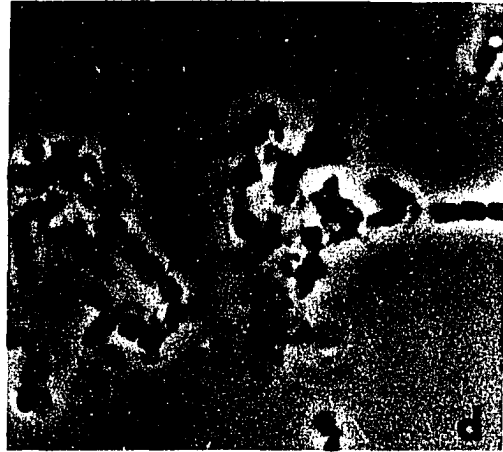
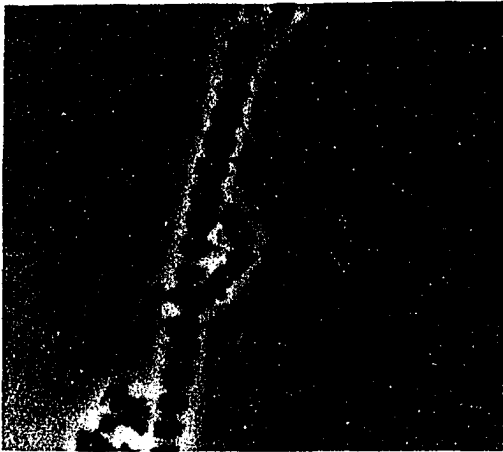
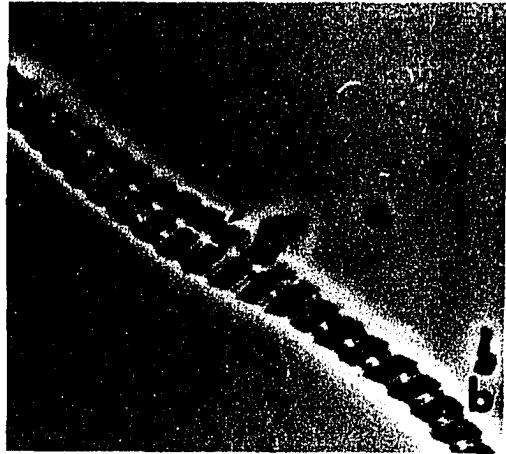
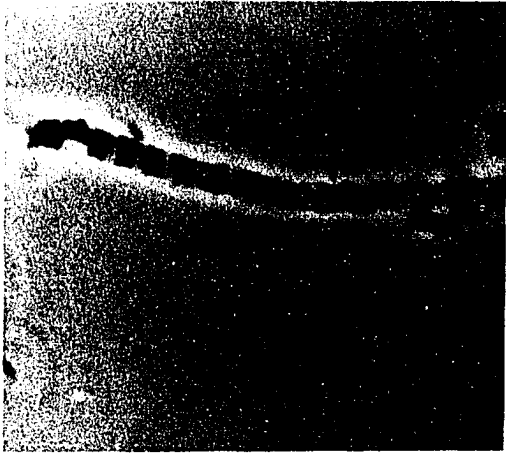


Figure 31. Phase contrast micrographs of myofibrils after incubation with purified CAF at 15° C and pH 5.5 and a brief homogenization

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 5.5, 0.1 mM EDTA, 5.1 mM CaCl₂, 5.0 mM MCE, at 15° C were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM. One ml of this suspension was diluted with 1.0 ml of 100 mM KCl, and then homogenized for 10 sec at 15,000 rpm as described in the Materials and Methods section. Micrographs a through f represent 0 (EDTA control), 2, 4, 8, 15, and 30 min incubations, respectively. The arrow in each indicates the position of a Z line or the position from which the Z line has been removed. The bar in micrograph f represents 5 μm. Magnification was X2000 in all figures.

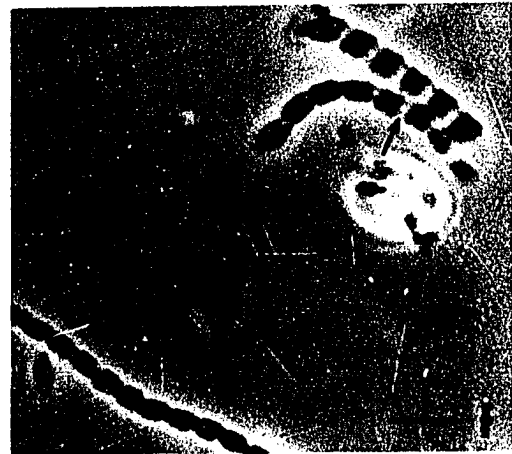
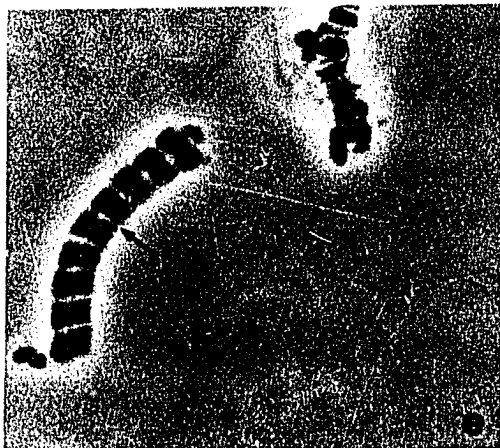
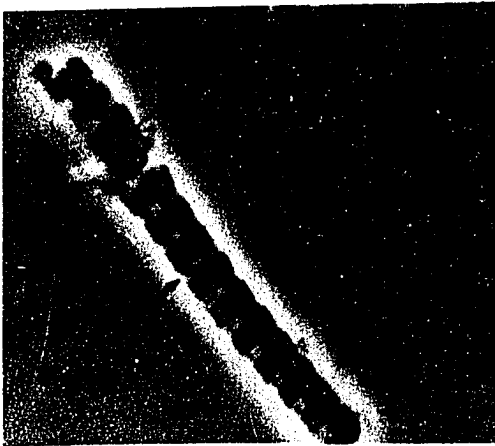
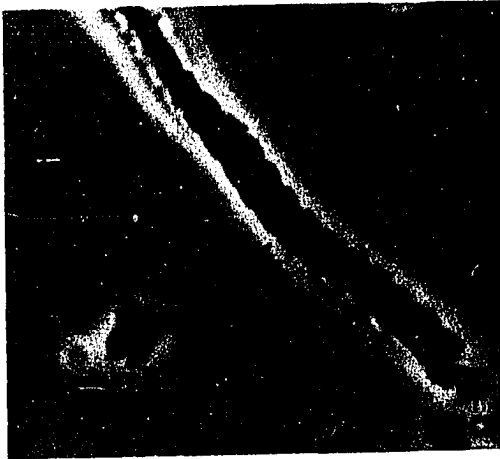
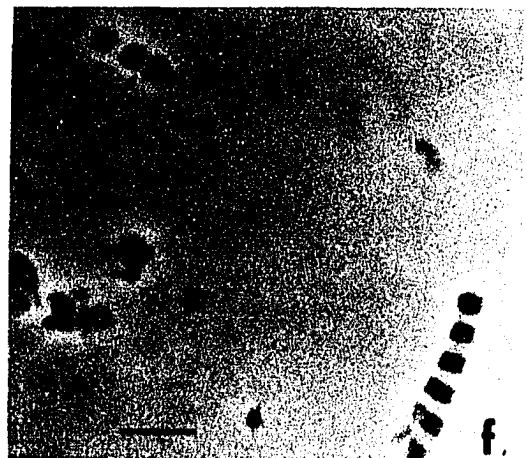
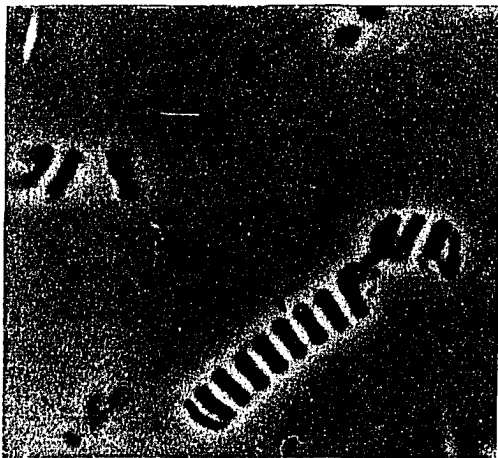
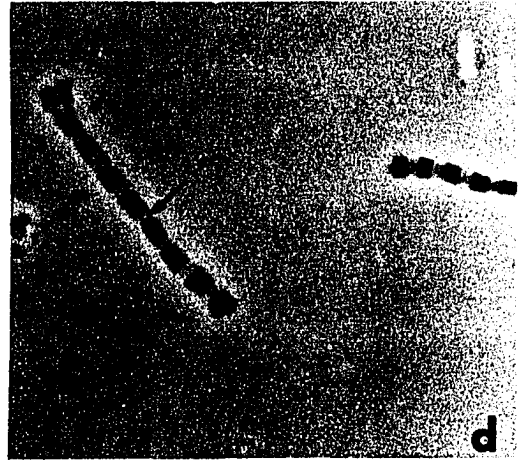
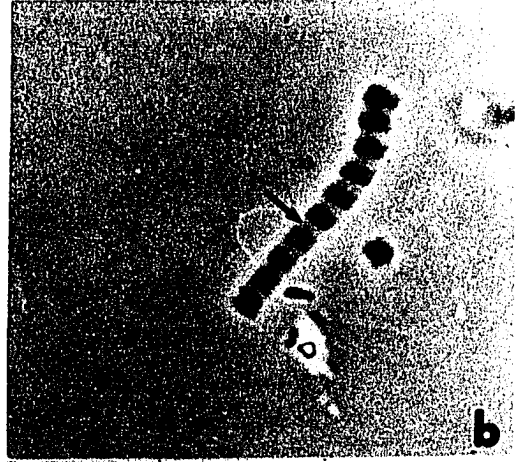
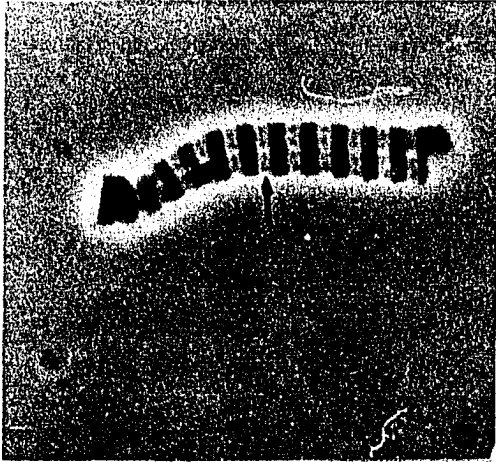


Figure 32. Phase contrast micrographs of myofibrils after incubation with purified CAF at 5° C and pH 7.5 and a brief homogenization

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 7.5, 0.1 mM EDTA, 5.1 mM CaCl₂, 5.0 mM MCE, at 5° C were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM. One ml of this suspension was diluted with 1.0 ml of 100 mM KCl, and then homogenized for 10 sec at 15,000 rpm as described in the Materials and Methods section. Micrographs a through f represent 0 (EDTA control), 2, 4, 8, 15, and 30 min incubations, respectively. The arrow in each indicates the position of a Z line or the position from which the Z line has been removed. The bar in micrograph f represents 5 μm. Magnification was X2000 in all figures.



or stretched appearance (see short myofibril at left). When the incubation with CAF was conducted at pH 6.5 and 5° C (Figure 33), the phase contrast micrographs of the myofibrils show that the loss of Z-line material required longer incubation times (8 to 15 min) than it had at higher pH values or at higher temperatures. By the end of the 30-min incubation at pH 6.5 and 5° C (Figure 33f), there was an increase in the proportion of fragmented myofibrils, but there were still many myofibrils having several sarcomeres. When the incubation was conducted at pH 5.5 and 5° C (Figure 34), the phase contrast micrographs of the myofibrils show that there was little loss of Z-line material or an increase in the proportion of fragmented myofibrils, even after 30 min of incubation (Figure 34f).

The extent of fragmentation of treated myofibrils was difficult to accurately assess because even untreated, freshly prepared myofibrils contained a population of myofibrils in which the number of sarcomeres per myofibril varied from 1 to 20 or more. In an attempt to more objectively determine if significant fragmentation had occurred as the result of CAF treatment and subsequent brief homogenization, 0-min (EDTA control) samples and samples from the 30-min incubation were compared. In this procedure, the number of sarcomeres per myofibril was counted in the phase contrast microscope in at least ten different fields. At least 30 (range = 30-50) total myofibrils were examined for each sampling pH and temperature. There were some biases in the method. Myofibrils greater than 15 sarcomeres long were rejected because they seldom remained in the plane of focus throughout

Figure 33. Phase contrast micrographs of myofibrils after incubation with purified CAF at 5° C and pH 6.5 and a brief homogenization

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 6.5, 0.1 mM EDTA, 5.1 mM CaCl₂, 5.0 mM MCE, at 5° C were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM. One ml of this suspension was diluted with 1.0 ml of 100 mM KCl, and then homogenized for 10 sec at 15,000 rpm as described in the Materials and Methods section. Micrographs a through f represent 0 (EDTA control), 2, 4, 8, 15, and 30 min incubations, respectively. The arrow in each indicates the position of a Z line or the position from which the Z line has been removed. The bar in micrograph f represents 5 μm. Magnification was X2000 in all figures.

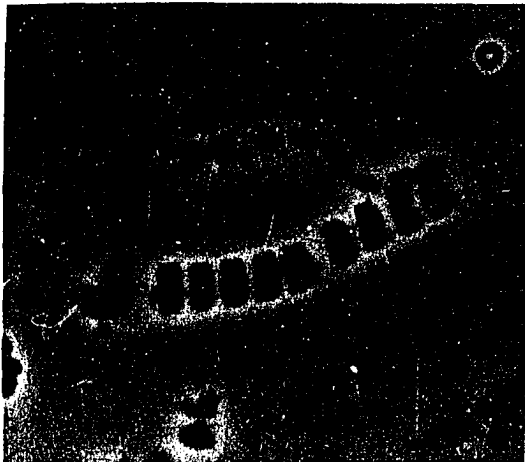
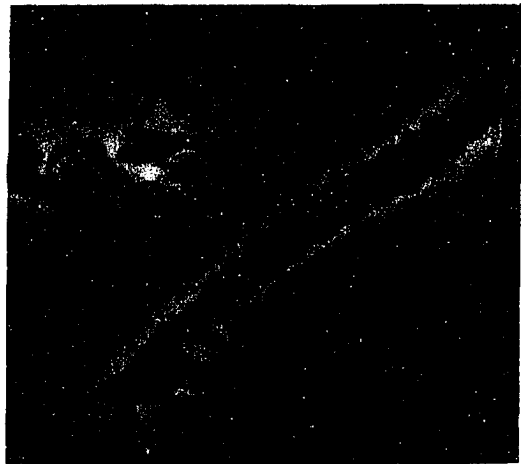
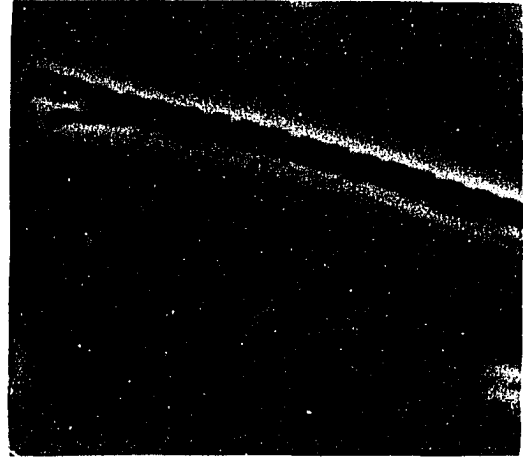
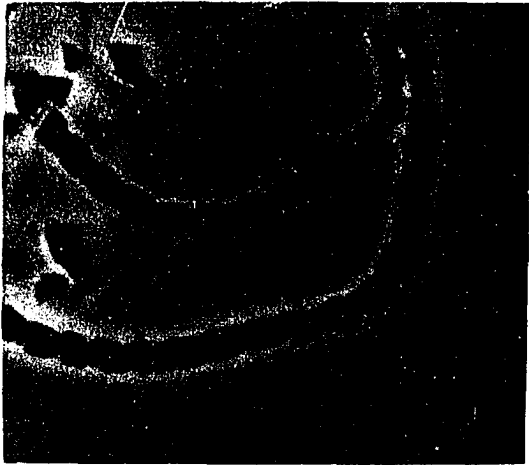
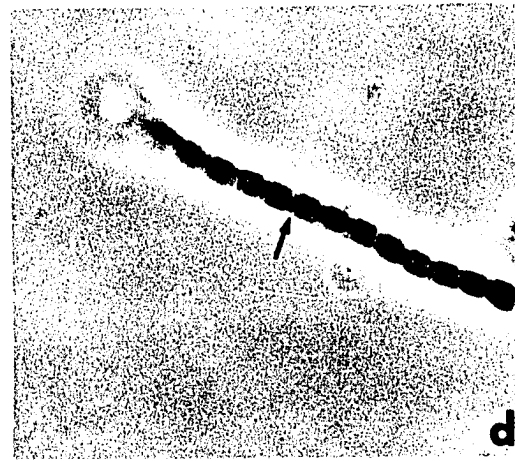
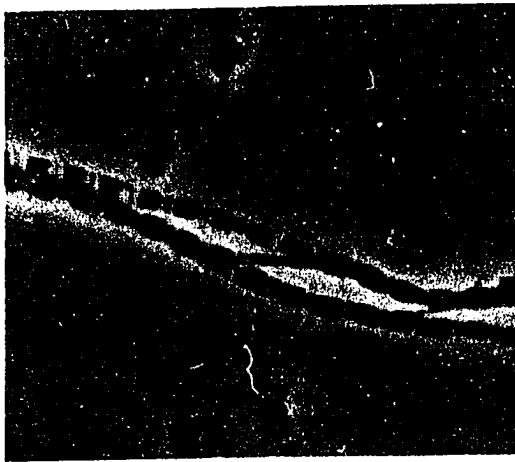
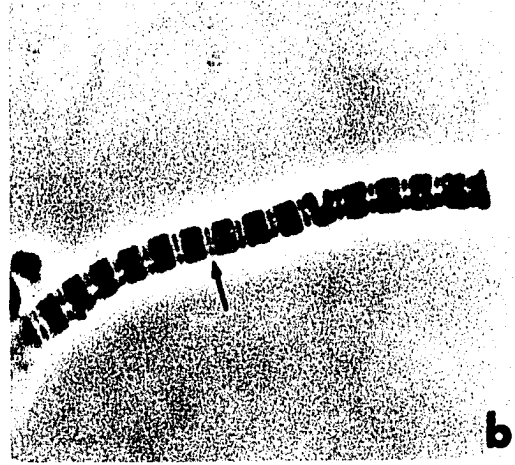


Figure 34. Phase contrast micrographs of myofibrils after incubation with purified CAF at 5° C and pH 5.5 and a brief homogenization

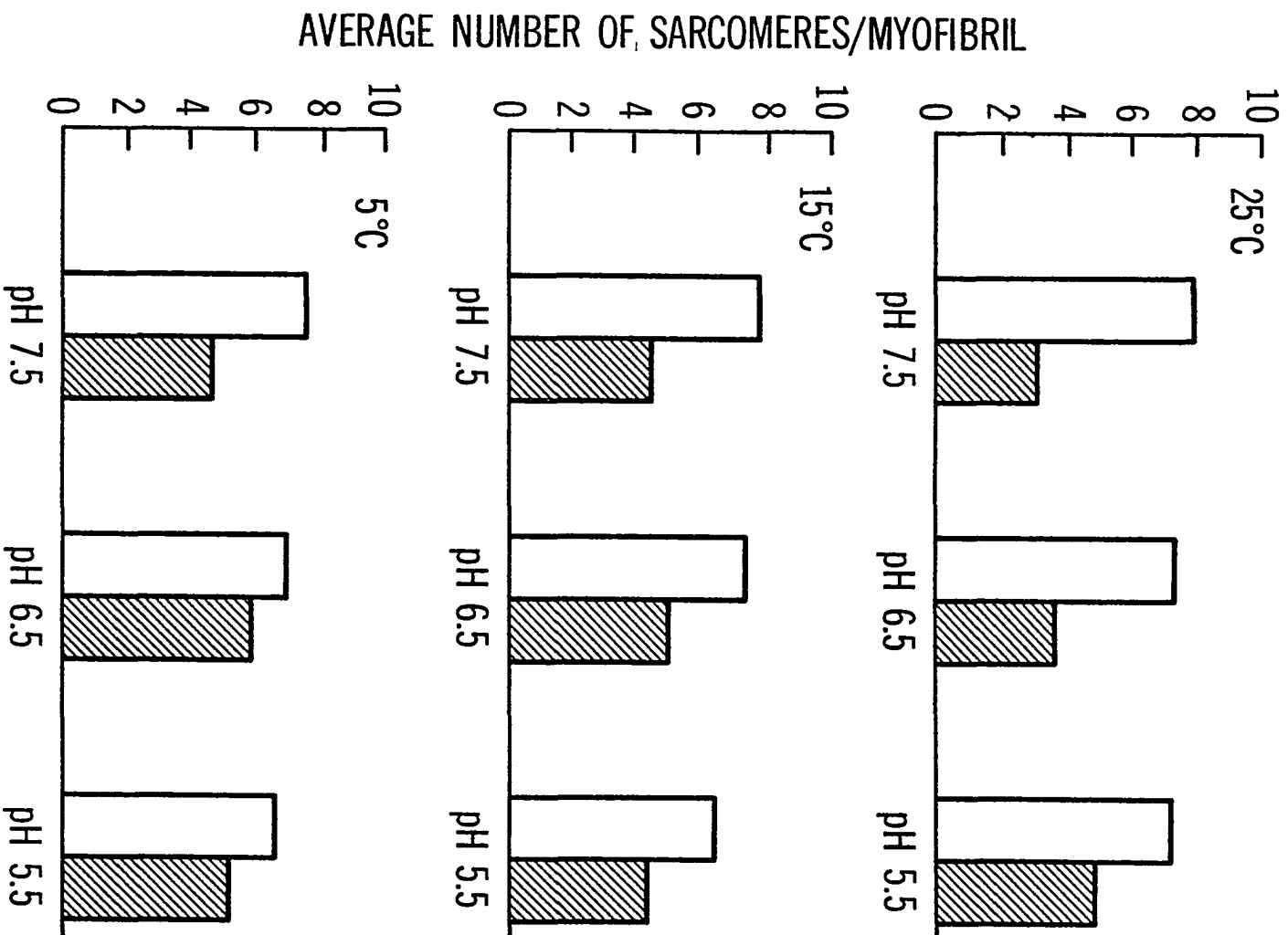
Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 5.5, 0.1 mM EDTA, 5.1 mM CaCl₂, 5.0 mM MCE, at 5° C were incubated with 40 μg of purified CAF. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM. One ml of this suspension was diluted with 1.0 ml of 100 mM KCl, and then homogenized for 10 sec at 15,000 rpm as described in the Materials and Methods section. Micrographs a through f represent 0 (EDTA control), 2, 4, 8, 15, and 30 min incubations, respectively. The arrow in each indicates the position of a Z line or the position from which the Z line has been removed. The bar in micrograph f represents 5 μm. Magnification was X2000 in all figures.



their length and were difficult to count. Myofibrils in or attached to "clumps" also were omitted. The number of myofibrils containing 1, 2, etc. sarcomeres was tallied. The total number of sarcomeres was divided by the total number of myofibrils counted to obtain the mean number of sarcomeres per myofibril for each incubation condition. The results in Figure 35 show the average number of sarcomeres per myofibril for the 0-min (EDTA control) and 30-min samples incubated at 25^o, 15^o, or 5^o C and at pH 7.5, 6.5, or 5.5. When the CAF incubations were done at 25^o C, there was little change in the controls at pH 7.5, 6.5, or 5.5. The sample incubated with CAF for 30 min at pH 7.5 showed a sharp decrease (from about 8 in the control to 3.5) in the average number of sarcomeres per myofibril. A fairly similar difference was observed between control and treated myofibrils at pH 6.5. At pH 5.5, a smaller decrease in the average number of sarcomeres per myofibril in the treated sample occurred compared to its control. The average number of sarcomeres per myofibril in the treated samples increased gradually with decreasing incubation pH at 25^o C. When myofibrils were incubated with CAF at 15^o C and pH 7.5, 6.5, or 5.5 and then homogenized, there was a smaller decrease in the average number of sarcomeres per myofibril for each of the treated samples compared to its control than was obtained in the samples incubated at 25^o C. When myofibrils were incubated with CAF at 5^o C and pH 7.5, 6.5, or 5.5 and then homogenized, the effect of the CAF was reduced yet further, although the treated samples still had fewer sarcomeres per myofibril than the respective controls.

Figure 35. Summary of degree of myofibril fragmentation after incubation with CAF at selected pH values and temperatures and a brief homogenization

Myofibrils were incubated with purified CAF at 25°, 15°, or 5° C and pH 7.5, 6.5, or 5.5 for 30 min and then subjected to a brief 10 sec homogenization. In the phase contrast microscope, the number of sarcomeres per myofibril was counted in at least ten different fields of the 0-min (EDTA control) sample and the sample treated with CAF for 30 min. The average number of sarcomeres per myofibril was calculated and is shown as open vertical bars for the 0-min samples and as shaded vertical bars for the 30-min samples.



C. Cathepsin D: Purification and Properties

A crude P₄₅₋₆₅ cathepsin D was prepared essentially by the method of Barrett et al. (1977) as outlined in Figure 36 and in the Materials and Methods section from the left ventricular portion of four to five bovine hearts that were obtained within one hour of slaughter. The extraction procedure yielded 2 to 3 g of protein in 200 to 400 ml of solution.

The crude P₄₅₋₆₅ cathepsin D was extensively dialyzed against 0.03 M Na barbital, pH 7.4, 0.6 M NaCl to remove all traces of the EDTA, clarified, and then loaded onto an affinity column of concanavalin A-Sepharose 4B. The elution profile is shown in Figure 37. Cathepsin D activity was found in the fraction eluted with the methylmannoside-containing solution. Material that was not bound to the column during the "washing" step had some hemoglobin hydrolyzing activity; however, this activity was not inhibited by pepstatin and, therefore, was not cathepsin D. The tubes within the vertical dashed lines were combined, called concanavalin A-Sepharose 4B purified cathepsin D, and used in the next purification step.

The concanavalin A-Sepharose 4B purified cathepsin D was dialyzed briefly (to limit autolysis) versus 0.1 M Na citrate, pH 3.0, 0.6 M NaCl to lower the pH to 3.0 and loaded onto an affinity column of pepstatin-Sepharose 4B. The elution profile is shown in Figure 38. Pepstatin is a peptide inhibitor of cathepsin D that readily binds the enzyme at pH 3.0 and that has been coupled to the Sepharose 4B as described in the Methods and Materials section. The column was washed

 Preparation of P₄₅₋₆₅ Crude Cathepsin D from Bovine Cardiac Muscle

Step I - Grind bovine cardiac muscle (2.5 kg)

- 1) Suspend in 2.5 volumes (v/w) of 0.15 M NaCl, 1 mM EDTA, 2% butanol, pH 7.2 by three 30 sec bursts on a Waring blender.
- 2) Adjust pH to 6.5 by addition of 1 M Tris.
- 3) Centrifuge at 14,000 x g_{max} for 15 min.

Sediment
(Discard)

Step II - Supernatant

- 1) Filter through glass wool.
- 2) Adjust pH to 4.5 by addition of acetic acid.
- 3) Let stand 12-16 hr at 25° C.
- 4) Centrifuge at 14,000 x g_{max} for 15 min.

Sediment
(Discard)

Step III - Supernatant

- 1) Add ammonium sulfate to 45% saturation.
- 2) Centrifuge at 14,000 x g_{max} for 15 min.

Sediment
(Discard)

Step IV - Supernatant

- 1) Add ammonium sulphate to 65% saturation.
- 2) Centrifuge at 14,000 x g_{max} for 15 min.

Supernatant
(Discard)

Step V - Sediment

- 1) Dissolve in 20 mM Na acetate, pH 5.0, 1 mM EDTA.
- 2) Dialyze versus the same buffer for 48 hr at 4° C.
- 3) Centrifuge at 143,000 x g_{max} for 60 min.

Sediment
(Discard)

Supernatant

Crude P₄₅₋₆₅ cathepsin D

Muscle tissue was obtained from the left ventricular portion of bovine hearts and processed as described in the Materials and Methods. All solution volumes are based on the wet weight of ground muscle tissue used in Step I.

Figure 36. Flow sheet for the preparation of crude P₄₅₋₆₅ cathepsin D from bovine cardiac muscle

Figure 37. Elution profile of the crude P₄₅₋₆₅ cathepsin D fraction from a 2.5 x 30 cm concanavalin A-Sepharose 4B column

The column was loaded with 3,256 mg of crude P₄₅₋₆₅ cathepsin D in 255 ml of solution. The column was first eluted with 30 mM Na barbital, pH 7.4, 0.6 M NaCl, 1 mM MCE, until the absorbance at 278 nm returned to a minimum. The material containing cathepsin D activity was then eluted using a solution of 100 mM Na phosphate pH 6.5, 200 mM α -methylmannoside, 0.6 M NaCl, 1 mM MCE. Flow rate was 55 ml/hr and 11 ml fractions were collected. Cathepsin D activity was determined using denatured hemoglobin as a substrate as described in the Materials and Methods and is indicated as a dashed line. Tubes within the profile represented by the two vertical dashed lines were pooled. This fraction was designated as concanavalin A-Sepharose 4B purified cathepsin D and was used in the next purification step.

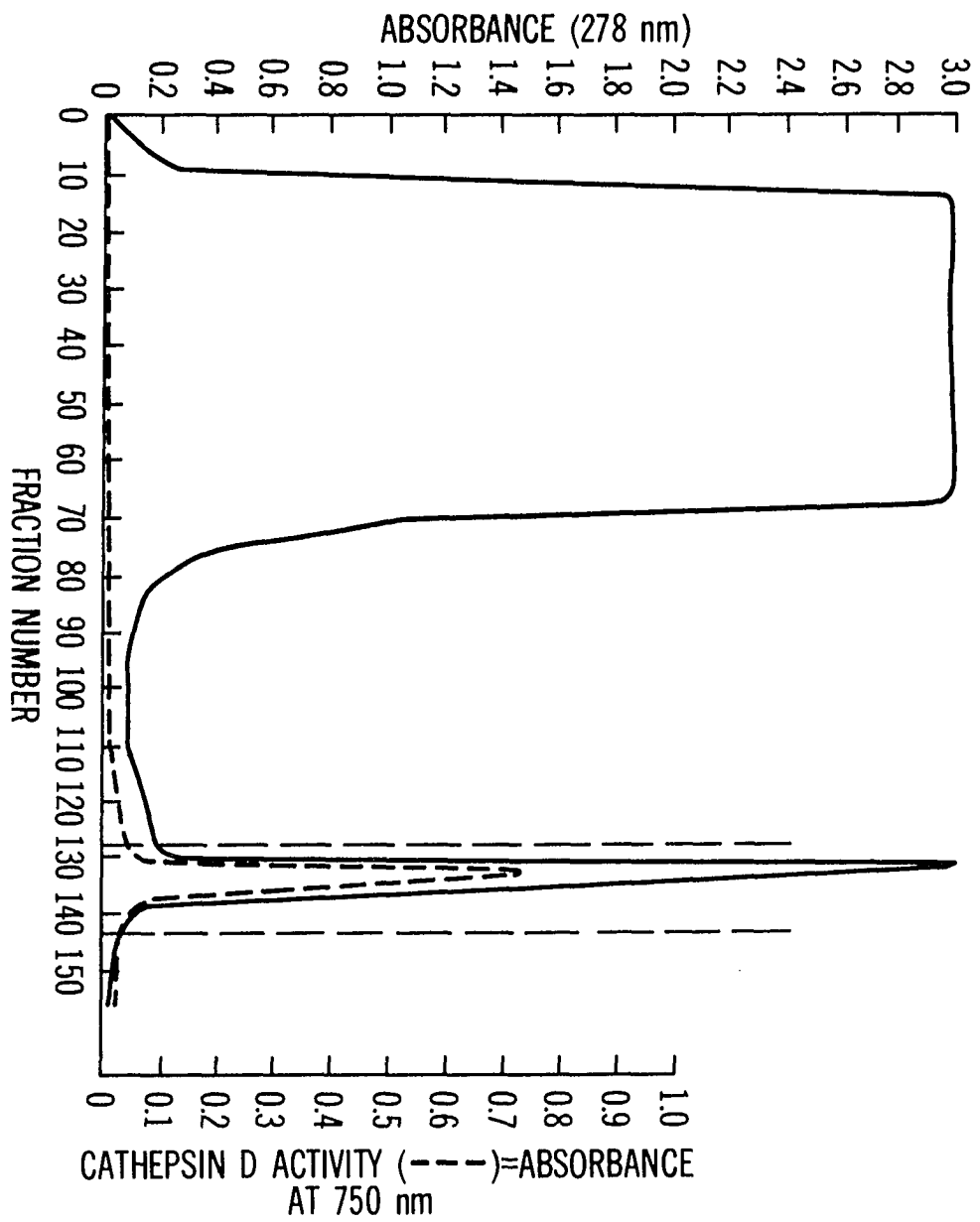
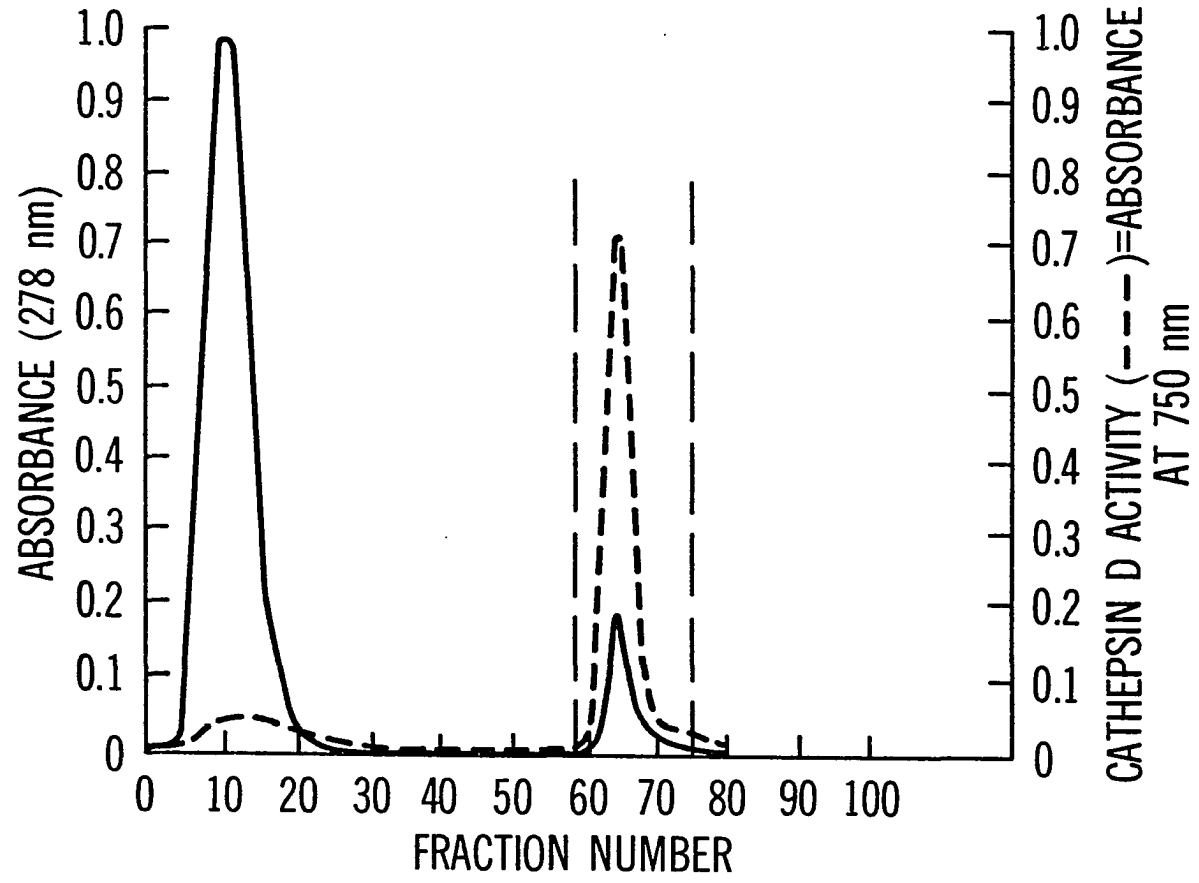


Figure 38. Elution profile of the concanavalin A-Sepharose 4B purified cathepsin D from a 2.5 x 12 cm pepstatin-Sepharose column

The column was loaded with 112 mg of concanavalin A-Sepharose 4B purified cathepsin D in 132 ml of solution that had been dialyzed at 4° C for 6 hr against 100 mM Na citrate, 0.6 M NaCl, pH 3.0. The column was washed with 100 mM Na citrate, 0.6 M NaCl, pH 3.0, until the absorbance at 278 nm returned to a minimum. The cathepsin D activity was then eluted with a solution of 100 mM NaHCO₃, pH 8.0, 0.6 M NaCl. Flow rate was 40 ml/hr and 10 ml fractions were collected. Cathepsin D activity was determined using denatured hemoglobin as substrate as described in the Materials and Methods section and is indicated as a dashed line. Tubes within the profile represented by the two vertical dashed lines were combined and called purified cathepsin D.



free of unbound material with 0.1 M Na-citrate, pH 3.0, 0.6 M NaCl. Cathepsin D activity was eluted from the column with a solution of 0.1 M NaHCO₃, 0.6 M NaCl, pH 8.0. The cathepsin D has a low affinity for pepstatin at pH values of 6.5 and above. The tubes contained within the vertical dashed lines were combined and called purified cathepsin D. Cathepsin D purified by this procedure yielded 3 to 4 mg of enzyme with a specific activity of 240 units/mg using the hemoglobin assay. One unit of activity was defined as 1 mg of tyrosine equivalent produced in one hr at pH 3.8 and 37° C as described in the Materials and Methods section. The purified enzyme preparation was tested for activity against Z-arg-arg and BANA, but no activity was detected. The lack of activity in the Z-arg-arg and BANA assays indicated the absence of other catheptic enzymes such as cathepsins B, H, or L.

The purified cathepsin D was examined by several electrophoretic methods under denaturing and nondenaturing conditions. When the enzyme was electrophoresed on a 10% polyacrylamide gel in a nondenaturing buffer system at pH 7.5 as shown in Figure 39, the gel contained three closely spaced bands of approximately equal intensity. When the cathepsin D was subjected to SDS-PAGE on a 10% polyacrylamide slab gel, two bands were observed with mobilities corresponding to molecular weights of about 47,000 for the major band and 29,500 for the minor band (Figure 39). In order to examine the subunit composition of the three species of cathepsin D observed under nondenaturing conditions, a two-dimensional gel electrophoresis system was used. Purified cathepsin D first was electrophoresed on a 10% polyacrylamide disc

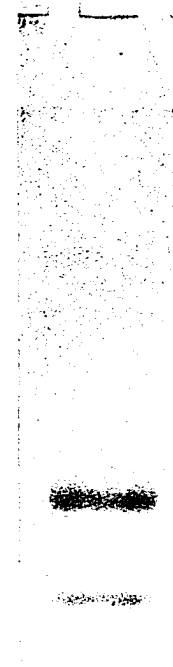
Figure 39. Polyacrylamide gel electrophoretic examination of purified cathepsin D under non-denaturing and denaturing conditions

Purified cathepsin D (16 μg) was electrophoresed under nondenaturing conditions at pH 7.5 on a 10% acrylamide gel (shown on the left) as described in the Materials and Methods section. The cathepsin D contained three closely spaced bands in approximately equal proportions. Purified cathepsin D (13.8 μg) also was electrophoresed under denaturing conditions in the presence of SDS on 10% acrylamide gel (shown on the right). The cathepsin D contained two major bands corresponding to molecular weights of about 47,000 and 29,500.

**pH 7.5
ELECTROPHORESIS**



**SDS
ELECTROPHORESIS**



47,000

29,500

gel using a nondenaturing buffer system at pH 7.5. The disc gel then was equilibrated in SDS buffer, placed on top of an SDS slab gel, and electrophoresed the second time as described in the Materials and Methods section. Figure 40 shows the results of the two-dimensional separation. The bands at the left of the gel correspond to molecular weight markers. It can be seen from this figure that the band nearest the top of the nondenaturing disc gel migrated as two spots, corresponding to molecular weights of about 47,000 and 29,500. The middle band on the nondenaturing gel migrated primarily as a spot corresponding to a molecular weight of about 47,000 and a very small amount of a spot corresponding to a molecular weight of 29,500. The third band on the disc gel migrated as one spot corresponding to a molecular weight of about 47,000.

D. The Effect of Purified Cathepsin D on Myofibrils

1. Protein released from myofibrils by cathepsin D treatment

Purified cathepsin D's ability to cause the release of soluble protein during incubation of myofibril suspensions was used as one measure of the enzyme's activity under varying conditions of pH and temperature. After the treatment with enzyme, the myofibrils were centrifuged and the supernatants were examined for protein by the Folin-Lowry method. Figure 41 shows the release of soluble protein after incubation with cathepsin D at 37° C and pH 5.5, 6.5, or 7.5. There was a significant amount of protein solubilized by cathepsin D at pH 5.5. However, little increase was observed in the amount of protein solubilized by cathepsin D after 120 min of incubation at pH

Figure 40. Two-dimensional PAGE analysis of purified cathepsin D

Purified cathepsin D (16 μ g) first was electrophoresed on a 10% acrylamide disc gel under nondenaturing conditions at pH 7.5. The disc gel was then incubated for one hr in 1.0% SDS, 0.1% MCE, placed horizontally on a 10% polyacrylamide slab gel, and subjected to SDS-PAGE as described in the Materials and Methods section. The large arrowhead indicates the position of the top of the disc gel. The bands at the left of the slab gel correspond to protein standards (approximately 2 μ g each) as follows: a) alpha-actinin, 100,000 daltons; b) BSA, 68,000 daltons; c) ovalbumin, 43,000 daltons; and d) chymotrypsinogen, 25,000. The arrows indicate spots on the slab gel derived from bands in the disc gel. The horizontal disc gel shown at the top is a stained duplicate of the disc gel used in the experiment. It has been positioned so that the stained bands in it are directly above the corresponding spots in the slab gel below.

P



S



q

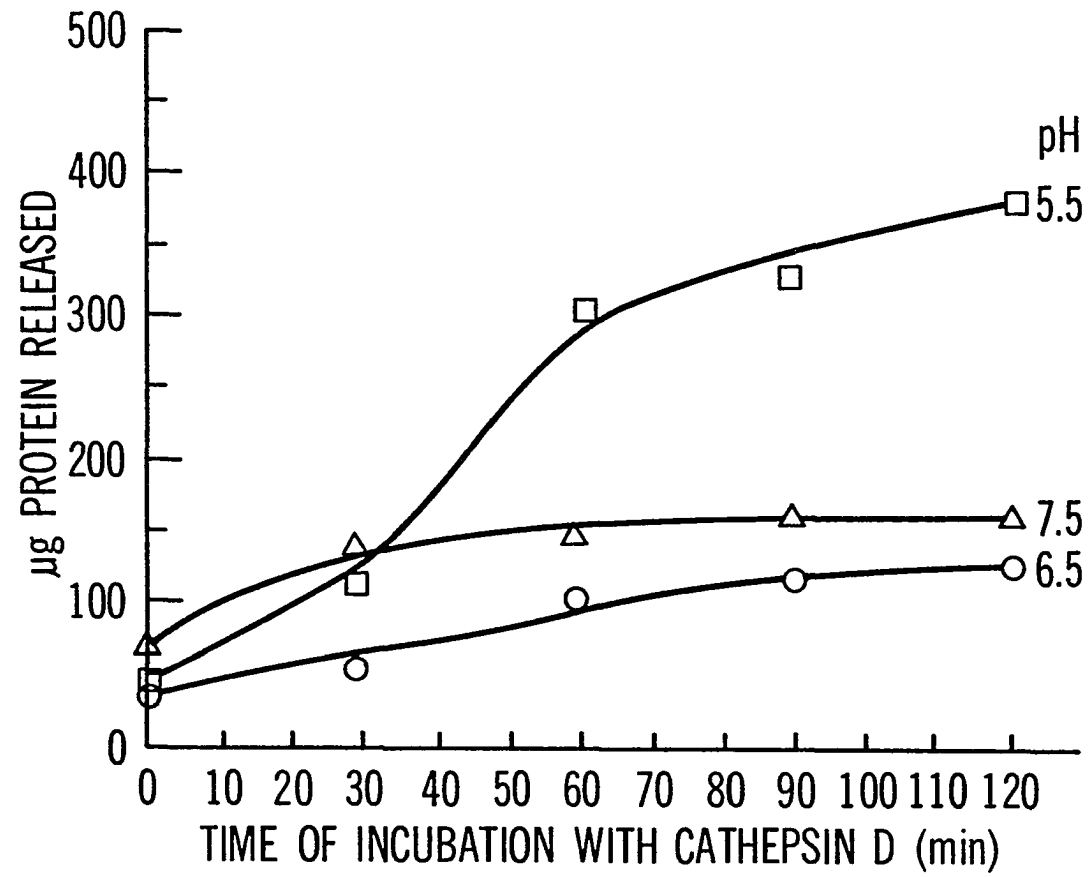
D



A

Figure 41. Protein released from myofibrils after incubation with purified cathepsin D at 37° C and pH 5.5, 6.5, or 7.5

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 5.5, 6.5, or 7.5, 1.0 mM EDTA, at 37° C were incubated with 40 μ g of purified cathepsin D. The reaction was stopped by the addition of 20 μ l of 10 mM pepstatin. Each sample was then sedimented at 50,000 x g_{max} for 15 min at 25° C. Protein remaining in the supernatant was measured by the Folin-Lowry method. The data points are averages of two determinations.



6.5 or 7.5. When the myofibrils were incubated with cathepsin D at 25° C and pH 5.5, as shown in Figure 42, there was a small amount of protein solubilized, especially in the first 30 min of incubation. There was a very small amount of protein solubilized from myofibrils by cathepsin D at pH 6.5. At pH 7.5, less protein was released than at pH 5.5, but more than at pH 6.5. There was, however, considerable scatter in the data obtained at pH 7.5. Finally, when myofibrils were incubated with cathepsin D at 15° C and pH 5.5, 6.5, or 7.5, as shown in Figure 43, cathepsin D treatment caused little solubilization of myofibrillar protein, even after 120 min of incubation.

2. SDS-PAGE analysis of myofibril fractions after cathepsin D treatment

Cathepsin D also was examined with respect to its ability to hydrolyze myofibrillar proteins under varying conditions of pH and temperature by SDS-PAGE (Figures 44-52). In these experiments, myofibrils were incubated with cathepsin D, centrifuged in order to separate supernatant and sediment fractions, and then examined by electrophoresis. Figures 44-46 show the results obtained at 37° C. Figure 44 contains the electrophoretic results obtained on supernatants and sediments from myofibrils incubated with cathepsin D at 37° C and pH 5.5. Lanes a through f contain supernatant fractions corresponding to the two controls (0 min) and to 30, 60, 90, and 120 min of incubation, respectively. Lanes g through l contain the corresponding sediment fractions. Examination of the supernatant lanes (a-f) shows that very little protein was solubilized by the cathepsin D except for some small molecular weight material that traveled with the tracking

Figure 42. Protein released from myofibrils after incubation with purified cathepsin D at 25° C and pH 5.5, 6.5, or 7.5

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 5.5, 6.5, or 7.5, 1.0 mM EDTA, at 25° C were incubated with 40 μ g of purified cathepsin D. The reaction was stopped by the addition of 20 μ l of 10 mM pepstatin. Each sample was then sedimented at 50,000 \times g_{max} for 15 min at 25° C. Protein remaining in the supernatant was measured by the Folin-Lowry method. The data points are averages of two determinations.

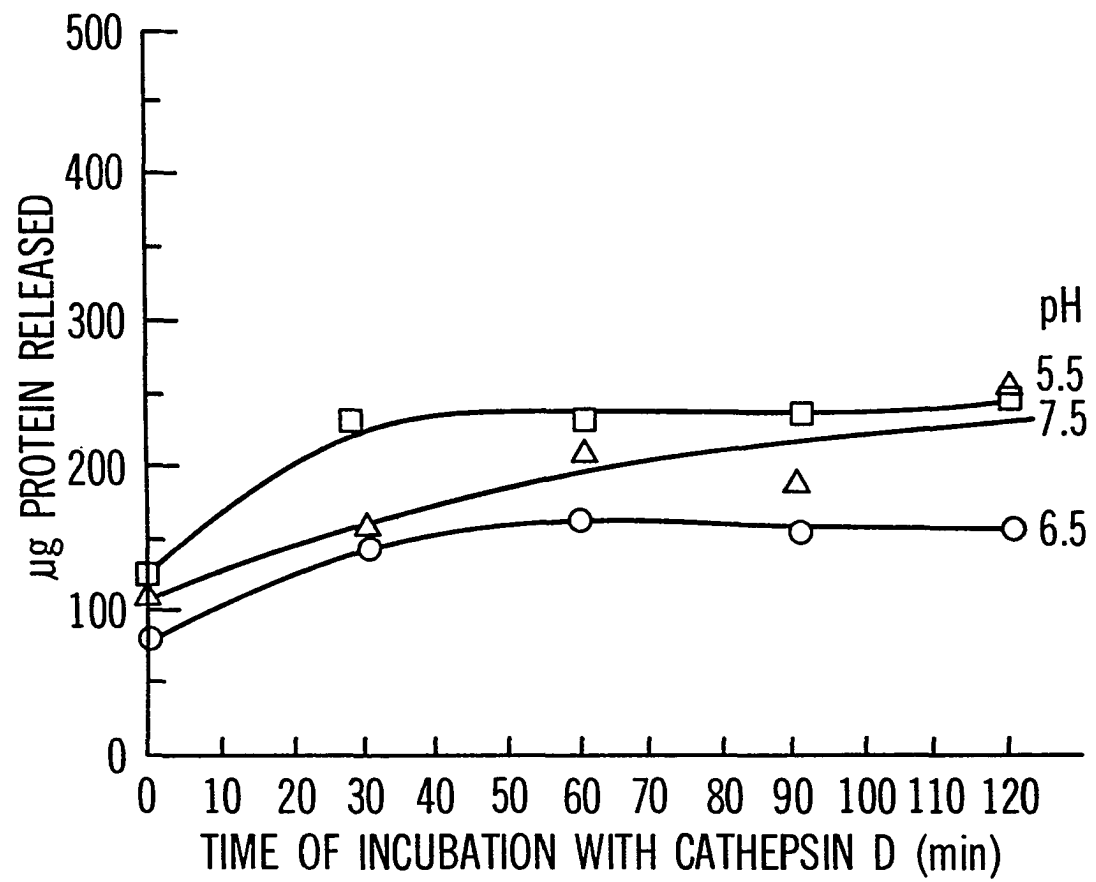


Figure 43. Protein released from myofibrils after incubation with purified cathepsin D at 15° C and pH 5.5, 6.5, or 7.5

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 5.5, 6.5, or 7.5, 1.0 mM EDTA, at 15° C were incubated with 40 μ g of purified cathepsin D. The reaction was stopped by the addition of 20 μ l of 10 mM pepstatin. Each sample was then sedimented at 50,000 \times g_{max} for 15 min at 15° C. Protein remaining in the supernatant was measured by the Folin-Lowry method. The data points are averages of two determinations.

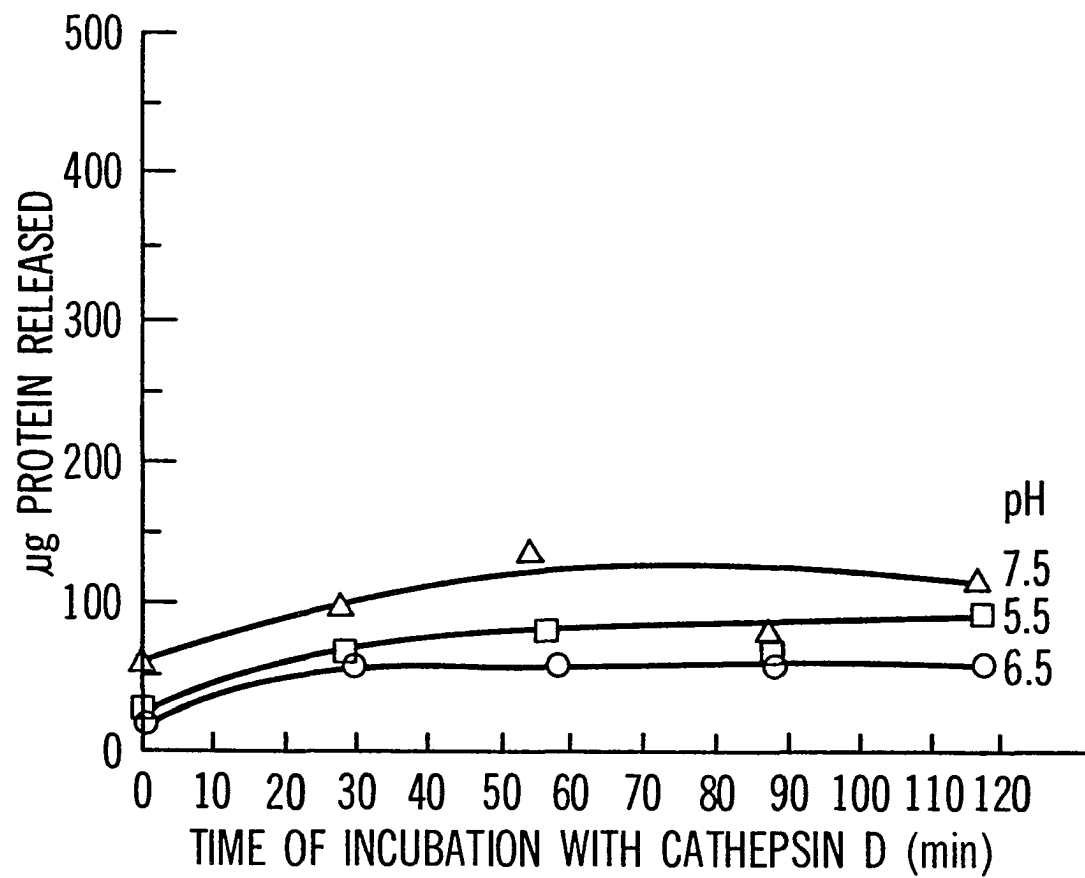


Figure 44. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified cathepsin D at 37° C and pH 5.5

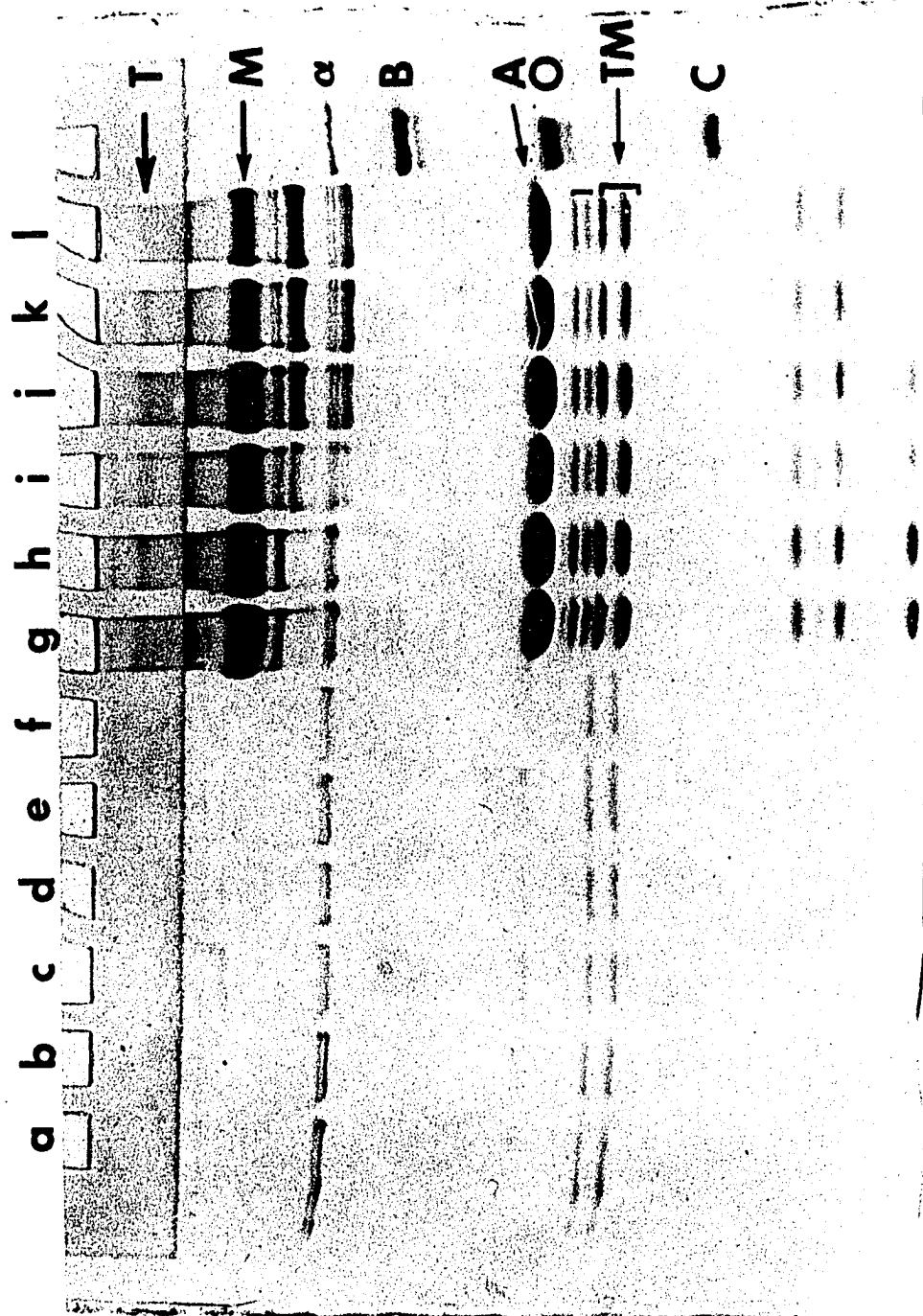
Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 5.5, 1.0 mM EDTA, were incubated with 40 μ g of purified cathepsin D at 37° C. The reaction was stopped by the addition of 20 μ l of 10 mM pepstatin and each sample was then centrifuged at 50,000 \times g_{\max} for 15 min at 25° C. Lanes a through f were loaded with 50 μ l of supernatant fractions corresponding to myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, and 30, 60, 90, and 120 min incubations with enzyme, respectively. Lanes g through l were loaded with 48 μ g of sediment fractions corresponding to myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, and 30, 60, 90, and 120 min incubations with enzyme, respectively (see Materials and Methods). Labels at the right of the gel are as follows: T, titin; M, myosin heavy chains; 155 K, the position of a 155,000-dalton fragment of myosin heavy chains; 90 K, a bracket indicates the position of a trio of bands of approximately 90,000 daltons; A, actin; TN T, a small bracket indicates troponin T isomers; TM, a bracket indicates the beta and alpha tropomyosin subunits; TN I, troponin I.

dye at the bottom of the gel (lanes c-f). This suggests that the increase observed in protein released under these conditions (Figure 44) was due primarily to release of very low molecular weight material. Some intact tropomyosin subunits were released, presumably by the low pH treatment, into all of the supernatant samples, including the controls. In the sediment lanes (g-l), even the two controls (lanes g and h) contained little titin. And that small amount was decreased further during the incubation. After 30 min of incubation (lane i), a great deal of degradation of myosin heavy chains had occurred and new bands were observed at about 155,000 and 90,000 daltons. There was a slight decrease in the actin band with increasing incubation time. Troponin T isomers were somewhat degraded. Tropomyosin was only slightly decreased in amount. Four to six faint bands appeared in the region of the gel below tropomyosin after 90 to 120 min of incubation. Troponin I and the strong band just above it (myosin light chain 1) also were partly degraded with increasing incubation time.

Figure 45 contains the electrophoretic results obtained on supernatants and sediments from myofibrils incubated with cathepsin D at 37° C and pH 6.5. The supernatant lanes (c-f) show that little change occurred in protein solubilized during the incubation when compared to control lanes (a and b). The sediment lanes show only a small amount of titin, even in the control lanes (g and h). During the digestion (lanes i-l), the amount of titin was decreased further. Myosin heavy chains were degraded much more slowly than when the incubation was done at pH 5.5 (compare lanes i-l in Figures 44 and 45).

Figure 45. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified cathepsin D at 37° C and pH 6.5

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 6.5, 1.0 mM EDTA, were incubated with 40 μ g of purified cathepsin D at 37° C. The reaction was stopped by the addition of 20 μ l of 10 mM pepstatin and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 25° C. Lanes a through f were loaded with 50 μ l of supernatant fractions corresponding to myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, and 30, 60, 90, and 120 min incubations with enzyme, respectively. Lanes g through l were loaded with 48 μ g of sediment fractions corresponding to myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, and 30, 60, 90, and 120 min incubations with enzyme, respectively (see Materials and Methods). Labels at the right of the gel are as follows: T, titin; M, myosin heavy chains; α , alpha-actinin; B, bovine serum albumin; A, actin; O, ovalbumin; the small bracket indicates troponin T isomers; TM, the larger bracket indicates beta and alpha tropomyosin subunits; C, chymotrypsinogen.



There was a slight decrease in actin and a distinct decrease in troponins T and I, tropomyosin, and myosin light chain 1 after 120 min of incubation. Figure 46 shows the electrophoretic results obtained on supernatants and sediments from myofibrils incubated with cathepsin D at 37° C and pH 7.5. The supernatant lanes show that little or no change occurred during the course of incubation (compare lanes c-f with control lanes a and b). The sediment lanes show that titin was slightly degraded, but not to the degree that had occurred at lower pH values. There was only a very slight decrease in amounts of troponins T and I and tropomyosin at the end of the 120 min incubation (lane 1).

Figures 47-49 show the results obtained at 25° C. Figure 47 contains the electrophoretic results obtained on supernatants and sediments from myofibrils incubated with cathepsin D at 25° C and pH 5.5. The supernatant lanes (a-f) show that there was little change during the incubation. A very small amount of low molecular weight material was evident at the bottom of the gel after 60 min of incubation (see lanes d-f). The lanes (g-l) of the sediments show that some degradation occurred in the titin (compare lanes k and l with g and h). Some of the myosin heavy chains were degraded to fragments of about 155,000 and 90,000 daltons after 90 to 120 min of incubation (lanes k and l), but not nearly to the degree that had occurred at the same pH at 37° C (Figure 44). Troponins T and I and tropomyosin subunits were only slightly degraded after 120 min of incubation. Figure 48 contains the electrophoretic results obtained on supernatants and sediments from myofibrils incubated with cathepsin D at 25° C and pH 6.5. The supernatant

Figure 46. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified cathepsin D at 37° C and pH 7.5

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 7.5, 1.0 mM EDTA, were incubated with 40 μ g of purified cathepsin D at 37° C. The reaction was stopped by the addition of 20 μ l of 10 mM pepstatin and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 25° C. Lanes a through f were loaded with 50 μ l of supernatant fractions corresponding to myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, and 30, 60, 90, and 120 min incubations with enzyme, respectively. Lanes g through l were loaded with 48 μ g of sediment fractions corresponding to myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, and 30, 60, 90, and 120 min incubations with enzyme, respectively (see Materials and Methods). Labels at the right of the gel are as follows: T, titin; M, myosin heavy chains; α , alpha-actinin; B, bovine serum albumin; A, actin; O, ovalbumin; the small bracket indicates troponin T isomers; TM, the larger bracket indicates beta and alpha tropomyosin subunits; C, chymotrypsinogen.

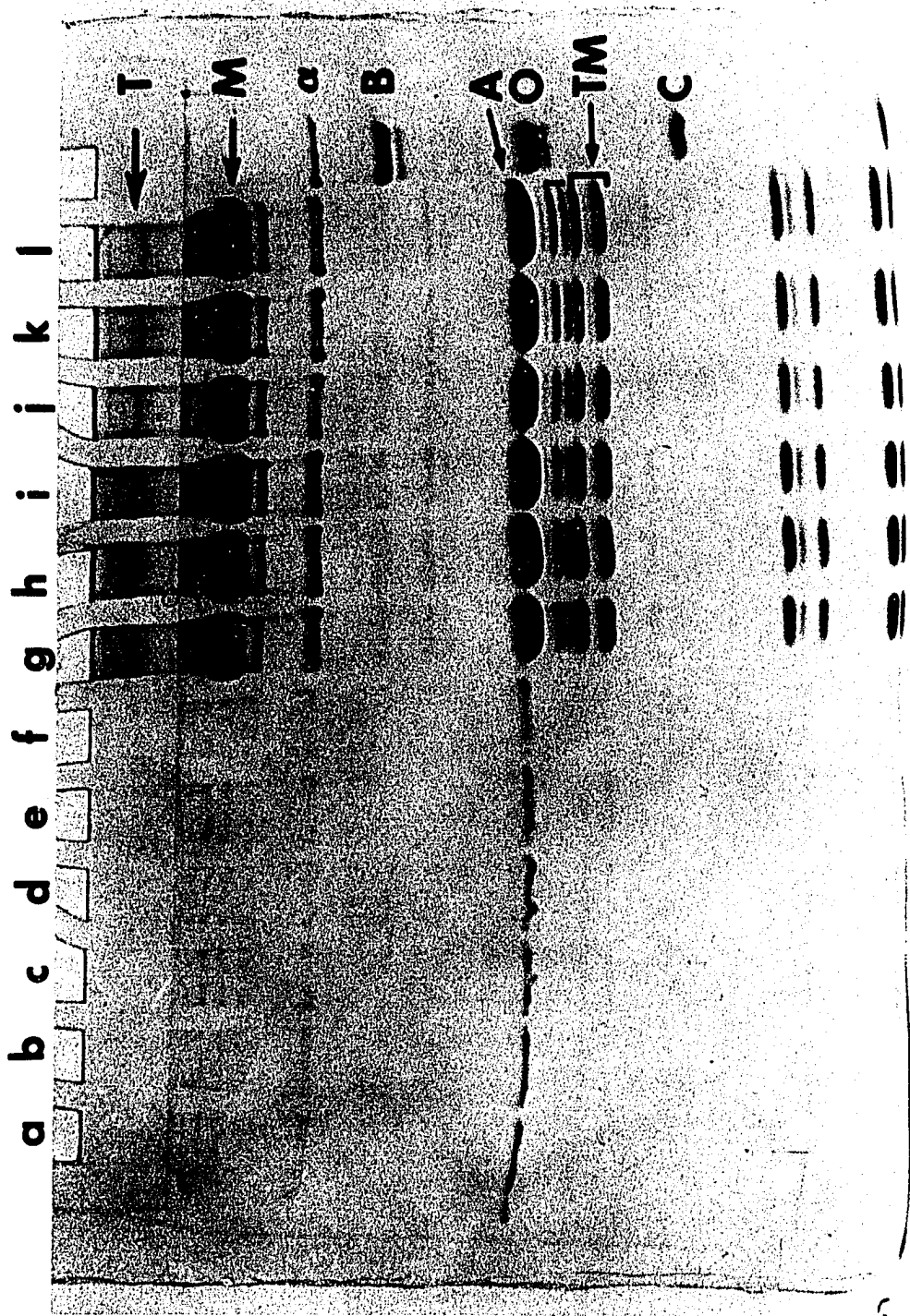


Figure 47. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified cathepsin D at 25° C and pH 5.5

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 5.5, 1.0 mM EDTA, were incubated with 40 μ g of purified cathepsin D at 25° C. The reaction was stopped by the addition of 20 μ l of 10 mM pepstatin and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 25° C. Lanes a through f were loaded with 50 μ l of supernatant fractions corresponding to myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, and 30, 60, 90, and 120 min incubations with enzyme, respectively. Lanes g through l were loaded with 48 μ g of sediment fractions corresponding to myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, and 30, 60, 90, and 120 min incubations with enzyme, respectively (see Materials and Methods). Labels at the right of the gel are as follows: T, titin; M, myosin heavy chains; α , alpha-actinin; B, bovine serum albumin; A, actin; O, ovalbumin; the small bracket indicates troponin T isomers; TM, the larger bracket indicates beta and alpha tropomyosin subunits; C, chymotrypsinogen.

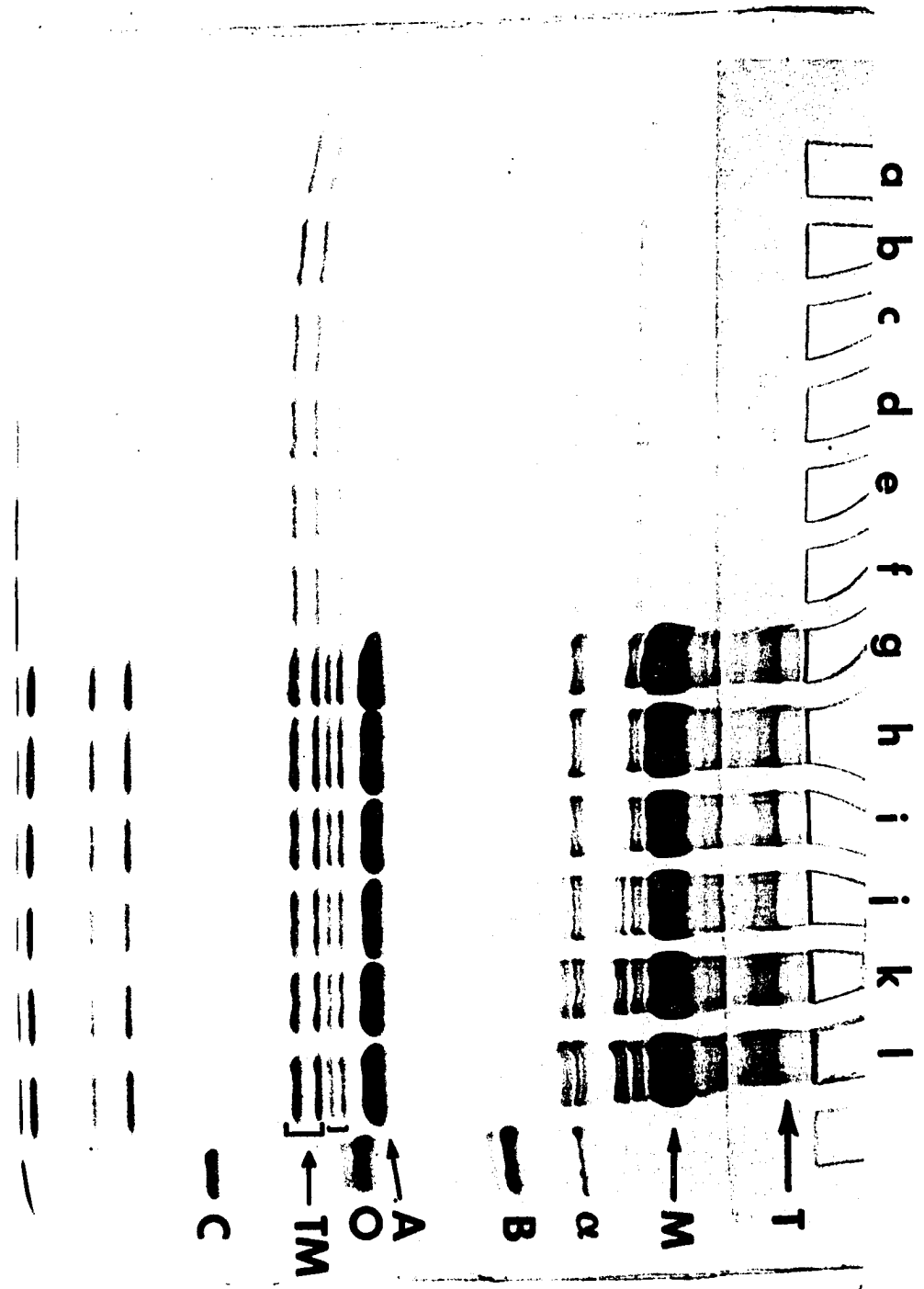


Figure 48. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified cathepsin D at 25° C and pH 6.5

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 6.5, 1.0 mM EDTA, were incubated with 40 μ g of purified cathepsin D at 25° C. The reaction was stopped by the addition of 20 μ l of 10 mM pepstatin and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 25° C. Lanes a through f were loaded with 50 μ l of supernatant fractions corresponding to myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, and 30, 60, 90, and 120 min incubations with enzyme, respectively. Lanes g through l were loaded with 48 μ g of sediment fractions corresponding to myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, and 30, 60, 90, and 120 min incubations with enzyme, respectively (see Materials and Methods). Labels at the right of the gel are as follows: T, titin; M, myosin heavy chains; α , alpha-actinin; B, bovine serum albumin; A, actin; O, ovalbumin; the small bracket indicates troponin T isomers; TM, the larger bracket indicates beta and alpha tropomyosin subunits; C, chymotrypsinogen.

lanes (a-f) show that little or no protein was released during the course of the incubation. The lanes (g-l) of the sediments show a decrease in intact titin with increasing incubation time, but no other marked changes. Figure 49 shows the electrophoretic results obtained on supernatants (lanes a-f) and sediments (lanes g-l) from myofibrils incubated with cathepsin D at 25° C and pH 7.5. There were few significant changes in appearance of either the supernatant or sediment lanes during the digestion.

Figure 50 contains the electrophoretic results obtained on supernatants and sediments from myofibrils incubated with cathepsin D at 15° C and pH 5.5. The supernatant lanes (a-f) show that very little protein had been solubilized. The sediment lanes (g-l) show a significant decrease occurred in intact titin with increasing incubation time (compare lane l with lanes g and h), but no obvious degradation of myosin heavy chains or other proteins had occurred in the sediments. Figure 51 shows the electrophoretic results obtained on supernatants and sediments from myofibrils incubated at 15° C and pH 6.5. The supernatant lanes (a-f) show that little or no protein was released under these incubation conditions. The sediment lanes (g-l) show that only a slight degradation had occurred in titin and possibly in troponins T and I. Figure 52 contains the electrophoretic results obtained on supernatants and sediments from myofibrils incubated with cathepsin D at 15° C and pH 7.5. Examination of the supernatant lanes (a-f) and sediment lanes (g-l) indicates that cathepsin D had little effect on myofibrillar proteins under these conditions.

Figure 49. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified cathepsin D at 25° C and pH 7.5

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 7.5, 1.0 mM EDTA, were incubated with 40 μ g of purified cathepsin D at 25° C. The reaction was stopped by the addition of 20 μ l of 10 mM pepstatin and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 25° C. Lanes a through f were loaded with 50 μ l of supernatant fractions corresponding to myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, and 30, 60, 90, and 120 min incubations with enzyme, respectively. Lanes g through l were loaded with 48 μ g of sediment fractions corresponding to myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, and 30, 60, 90, and 120 min incubations with enzyme, respectively (see Materials and Methods). Labels at the right of the gel are as follows: T, titin; M, myosin heavy chains; α , alpha-actinin; B, bovine serum albumin; A, actin; O, ovalbumin; the small bracket indicates troponin T isomers; TM, the larger bracket indicates beta and alpha tropomyosin subunits; C, chymotrypsinogen.

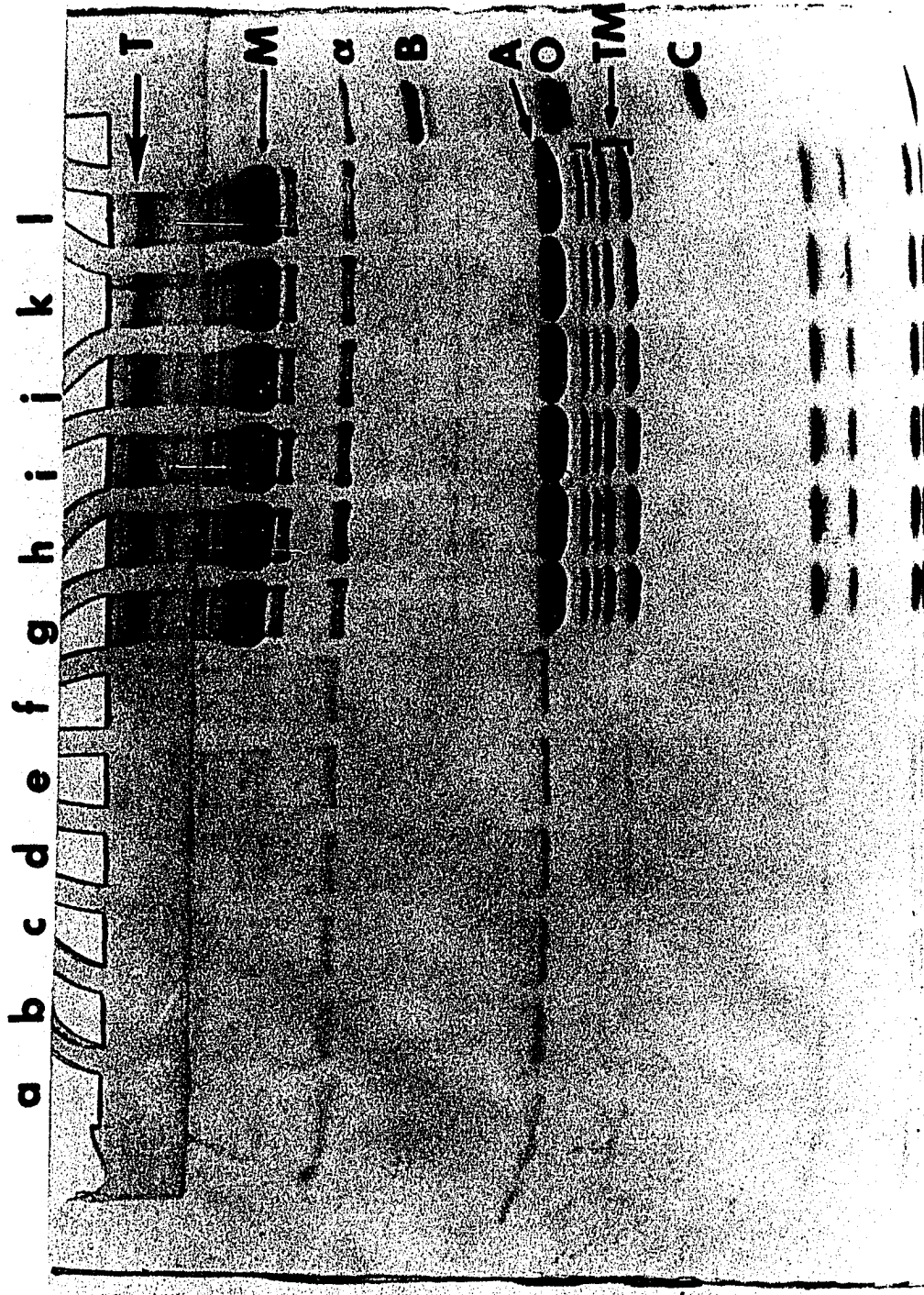


Figure 50. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified cathepsin D at 15° C and pH 5.5

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 5.5, 1.0 mM EDTA, were incubated with 40 μ g of purified cathepsin D at 15° C. The reaction was stopped by the addition of 20 μ l of 10 mM pepstatin and each sample was then centrifuged at 50,000 x g_{\max} for 15 min at 15° C. Lanes a through f were loaded with 50 μ l of supernatant fractions corresponding to myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, and 30, 60, 90, and 120 min incubations with enzyme, respectively. Lanes g through l were loaded with 48 μ g of sediment fractions corresponding to myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, and 30, 60, 90, and 120 min incubations with enzyme, respectively (see Materials and Methods). Labels at the right of the gel are as follows: T, titin; M, myosin heavy chains; α , alpha-actinin; A, actin; TN T, the small bracket indicates troponin T isomers; TM, a bracket indicates the beta and alpha tropomyosin subunits; C, chymotrypsinogen.

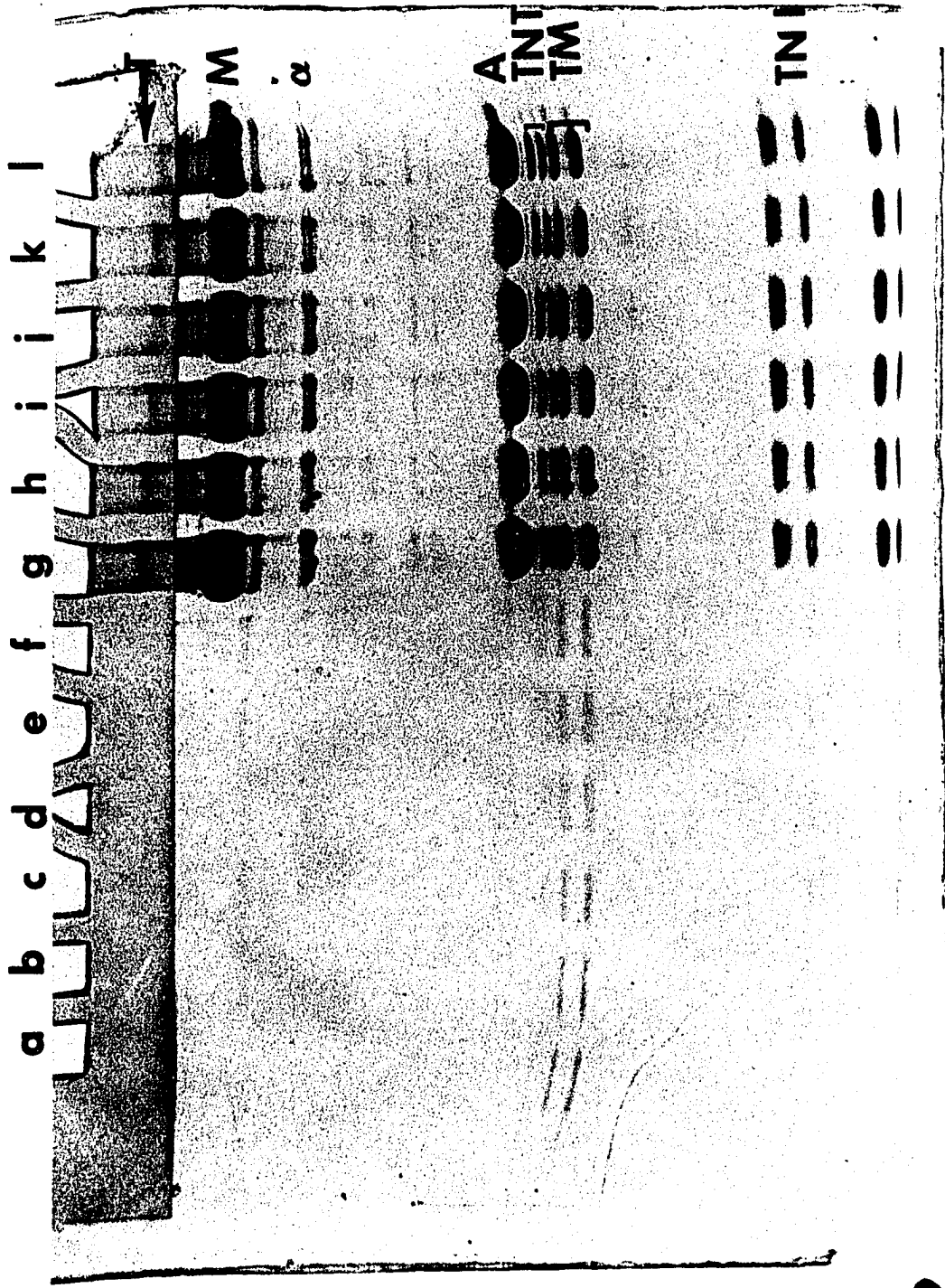


Figure 51. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified cathepsin D at 15° C and pH 6.5

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 6.5, 1.0 mM EDTA, were incubated with 40 μ g of purified cathepsin D at 15° C. The reaction was stopped by the addition of 20 μ l of 10 mM pepstatin and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 15° C. Lanes a through f were loaded with 50 μ l of supernatant fractions corresponding to myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, and 30, 60, 90, and 120 min incubations with enzyme, respectively. Lanes g through l were loaded with 48 μ g of sediment fractions corresponding to myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, and 30, 60, 90, and 120 min incubations with enzyme, respectively (see Materials and Methods). Labels at the right of the gel are as follows: T, titin; M, myosin heavy chains; α , alpha-actinin; B, bovine serum albumin; A, actin; O, ovalbumin; the small bracket indicates troponin T isomers; TM, the larger bracket indicates beta and alpha tropomyosin subunits; C, chymotrypsinogen.

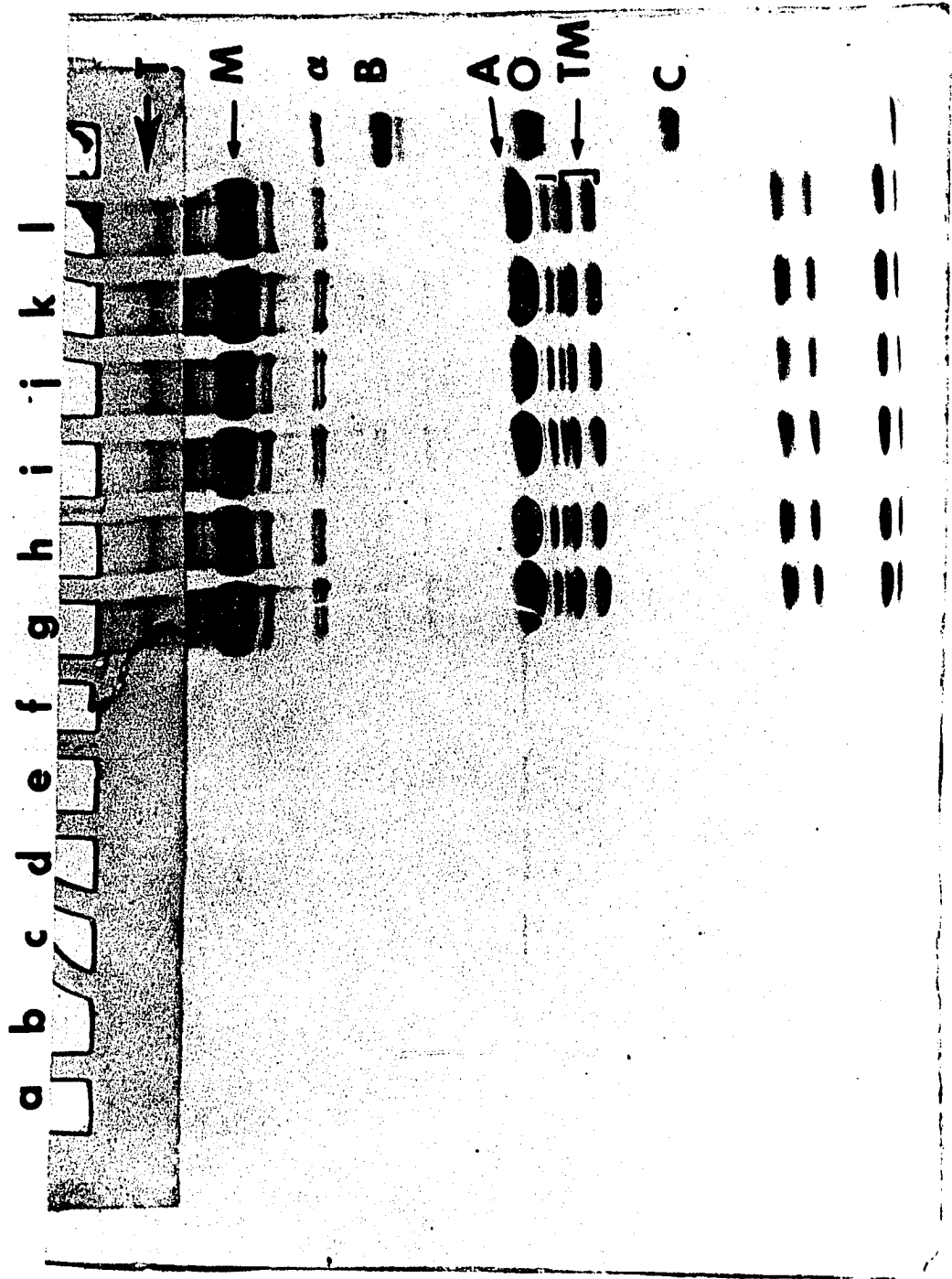
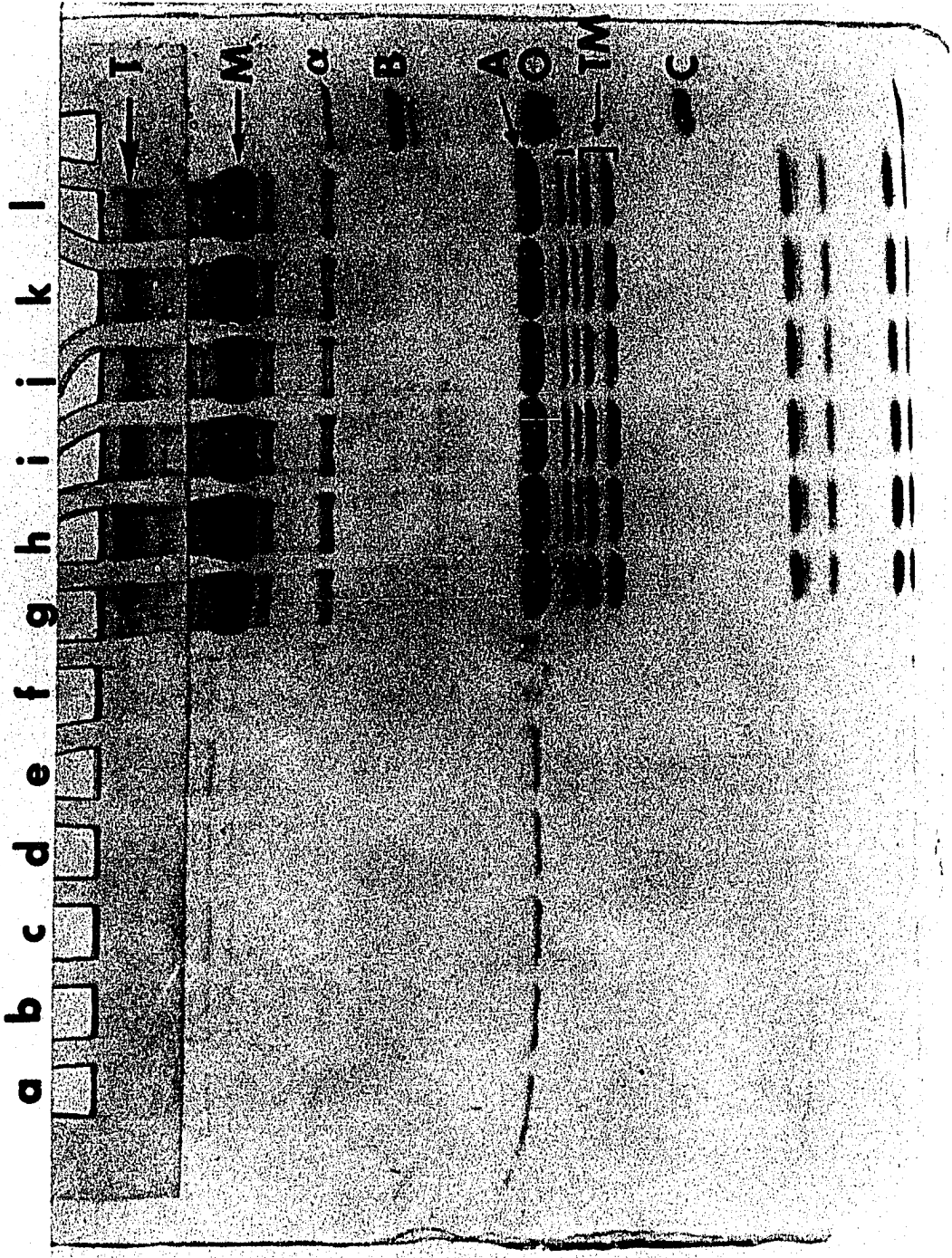


Figure 52. Ten percent SDS-PAGE slab gel, containing a 5% stacking gel, of supernatant and sediment fractions obtained from myofibrils that had been incubated with purified cathepsin D at 15° C and pH 7.5

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 7.5, 1.0 mM EDTA, were incubated with 40 μ g of purified cathepsin D at 15° C. The reaction was stopped by the addition of 20 μ l of 10 mM pepstatin and each sample was then centrifuged at 50,000 x g_{max} for 15 min at 15° C. Lanes a through f were loaded with 50 μ l of supernatant fractions corresponding to myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, and 30, 60, 90, and 120 min incubations with enzyme, respectively. Lanes g through l were loaded with 48 μ g of sediment fractions corresponding to myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, and 30, 60, 90, and 120 min incubations with enzyme, respectively (see Materials and Methods). Labels at the right of the gel are as follows: T, titin; M, myosin heavy chains; α , alpha-actinin; B, bovine serum albumin; A, actin; O, ovalbumin; the small bracket indicates troponin T isomers; TM, the larger bracket indicates beta and alpha tropomyosin subunits; C, chymotrypsinogen.



3. Fragmentation of myofibrils following cathepsin D digestion

Cathepsin D also was examined with respect to its effectiveness in altering the structural integrity of the myofibril under varying conditions of pH and temperature. Following incubation with cathepsin D under selected conditions of time, temperature, and pH, the myofibrils were subjected to a brief (10 sec) homogenization and then examined in the phase contrast microscope to ascertain the degree of fragmentation caused by cathepsin D. Figure 53 contains representative micrographs of myofibrils that had been incubated with cathepsin D at 37° C and pH 5.5. The arrowheads in the figures mark the position of an A band. Figure 53a and c show the appearance of the 0-min controls (myofibrils plus pepstatin-no enzyme and myofibrils plus pepstatin plus enzyme, respectively). Figure 53e and g show the appearance of myofibrils that have been incubated with cathepsin D for 60 and 120 min, respectively. The A band region progressively narrowed with increase in incubation time. However, there was still some Z-line material left after 120 min of incubation and there was no obvious increase in number of short (fragmented) myofibrils. Figure 53b, d, f, and h contain phase contrast micrographs of myofibrils incubated at 37° C and pH 6.5. Figure 53b and d contain the two controls. There appeared to be a small change (narrowing) in the A band after 120 min (Figure 53h) of treatment. Samples incubated for 60 (Figure 53f) or 120 min (Figure 53h) still had Z lines. Nor was there any obvious increase in fragmentation during the incubation. Figure 54a, c, e, and g contain phase contrast micrographs of myofibrils incubated at 37° C and pH 7.5. At this pH, there

Figure 53. Phase contrast micrographs of myofibrils after incubation with purified cathepsin D at 37° C and pH 5.5 or 6.5 and a brief homogenization

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 5.5 or 6.5, 1 mM EDTA, were incubated with 40 μ g of purified cathepsin D at 37° C. The reaction was stopped by the addition of 20 μ l of 10 mM pepstatin. One ml of this suspension was diluted with 1.0 ml of 100 mM KCl, and then homogenized for 10 sec at 15,000 rpm as described in the Materials and Methods section. Micrographs a, c, e, and g represent myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, 60, and 120 min incubations with enzyme, respectively, at 37° C and pH 5.5. Micrographs b, d, f, and h represent myofibrils plus pepstatin-no enzyme control, myofibrils plus enzyme plus pepstatin control, 60, and 120 min incubations with enzyme, respectively, at 37° C, and pH 6.5. The arrowhead in each indicates the position of an A band in the myofibril. The bar in micrographs g and h represents 5.0 μ m. Magnification was X2000 in all figures.

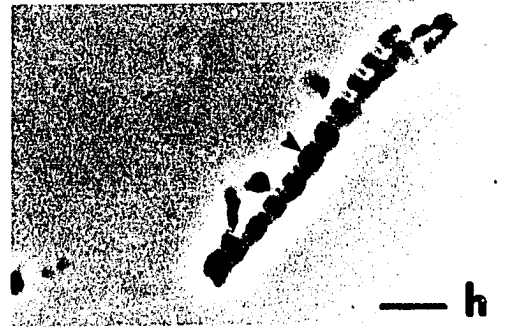
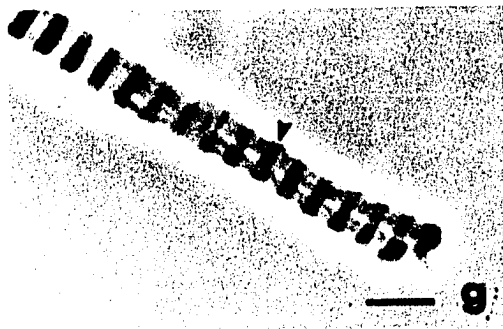
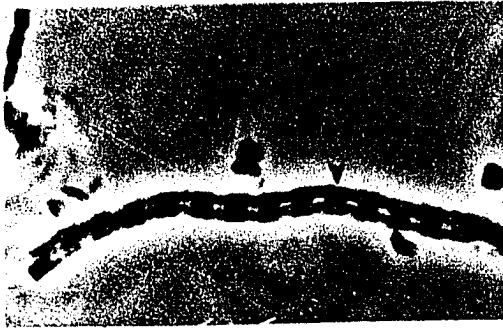
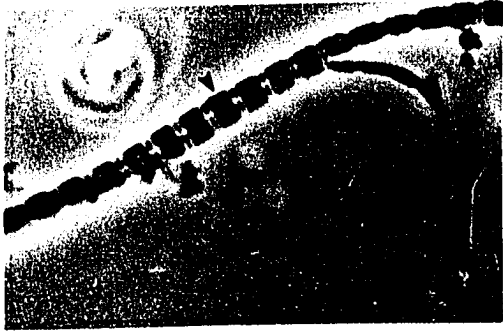
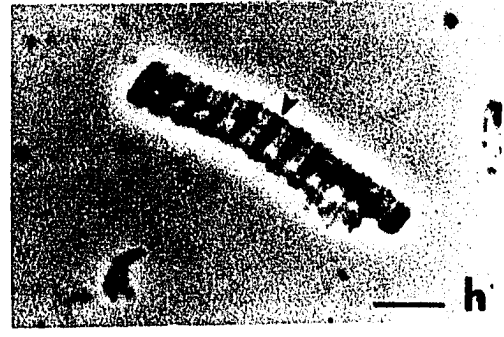
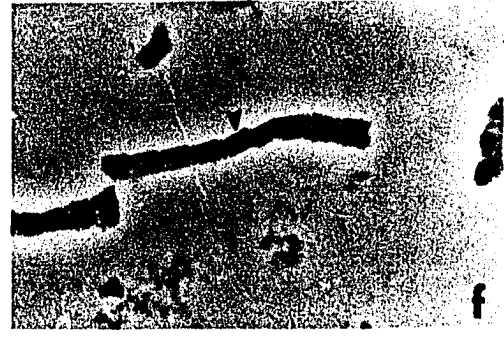
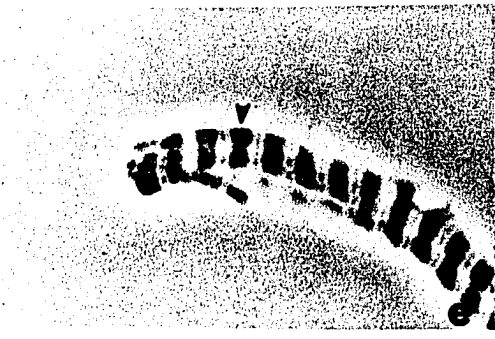
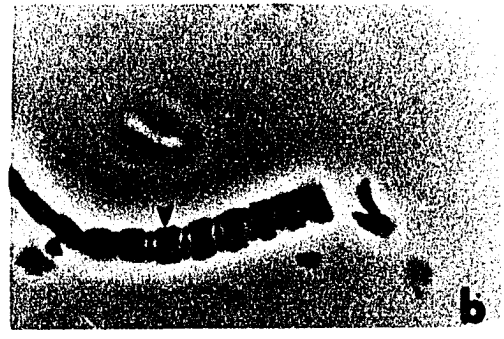


Figure 54. Phase contrast micrographs of myofibrils after incubation with purified cathepsin D at 37° C and pH 7.5 or at 25° C and pH 5.5 and a brief homogenization

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 7.5 (or 5.5), 1 mM EDTA, were incubated with 40 μ g of purified cathepsin D at 37° C (or 25° C). The reaction was stopped by the addition of 20 μ l of 10 mM pepstatin. One ml of this suspension was diluted with 1.0 ml of 100 mM KCl, and then homogenized for 10 sec at 15,000 rpm as described in the Materials and Methods section. Micrographs a, c, e, and g represent myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, 60, and 120 min incubations with enzyme, respectively, at 37° C and pH 7.5. Micrographs b, d, f, and h represent myofibrils plus pepstatin-no enzyme control, myofibrils plus enzyme plus pepstatin control, 60, and 120 min incubations with enzyme, respectively, at 25° C and pH 5.5. The arrowhead in each indicates the position of an A band in the myofibril. The bar in micrographs g and h represents 5.0 μ m. Magnification was X2000 in all figures.



were few if any marked structural changes after 60 (Figure 54e) or 120 min (Figure 54g) of incubation compared to the controls (Fig. 54a and c).

Figure 54b, d, f, and h show phase contrast micrographs of myofibrils that had been incubated with cathepsin D at 25^o C and pH 5.5. In comparison to the controls (Figure 54b and d), the A bands of the myofibrils after 120 min of treatment (Figure 54h) appeared somewhat narrow. The Z lines may have been degraded slightly as well, but some Z-line substance remained and there was no obvious increase in degree of fragmentation. Figure 55a, c, e, and g contain phase contrast micrographs of myofibrils that had been incubated at 25^o C and pH 6.5. After 120 min of incubation (Figure 55g), some loss of Z-line and A-band material appeared (compare to controls in Figure 55a and c). Figure 55b, d, f, and h show phase contrast micrographs of myofibrils that had been incubated at 25^o C and pH 7.5. There was little or no significant change in appearance of the myofibrils, except possibly for loss of some Z-line material in some myofibrils, after 120 min (Figure 55h) of incubation (compare to controls in Figure 55b and d).

Figure 56 shows phase contrast micrographs of myofibrils that had been incubated at 15^o C and pH 5.5 (Figure 56a, c, e, g) or pH 6.5 (Fig. 56b, d, f, h). At this temperature, little observable change in appearance of the myofibrils had occurred during incubation with the cathepsin D.

The extent of myofibril fragmentation caused by treatment with cathepsin D was difficult to accurately assess because even untreated, freshly prepared myofibrils contained a distribution of myofibrils in which the number of sarcomeres per myofibril varied from 1 to 20 or more.

Figure 55. Phase contrast micrographs of myofibrils after incubation with purified cathepsin D at 25° C and pH 6.5 or 7.5 and a brief homogenization

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 6.5 or 7.5, 1 mM EDTA, were incubated with 40 µg of purified cathepsin D at 25° C. The reaction was stopped by the addition of 20 µl of 10 mM pepstatin. One ml of this suspension was diluted with 1.0 ml of 100 mM KCl, and then homogenized for 10 sec at 15,000 rpm as described in the Materials and Methods section. Micrographs a, c, e, and g represent myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, 60, and 120 min incubations with enzyme, respectively, at 25° C and pH 6.5. Micrographs b, d, f, and h represent myofibrils plus pepstatin-no enzyme control, myofibrils plus enzyme plus pepstatin control, 60, and 120 min incubations with enzyme, respectively, at 25° C and pH 7.5. The arrowhead in each indicates the position of an A band in the myofibril. The bar in micrographs g and h represents 5.0 µm. Magnification was X2000 in all figures.

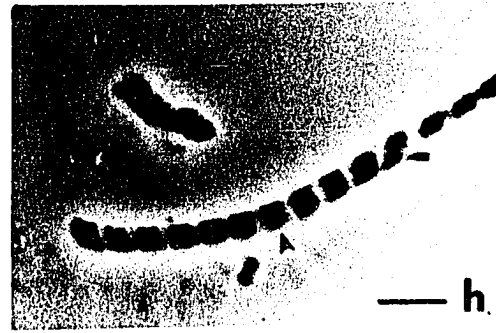
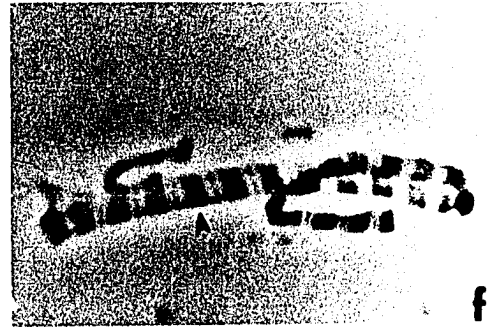
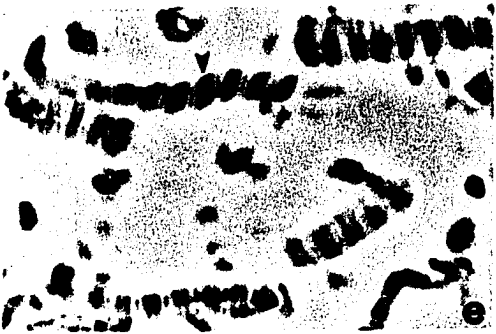
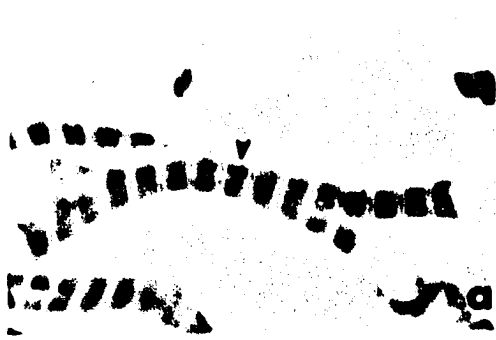


Figure 56. Phase contrast micrographs of myofibrils after incubation with purified cathepsin D at 15° C and pH 5.5 or 6.5 and a brief homogenization

Assay conditions: 4 mg of bovine skeletal myofibrils in 2.0 ml of 100 mM KCl, 50 mM imidazole-HCl, pH 5.5 or 6.5, 1 mM EDTA, were incubated with 40 μ g of purified cathepsin D at 15° C. The reaction was stopped by the addition of 20 μ l of 10 mM pepstatin. One ml of this suspension was diluted with 1.0 ml of 100 mM KCl, and then homogenized for 10 sec at 15,000 rpm as described in the Materials and Methods section. Micrographs a, c, e, and g represent myofibrils plus pepstatin-no enzyme control, myofibrils plus pepstatin plus enzyme control, 60, and 120 min incubations with enzyme, respectively, at 15° C and pH 5.5. Micrographs b, d, f, and h represent myofibrils plus pepstatin-no enzyme control, myofibrils plus enzyme plus pepstatin control, 60, and 120 min incubations with enzyme, respectively, at 15° C, and pH 6.5. The arrowhead in each indicates the position of an A band in the myofibril. The bar in micrographs g and h represents 5.0 μ m. Magnification was X2000 in all figures.



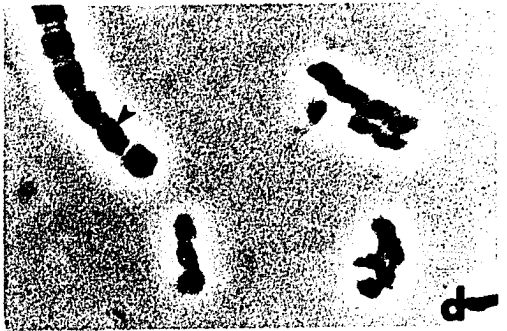
a



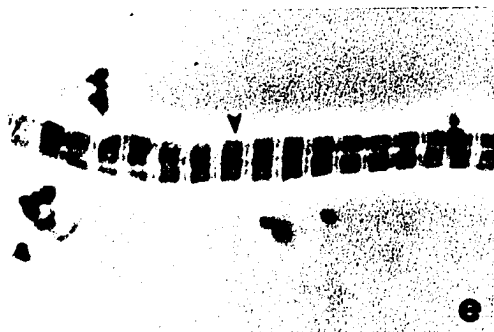
b



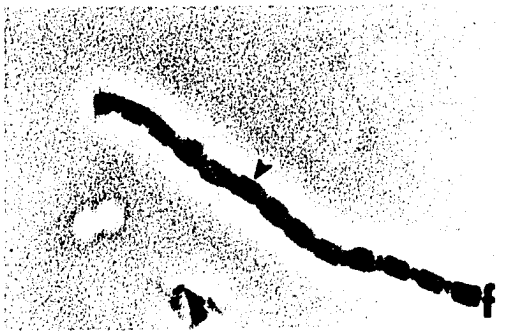
c



d



e



f



— g

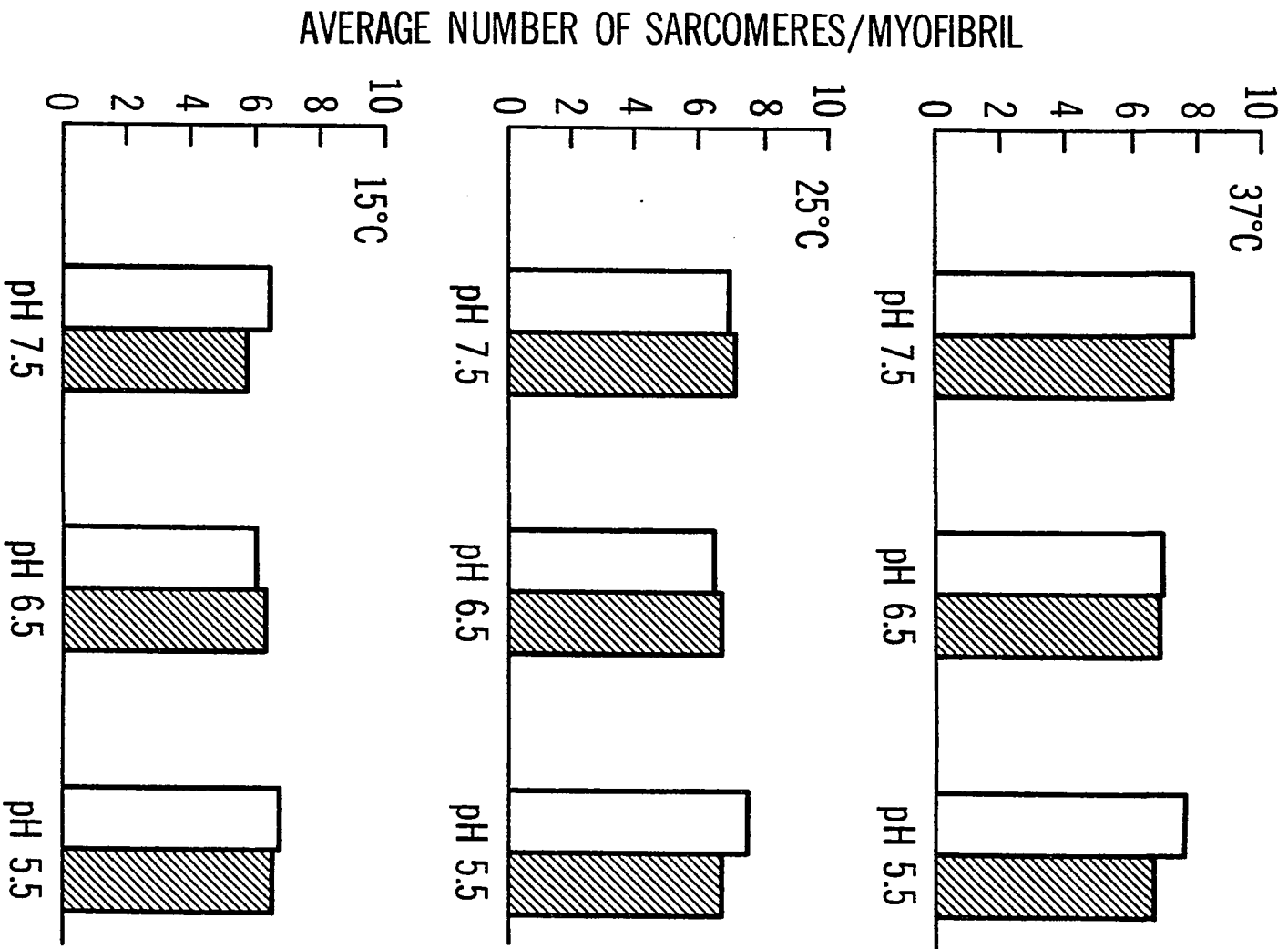


— h

In an attempt to more objectively determine if cathepsin D treatment caused significant fragmentation compared to the controls, 0-min (myofibrils plus pepstatin-no enzyme) samples and samples from the 120-min incubations were compared (Figure 57). The procedure followed was the same used in the CAF comparisons (Figure 35). The results in Figure 57 show the average number of sarcomeres per myofibril for the 0-min control and for the 120-min samples, incubated at 37^o, 25^o, or 15^o C and at pH 7.5, 6.5, or 5.5. There were no obvious differences in degree of fragmentation (sarcomeres per myofibril) between the control and treated samples.

Figure 57. Summary of degree of myofibril fragmentation after incubation with cathepsin D at selected pH values and temperatures and a brief homogenization

Myofibrils were incubated with purified cathepsin D at 37°, 25°, or 15° C, and pH 5.5, 6.5, or 7.5 for 120 min and then subjected to a brief 10-sec homogenization. In the phase contrast microscope, the number of sarcomeres per myofibril was counted in at least ten fields of the 0-min (myofibril plus pepstatin-no enzyme (control) sample and the sample treated with cathepsin D for 120 min. The average number of sarcomeres per myofibril was calculated and is shown as open vertical bars for the 0-min samples and as shaded vertical bars for the 120-min samples.



V. DISCUSSION

Two of the endogenous proteolytic enzymes that have been proposed as playing important roles in changes occurring in muscle postmortem are CAF and cathepsin D (Dutson, 1983; Goll et al., 1983a). CAF is not enclosed in lysosomes, whereas cathepsin D is a lysosomal enzyme (Bird et al., 1980; Goll et al., 1983b). As is the case for all enzymes, CAF and cathepsin D have their own respective set of optimum conditions that are needed for maximum activity in vitro. The postmortem environment in muscle cells, however, changes considerably during the first 24 to 48 hr after death of the animal. And, the conditions present in postmortem muscle seemingly are never ideal for maximum activity of either enzyme. The major purpose of this dissertation research was to determine the ability of purified CAF and purified cathepsin D to hydrolyze myofibrillar proteins and to cause structural alterations in myofibrils under in vitro conditions resembling those found in postmortem muscle. Before studies involving the effects of CAF and cathepsin D on myofibrillar proteins and structure could be done satisfactorily, purified preparations of each enzyme had to be prepared.

A. Purification and Characterization of CAF

The methods used in this study for the preparation of cardiac CAF resulted in a greater yield (approximately 5 $\mu\text{g/g}$ ground muscle) than those outlined for its purification from porcine skeletal muscle (approximately 0.8 $\mu\text{g/g}$ muscle; Dayton et al., 1975, 1976a). There are several possible explanations for this increase in yield. First, it

has been reported (Dean, 1980; Millward, 1980) that cardiac muscle contains a greater amount of proteolytic enzymes in general than does skeletal muscle. Second, the improvement in chromatographic procedures as described by D. E. Goll¹ eliminated the need for concentration steps between columns by ammonium sulfate precipitation. Each precipitation of CAF with ammonium sulfate results in some loss of total units of activity. Finally, the shortening of time required for preparation of purified CAF by the methods used herein offers the advantage of reduced exposure of the enzyme to the denaturing elements present in any in vitro isolation.

In the chromatography of P₀₋₄₀ crude CAF on DEAE-cellulose, the fractions containing CAF activity were eluted late in the profile. Although no KCl concentration determinations were made, it can be estimated from the position of the active fractions in the profile that the KCl concentration was near 300 mM. This is in reasonable agreement with reported values of 220 to 280 mM KCl for skeletal muscle CAF (Goll et al., 1983a). The phenyl-Sepharose and antipain-Sepharose columns were quite effective in producing a highly homogeneous enzyme.

The extraction and purification procedures utilized resulted in purified CAF that generally contained 20 to 30 mg of protein (from 5 kg of ground muscle) with a specific activity of 200 to 300 units/mg. The preparative procedures were quite reproducible in the three CAF preparations made for the studies described in this dissertation. My preparation contained only the high-Ca²⁺ form of the enzyme (see

¹Goll, D. E., University of Arizona, Tucson, Arizona, 1982.

Goll et al., 1983b for complete description of CAF forms). Almost no enzymic activity was found when the purified CAF was assayed against casein in the presence of $100 \mu\text{M Ca}^{2+}$; thus, the low- Ca^{2+} CAF form was essentially absent. It has been reported that most muscle sources contain both high- and low- Ca^{2+} forms of CAF and that beef skeletal muscle is one of the richest sources of the low- Ca^{2+} form (Goll et al., 1983b). Therefore, it originally seemed likely that the low- Ca^{2+} form was present in the original extract from bovine cardiac muscle, but that it was lost in subsequent purification steps. Examination of the relative CAF activity profile shown in the DEAE-cellulose purification (Figure 2), however, showed no obvious peak of CAF activity early in the elution profile where the low- Ca^{2+} form of CAF would be eluted (Szpacenko et al., 1981; Goll et al., 1983b). Thus, it appears that bovine cardiac muscle contains primarily the high- Ca^{2+} form of CAF.

Purified CAF was characterized by electrophoresis under non-denaturing and denaturing conditions. When examined under nondenaturing conditions on a 7.5% acrylamide gel, the CAF showed one major band and one significant lighter band that migrated just below the major band. When the same CAF preparation was electrophoresed on a 10% acrylamide gel containing SDS, it showed primarily two bands corresponding to protein subunits of 80,000 and 30,000 molecular weight. These results were in good agreement with studies on skeletal muscle CAF by other investigators (Dayton et al., 1976a; Goll et al., 1983b), and demonstrated the high purity of the enzyme isolated in this study.

In order to examine the subunit composition of bands observed with CAF separated on nondenaturing gels, a two-dimensional gel system was employed. This technique showed that the major band observed in the nondenaturing system (first dimension) was composed of two spots (second dimension) with molecular weights of approximately 80,000 and 30,000. The minor band observed in the nondenaturing gel was composed of the 80,000 molecular weight subunit and a small amount of an 18,000-dalton component. No attempt was made to determine the relative molar ratio of the 80,000 and 18,000 dalton subunits. It has been reported (Edmunds et al., 1984) that bovine skeletal high-Ca²⁺ CAF contains subunits of about 80,000 and 30,000 molecular weight (78,000 and 28,000, respectively, on Laemmli gels). Under conditions of very limited autolysis, the 30,000-dalton component is degraded to a major fragment of 18,000 daltons (Goll et al., 1983b; Edmunds et al., 1984). Therefore, the results found herein with cardiac muscle CAF suggested that the major band observed in nondenaturing gels was the native high-Ca²⁺ CAF and that the lighter band below it on denaturing gels represented the slightly autolyzed form of the enzyme (i.e., autolyzed high-Ca²⁺ CAF). Both forms are seemingly identical in their specific activities and substrate specificities (Goll et al., 1983b).

A new and very useful method was developed for the simple and rapid assay of CAF-containing samples as described in the Materials and Methods section. The casein agarose plate method (spot assay method) offered the advantage of permitting one to examine a large number of samples in a short period of time, with good sensitivity.

A conventional casein assay (tube assay) requires incubation of each fraction in a tube, precipitation, centrifugation, and measurement of the absorbance at 278 nm. Thus, the tube assay method requires significantly more time and sample than does the agarose plate method. A limitation to the sensitivity of the casein agarose plate method occurs in the presence of large quantities of other proteins, which cause dark staining circles in the assay that mask the activity of CAF. This situation was encountered especially in assays done on P₀₋₄₀ crude CAF fractions. It was overcome, however, simply by diluting the sample 1:10 with water before spotting.

B. Examination of the Effect of Purified CAF on Myofibrils

The studies described herein on the effect of CAF on myofibrils were repeated three times. All of the results from the repetitions were similar. But, there were small differences in myofibril preparations, appearances of samples examined by SDS-PAGE, etc. Thus, a representative set of results was shown herein. The results obtained by SDS-PAGE analysis (Figures 11-25) of supernatant and sediment fractions prepared from myofibrils that had been treated with CAF and shown herein were from the same preparations of myofibrils and CAF used to measure the amount of protein released by CAF (Figures 8-10). This enabled more direct comparisons. Myofibrils examined for fragmentation by phase contrast microscopy were treated with CAF separately from those used for SDS-PAGE analysis or release of soluble protein because of time constraints. It was found that CAF was effective in releasing

soluble protein from myofibrils when incubated under a wide range of pH and temperature conditions (Figures 8-10).

Substantial amounts of protein were released at 25^o C and pH 7.5 to 5.5. Although there was a decrease in the amount of protein released as the pH or temperature was decreased, significant amounts were still released by CAF treatment at 15^o C or 5^o C and pH 6.0 or 6.5, respectively. These conditions would be present during the time that many changes occur in postmortem muscle. The activity at 5^o C and pH 5.5, conditions similar to those in well-chilled postmortem muscle, was small. It is not known if this small amount of CAF activity would, over a long period of time (several days), continue to contribute significantly to further changes occurring in myofibrils during aging of meat.

A limited study on the release of soluble protein from myofibrils incubated with CAF has previously been reported by Suzuki and Goll (1974). Those investigators observed that incubation of rabbit skeletal muscle myofibrils at pH 7.0 and 25^o C with a crude CAF preparation resulted in the release of some soluble protein. In subsequent studies, it was shown by SDS-PAGE that the solubilized fraction contained components with molecular weights of about 34,000, 100,000, and 300,000 to 400,000 (Suzuki et al., 1975; Suzuki et al., 1978b). The 100,000 dalton component was tentatively identified as alpha-actinin. Suzuki et al. (1978a) showed that when previously low-ionic-strength, Z-line-extracted myofibrils were incubated with a partially purified sample of protein released from myofibrils by CAF, the Z lines of these myofibrils were partly reconstituted. This suggests that at least some

of the proteins released by CAF originated from the Z line. More recently, the high molecular weight component (300,000-400,000) was isolated from the protein solubilized by incubation of myofibrils with CAF and was named Z nin (Suzuki et al., 1982).

The results concerning release of solubilized protein from myofibrils by CAF presented by Suzuki et al. (1978a,b) are fairly similar to the results presented in this study. However, the work done by those investigators was done with crude enzyme preparations that may have contained other enzymes. This made it difficult to confirm that the observed effects actually resulted from the action of CAF. The work presented herein with purified CAF enzyme demonstrated that CAF is effective in releasing soluble protein from myofibrils in vitro and that it is effective over a fairly wide range of pH and temperature conditions.

The components of released protein were shown by SDS-PAGE analysis of supernatant fractions prepared from the treated myofibrils to contain soluble, putative high molecular weight fragments of titin, intact alpha-actinin, and some other unidentified high molecular weight proteins. In some incubations done at low pH (e.g., pH 6.0-5.5; Figures 14-15), a small amount of thin filament proteins such as troponin T and tropomyosin was also solubilized, even in the control samples. A possible explanation for this result was that the low pH alone was changing the solubility/extraction properties of the myofibrils and, thus, inducing the release of some thin filament proteins. Alpha-actinin appeared to be the major single constituent in the protein

released by CAF. It did not appear to be degraded. These results showing that CAF can release intact alpha-actinin are in agreement with those of Dayton et al. (1975).

The sediment fractions prepared by centrifugation of myofibrils that had been incubated with CAF were also examined by SDS-PAGE. These results showed that the recently discovered, high molecular weight myofibrillar protein titin (Wang et al., 1979) appeared to be the most susceptible component to CAF-induced degradation. The SDS-PAGE and immunoblot studies showed that intact titin migrated into the 5% acrylamide stacking gel region. Several soluble titin fragments were generated during the incubation. However, some slightly degraded, but still very high molecular weight, titin fragments also remained with the insoluble sediments. The results herein showing that CAF can degrade titin are interesting because Lusby et al. (1983) recently have shown that titin is degraded in bovine muscle stored postmortem.

The SDS-PAGE procedure employed in my studies was able to effectively resolve the high molecular weight protein titin, but it did not adequately resolve the protein nebulin (Wang and Williamson, 1980). In results not shown here, it was found that purified bovine skeletal nebulin migrated to the region of the interface between the stacking and separating gels in the SDS-PAGE system used in my studies. Therefore, it was not possible to observe alterations in nebulin that might have been caused by incubation with CAF.

Other alterations observed in the results of SDS-PAGE analysis of sedimented fractions were: 1) The loss of alpha-actinin coincided

with its release into the supernatant fractions; 2) degradation of troponin T, tropomyosin and troponin I; and 3) the appearance of a trio of bands in the 30,000-dalton region of the slab gels. These results are in agreement with those of other researchers (e.g., Dayton et al., 1975; Olson et al., 1977). The appearance of the 30,000-dalton bands is of possible importance to meat science because they appear as a result of the action of CAF (Olson et al., 1977). These bands appear in SDS-PAGE gels of muscle samples aged postmortem and, thus, could be used as an indicator of postmortem CAF activity. Desmin has been shown to be degraded by CAF (O'Shea et al., 1979), but desmin was not observed in my studies because of its low concentration in skeletal muscle myofibril fractions (O'Shea et al., 1981).

In general, the extent of changes observed in results obtained by SDS-PAGE analysis of samples from CAF-treated myofibrils (Figures 11-25) reflected the same time course, pH, and temperature effects seen in the studies on quantitation of protein released (Figures 8-10). Decrease in either pH from 7.5 or in temperature from 25^o C reduced the observed changes. However, significant changes were still seen during the course of digestions done at 15^o C and pH 6.0-6.5 or at 5^o C and pH 6.5-7.0.

The loss of Z-line integrity has been proposed as one cause for the increase in tenderness that occurs in postmortem muscle (Stromer et al., 1967; Davey and Gilbert, 1969; Henderson et al., 1970; and Penny, 1970). The loss of Z line integrity may result in many breaks along the myofibril. Parrish et al. (1973) found that the myofibrils prepared

from tender muscles had shorter myofibril fragments than myofibrils prepared from less tender muscles. And, Olson et al. (1976) have reported a high correlation coefficient (-0.75) between myofibril fragmentation and Warner-Bratzler shear values for bovine longissimus muscle. Therefore, the results obtained by phase contrast microscope examination of CAF-treated and homogenized myofibril samples shown within this study (Figures 27-35) are significant with respect to understanding the events contributing to postmortem tenderization. My results showed that CAF can cause an increase in loss of Z line integrity and in myofibril fragmentation in vitro within a fairly wide range of pH and temperature conditions. This evidence adds further support to the suggestion that CAF has a principle role in the post-mortem tenderization process (Goll et al., 1983a; Olson et al., 1976). Additional studies will be required to answer questions such as the Ca^{2+} concentration required for activation of CAF postmortem and how the CAF/CAF inhibitor interaction is controlled and modified under postmortem conditions.

C. Purification and Characterization of Cathepsin D

The results of my study concerning purification of cathepsin D and its effects on myofibrils should be considered preliminary in nature. Three enzyme preparations of cathepsin D were prepared and tested. But only the last preparation made was of high purity and high specific activity. Thus, the results shown herein were all done with a single enzyme preparation. The cathepsin D isolation (crude P₄₅₋₆₅ cathepsin D) from bovine cardiac muscle was performed as described by

Barrett (1977) for the rat skeletal muscle enzyme and as outlined in Figure 36. Chromatographic procedures used were based on those described by Afting and Becker (1981) for the purification of cathepsin D from porcine myometrium. Concanavalin A-Sepharose chromatography of crude P₄₅₋₆₅ cathepsin D was very effective in separating cathepsin D from other proteins. Subsequent affinity chromatography on pepstatin-Sepharose 4B resulted in a highly homogeneous preparation of cathepsin D in comparison to the purified cathepsin D enzymes prepared by others (Robbins et al., 1979; Okitani et al., 1981; Matsumoto et al., 1982). The use of urea-containing buffer, as described by Afting and Becker (1981), in the pepstatin affinity chromatography step was not required. The use of cardiac muscle as a source of cathepsin D resulted in a higher yield (approximately 1 $\mu\text{g/g}$ of fresh ground tissue) of enzyme than in procedures using rat skeletal muscle (approximately 0.1 $\mu\text{g/g}$ of fresh ground tissue; Schwartz and Bird, 1977).

Purified cathepsin D was subjected to electrophoresis under denaturing and nondenaturing conditions. Under nondenaturing conditions at pH 7.5, the purified cathepsin D exhibited three closely spaced bands (Figure 39). However, SDS-PAGE analysis showed the presence of only two bands of approximately 47,000 and 29,500 molecular weight (Figure 39). In an attempt to further characterize the three bands seen in nondenaturing gels, cathepsin D also was examined by a two-dimensional electrophoretic procedure in which nondenaturing electrophoresis was performed first in a disc gel. The gel was then equilibrated in SDS-buffer and electrophoresed in the second dimension

by SDS-PAGE (denaturing conditions) on a slab gel (Figure 40). In this way, it was possible to examine the subunit composition of native cathepsin D bands observed on nondenaturing gels. The results showed that the fastest migrating band in the nondenaturing disc gel contained only the 47,000-dalton subunit. The slowest migrating band in the disc gel contained both 47,000- and 29,500-dalton subunits.

One group of investigators has reported that cathepsin D isolated from porcine spleen contained three to five isozymes with native molecular weights of approximately 50,000 (Huang et al., 1979). Other investigators have reported the presence of a 100,000-dalton native species, which they suggested was a proenzyme form of cathepsin D (Puizdare and Turk, 1981). The subunit molecular weights of porcine spleen cathepsin D reported by Puizdare and Turk (1981) were estimated to be 35,000 and 15,000 using the electrophoresis system described by Weber and Osborn (1969). Ogunro et al. (1979, 1982) found that cathepsin D purified from canine cardiac muscle had a native molecular weight of approximately 50,000 as measured by gel filtration. Canine cardiac cathepsin D was composed of two subunits with molecular weights of approximately 32,000 and 14,000 (Ogunro et al., 1980; Sameral et al., 1981) by SDS-PAGE. Multiple isozymes of rat skeletal muscle cathepsin D were observed by isoelectric focusing (Schwartz and Bird, 1977).

Clearly, direct comparisons and reconciliation of the electrophoretic results presented in my study on cathepsin D with those of other investigators are difficult. This may partly result from differences in SDS-PAGE systems used. In general, most other

investigators have observed two bands, corresponding to molecular weights of approximately 30,000 to 35,000 and 13,000 to 15,000, for purified cathepsin D subjected to SDS-PAGE. The SDS-PAGE results of cathepsin D presented herein in Figure 39 showed two bands with molecular weights of approximately 47,000 and 29,500. Although the reasons for this discrepancy in estimated subunit molecular weights are not clear, it is apparent that the molecular properties of cathepsin D remain controversial and unclear. This seems surprising because cathepsin D has been prepared from many sources and studied for over 20 years.

D. Effect of Cathepsin D on Myofibrils

Cathepsin D was examined with respect to its ability to release soluble protein from myofibrils over a fairly wide range of pH and temperature conditions. The results (Figures 41-43) indicated that, compared to CAF, cathepsin D caused only a small release of soluble protein. Only when cathepsin D was incubated at pH 5.5 and 37^o C was there a significant release of protein. SDS-PAGE analysis showed that the solubilized fractions (supernatants resulting from centrifugation) of the treated myofibrils contained primarily very low molecular weight products. Incubation at higher pHs or at lower temperatures resulted in very little solubilized protein.

SDS-PAGE analysis of the sedimented myofibrils obtained after cathepsin D incubation showed that myosin heavy chains were extensively degraded at 37^o C and pH 5.5 (Figure 44) to fragments with molecular weights of approximately 155,000 and 90,000. A small amount of this type of degradation occurred at 37^o C and pH 6.5 and at 25^o C and

pH 5.5. At lower temperatures and/or higher pH combinations, no significant degradation of myosin heavy chains occurred as a result of cathepsin D treatment. The pattern of myosin heavy chain degradation seen at high temperature and low pH was similar to that observed by Schwartz and Bird (1977). Those investigators reported that cathepsin D treatment of myosin produced myosin heavy chain fragments of 150,000 to 175,000 daltons and of 110,000 daltons.

The amount of titin in these myofibrils observed by SDS-PAGE was somewhat variable; however, cathepsin D did degrade it. Maruyama et al. (1981a) also have found that titin is degraded by cathepsin D.

Degradation of actin by cathepsin D treatment at high temperature and low pH also was observed in this study (Figure 44) and by Schwartz and Bird (1977). Tropomyosin and troponins T and I were slowly degraded by my purified cathepsin D at low pH and high temperature. Similar results were obtained by using rabbit skeletal muscle cathepsin D treatment of rabbit skeletal myofibrils by Matsumoto et al. (1982). However, in results not shown herein, I found that purified cathepsin D was not effective in degrading purified bovine skeletal muscle troponin subunits or tropomyosin (at pH 5.5 and 37^o C). The reason for these differences is unclear. Perhaps the conformation of the troponin and tropomyosin substrates is different in the myofibril. It has also been reported that rabbit skeletal muscle cathepsin D degraded alpha-actinin (Okitani et al., 1981; Matsumoto et al., 1982); however, no similar effects on alpha-actinin were observed with cathepsin D in my study. Because their studies may have been done

with impure enzymes, the effects ascribed to cathepsin D may have been due to other contaminating proteases.

The range of conditions in which cathepsin D was effective in causing significant myofibrillar alterations was rather narrow. Little activity was observed at pH 6.5 and above at 25° C (Figure 49) or at lower temperature coupled with any pH.

Cathepsin D caused significant structural alterations in myofibrils at pH 5.5 and 37° C as judged by phase contrast microscopy (Figure 53). The treated myofibrils exhibited unusually narrow A bands and some, but not all, of the Z line material was lost. The degree of A band narrowing observed correlated well with the degradation of myosin heavy chains observed on SDS-PAGE gels. However, even when these myofibrils were subjected to brief homogenization, there was no obvious increase in degree of fragmentation (Figure 57). This suggested that the cathepsin D treatment had not sufficiently degraded the Z line and/or other parts of the myofibril to cause fragmentation. This latter result is important to meat science because some investigators have proposed that cathepsin D is a causative agent in the postmortem tenderization process (Robbins et al., 1979; Dutson, 1983). In view of the results from in vitro assays of the effect of purified cathepsin D on myofibrils found herein, it seems doubtful that this enzyme plays a principal role in the postmortem tenderization process. A similar suggestion recently has been made by Goll et al. (1983a), who suggested that CAF, but not cathepsin D, is an important enzyme in terms of the proteolysis that occurs in muscle postmortem. In further support of

the notion that CAF is involved in the proteolysis that occurs in myofibrillar proteins and structure in postmortem muscle but that cathepsin D is not, are the results showing that the changes caused by CAF (e.g., loss of Z lines, breakdown of titin, troponin T, etc.) but not the changes caused by cathepsin D (e.g., degradation of myosin or actin) mimic the changes observed in muscle stored postmortem (Olson and Parrish, 1977; Maruyama, 1981b; Goll et al., 1983a). Breakdown of myosin heavy chains, actin, etc. does not occur in muscle stored postmortem (Dayton et al., 1976b; Olson et al., 1976) unless the muscle is stored at abnormally high temperatures (Goll et al., 1983a).

VI. SUMMARY AND CONCLUSIONS

Cathepsin D and CAF are two proteolytic enzymes present in muscle cells that may play roles in causing some of the changes that occur in postmortem muscle. However, nearly all studies done to demonstrate the effect of CAF or cathepsin D on myofibrillar structure and proteins have been performed at or near optimal conditions for the enzymes. Those conditions, however, are not found in muscle cells postmortem. My study was designed to assess the effectiveness of these enzymes under conditions that more closely resemble those found in the muscle cell postmortem. Many past studies also have been done using "crude" or "partially purified" enzyme preparations, which may have contained more than one proteolytic enzyme. Considerable effort was made in my studies to prepare and use highly homogeneous preparations of enzymes so that any observed effects caused by incubation of myofibrils with CAF or cathepsin D were due to the respective enzyme.

A. Studies with CAF

A P₀₋₄₀ crude CAF fraction was isolated from bovine heart using the procedures described by Dayton et al. (1975) and purified by three successive chromatographic steps. These procedures routinely yielded 20 to 30 mg (from 5 kg of ground tissue) of highly homogeneous enzyme. Most of the CAF protein consisted of molecules that contained both 80,000- and 30,000-dalton subunits. A smaller amount of autolyzed CAF protein, which contained 80,000- and 18,000-dalton subunits, also was present. The CAF enzyme required millimolar concentrations of

Ca²⁺ ions; thus, the preparation consisted of the high-Ca²⁺ form of CAF (Goll et al., 1983b).

Purified CAF caused a number of effects on myofibrillar proteins and structure. The major effects were as follows:

1) CAF treatment of bovine skeletal myofibrils caused a large release of soluble protein from the myofibrils. CAF was most effective in causing this release at pH 7.5 and 25° C; however, significant release still occurred at pH 5.5 and 25° C, pH 6.0 to 6.5 and 15° C, or pH 6.5 and 5° C.

2) SDS-PAGE examination of the protein fractions solubilized (collected as supernatants after centrifugation) by incubation with CAF contained intact alpha-actinin, titin fragments, and several unidentified high molecular weight (above 180,000) proteins.

3) Examination of the sediments obtained by centrifugation of myofibrils that had been treated with CAF showed that titin was the most rapidly degraded myofibrillar protein. Alpha-actinin was decreased in amount. Tropomyosin and troponins T and I were at least partly degraded. A trio of bands that migrated in the 30,000 molecular weight region of the gel appeared during the digestion.

4) The ability of CAF to cause alterations in integrity of myofibrillar protein molecules under varying conditions of pH and temperature paralleled the results observed for the release of soluble protein by CAF. The alterations were greatest at pH 7.0-7.5 and 25° C, but significant activity remained at pH 6.0-6.5 and 15° C, or at pH 6.5-7.0 at 5° C.

5) When myofibrils were incubated with CAF and subjected to a brief homogenization, CAF clearly caused an increase in degree of myofibril fragmentation (shorter myofibrils). CAF was most effective in causing the myofibrillar fragmentation at pH 7.5 and 25^o C; however, there was still a significant amount of CAF-induced fragmentation at 25^o C and pH 5.5, 15^o C and pH 6.5, and 5^o C and pH 7.5.

These results indicate that CAF digestion can cause a number of alterations in myofibrillar proteins and structure within a range of pH and temperature conditions similar to those present for at least several hours in muscle cells after death. Thus, this study provides additional support for CAF's involvement in some of the changes that occur in postmortem muscle.

B. Studies with Cathepsin D

Although cathepsin D has been studied by many investigators over the last 20 to 30 years, it remains a poorly characterized enzyme, especially as isolated from skeletal muscle. Preliminary studies were conducted on cathepsin D as part of my dissertation research. In my study, cathepsin D was extracted from bovine heart muscle as described by Barrett (1977) and purified by modification of the chromatographic procedures described by Afting and Becker (1981). These procedures resulted in 3 to 4 mg of highly homogeneous protein with a high specific activity. The cathepsin D contained three closely spaced bands when examined by electrophoresis in nondenaturing buffers. Examination by SDS-PAGE showed that the cathepsin D contained two subunits with molecular weights of approximately 47,000 and 29,500.

In comparison to CAF, cathepsin D's effect on myofibrils was very limited. The enzyme presumably would have been more effective if incubated at pH values lower than 5.5; however, pH values below 5.5 would seldom occur normally in postmortem muscle. The major effects of cathepsin D on myofibrillar proteins and structure under selected conditions of pH and temperature were as follows:

1) Cathepsin D treatment of bovine skeletal myofibrils caused a significant release of soluble protein from the myofibrils after 30 to 60 min of incubation at pH 5.5 and 37^o C. However, little or no release of soluble protein above that found in controls occurred at the other higher pH and lower temperature values tested in this study, even after 120 min of incubation.

2) SDS-PAGE examination of the protein fractions solubilized/ released (collected as supernatants after centrifugation) from myofibrils by incubation with cathepsin D at pH 5.5 and 37^o C showed that they contained mostly very low molecular weight material (less than 15,000).

3) Examination by SDS-PAGE of the sediments obtained by centrifugation of myofibrils that had been treated with cathepsin D at pH 5.5 and 37^o C showed that titin was degraded and that myosin heavy chains were degraded to major fragments of approximately 155,000 and 90,000 daltons. Actin, tropomyosin, troponins T and I, and myosin light chain 1 also were slightly degraded. When myofibrils were incubated with cathepsin D at pH 6.5 and 37^o C, the extent of these changes was greatly reduced (e.g., only a small amount of myosin heavy chains was

degraded, etc.). Cathepsin D showed even less activity at pH 5.5 and 25° C. Incubations done at combinations of lower temperature/higher pH showed little or no activity, except possibly in degradation of titin.

4) When myofibrils were incubated with cathepsin D at pH 5.5 and 37° C and subjected to a brief homogenization, cathepsin D caused little or no increase in fragmentation (shorter myofibrils) as observed by phase contrast microscopy. Under these conditions, cathepsin D did alter the A band regions (appeared more narrow), but this did not result in more fragmentation. Treatment of myofibrils with cathepsin D at higher pH/lower temperature conditions caused progressively less change in the A band and, as before, no obvious changes in the degree of myofibril fragmentation.

The results of this part of my study showed that cathepsin D was active in a much more limited range of ionic conditions than was CAF. And, the alterations caused seemingly had no effect on myofibril fragmentation. In the normal course of pH decline that occurs in post-mortem muscle, a pH of 5.5 would seldom be reached when the temperature is still near 37° C. Thus, it seems unlikely that cathepsin D plays a principal role in the changes that occur in myofibrillar proteins and structure in postmortem muscle. If decline in pH is accelerated while the temperature remains high, some degradation of myosin, titin, etc. could occur. However, these changes may not cause an increase in fragmentation of myofibrils.

VII. BIBLIOGRAPHY

- Afting, E. G., and M. L. Becker. 1981. Two-step affinity-chromatographic purification of cathepsin D from pig myometrium with high yield. *Biochem. J.* 197:519-522.
- Arakawa, N., C. Inagaki, T. Kitamura, S. Fujiki, and M. Fujimaki. 1976. Some possible evidence for an alteration in actin-myosin interaction in stored muscle. *Agr. Biol. Chem.* 40:1445-1447.
- Arakawa, N., D. E. Goll, and J. Temple. 1970. Molecular properties of postmortem muscle. 8. Effect of postmortem storage on α -actinin and the tropomyosin-troponin complex. *J. Food Sci.* 35:703-711.
- Azanza, J. L., J. Raymond, J. M. Robin, P. Cottin, and A. Ducastaing. 1979. Purification and some physical-chemical and enzymic properties of a calcium ion-activated neutral proteinase from rabbit skeletal muscle. *Biochem. J.* 183:339-347.
- Bailey, A. J., and T. J. Sims. 1977. Meat tenderness. Distribution of molecular species of collagen in bovine muscle. *J. Sci. Fd. Agr.* 28:565-570.
- Barth, R., and J. S. Elce. Immunofluorescent localization of a Ca^{2+} -dependent neutral protease in hamster muscle. *Am. J. Physiol.* 240: E493-E498.
- Barrett, A. J. 1972. Lysosomal enzymes. Pages 46-136 in J. T. Dingle, ed. *Lysosomes: A laboratory handbook*. 2nd Edition. North Holland Publishing Co., Amsterdam, Netherlands.
- Barrett, A. J. 1976. An improved color reagent for use in Barrett's assay of cathepsin B. *Anal. Biochem.* 76:374-376.
- Barrett, A. J. 1977. Cathepsin B and other thiol proteinaes. Pages 181-201 in A. J. Barrett, ed. *Proteases in mammalian cells and tissues*. North Holland Publishing Co., Amsterdam, Netherlands.
- Barrett, A. J., and M. F. Heath. 1977. Lysosomal enzymes in lysosomes: A laboratory handbook. Pages 19-148 in J. T. Dingle, Ed. 2nd Edition. North Holland Publishing Co., Amsterdam, Netherlands.
- Bate-Smith, E. C., and J. R. Bendall. 1949. Factors determining the time course of rigor mortis. *J. Physiol.* 110:47.
- Behnke, J. R., O. Fennema, and R. G. Cassens. 1973. Quality changes in prerigor beef at 3^oC. *J. Food Sci.* 38:539-541.

- Bhan, A., A. Malhotra, and V. B. Hatcher. 1978. Partial characterization of a protease from cardiac myofibrils of dystrophic hamsters. Pages 607-618 in H. L. Segal and D. J. Doyle, eds. Protein turnover and lysosome function. Academic Press, Inc., New York.
- Bird, J. W. C. 1975. Skeletal muscle lysosomes. Pages 75-110 in J. T. Dingle and R. T. Dean, eds. Lysosomes in biology and pathology. Vol. 4. North Holland Publishing Co., Amsterdam, Netherlands.
- Bird, J. W. C., A. M. Spanier, and W. N. Schwartz. 1978. Cathepsins B and D: Proteolytic activity and ultrastructural localization in skeletal muscle. Pages 589-604 in H. L. Segal and D. J. Doyle, eds. Protein turnover and lysosomal function. Academic Press, Inc., New York.
- Bird, J. W. C., and J. H. Carter. 1980. Proteolytic enzymes in striated and non-striated muscle. Pages 51-85 in K. Wildenthal, ed. Degradative processes in heart and skeletal muscle. Elsevier/North Holland Biomedical Press, New York.
- Bird, J. W. C., and W. N. Schwartz. 1977. Intracellular protein catabolism in muscle. Pages 167-182 in V. Turk and N. Marks, eds. Intracellular protein catabolism II. Plenum Press, New York.
- Bird, J. W. C., J. H. Carter, R. E. Triemer, R. M. Brooks, and A. M. Spanier. 1980. Proteinases in cardiac and skeletal muscle. Fed. Proc. 39:20-25.
- Bird, J. W. C., F. J. Roisen, G. Yorke, J. A. Lee, M. A. McElligott, D. F. Triemer, and A. St. John. 1981. Lysosomes and proteolytic enzyme activities in cultured striated muscle cells. J. Histochem. Cytochem. 29:431-439.
- Briskey, E. J. 1967. Myofibrillar proteins of skeletal muscle. Proc. Meat Ind. Res. Conf. 20:1-28.
- Busch, W. A. 1969. Subcellular events associated with the development and release of isometric tension in postmortem bovine, rabbit and porcine skeletal muscle. Ph.D. Thesis. Iowa State University, Ames, Iowa.
- Busch, W. A., D. E. Goll, F. C. Parrish, Jr. 1972a. Molecular properties of postmortem muscle. Isometric tension development and decline in bovine, porcine, and rabbit muscle. J. Food Sci. 37: 289-299.

- Busch, W. A., M. H. Stromer, D. E. Goll, and A. Suzuki. 1972b. Ca^{++} specific removal of Z lines from rabbit skeletal muscle. *J. Cell Biol.* 52:367-381.
- Canonico, P. G., and J. W. C. Bird. 1970. Lysosomes in skeletal muscle tissue. *J. Cell Biol.* 45:321-333.
- Cohen, C., and A. G. Szent-Gyorgyi. 1957. Optical rotation and helical configuration in α -proteins. *J. Am. Chem. Soc.* 79:248.
- Collins, J. H., and M. Elzinga. 1975. The primary structure of actin from rabbit skeletal muscle. Completion and analysis of the amino acid sequence. *J. Biol. Chem.* 250:5915-5920.
- Cottin, P., P. L. Vidalenc, and A. Ducasting. 1981. Ca^{+2} -dependent association between a Ca^{2+} -activated neutral proteinase (CaANP) and its specific inhibitor. *FEBS Letters* 2:221-224.
- Craig, R., and G. Offer. 1976. The isolation of C-protein in rabbit skeletal muscle. *Proc. R. Soc. London B*, 192:451-461.
- Crick, F. H. C. 1953. The packing of α -helices: Simple coiled cells. *Acta Crystallog.* 6:689-697.
- Cummins, P., and S. J. Perry. 1973. The subunits and biological activity of polymorphic forms of tropomyosin. *Biochem. J.* 133:765-777.
- Davey, C. L., and K. V. Gilbert. 1966. Studies in meat tenderness. II. Proteolysis and the aging of beef. *J. Food Sci.* 31:135-140.
- Davey, C. L., and K. V. Gilbert. 1969. Studies in meat tenderness. 7. Changes in the fine structure of meat during aging. *J. Food Sci.* 34:69-74.
- Davey, C. L., and K. V. Gilbert. 1974. Temperature-dependent cooking toughness in beef. *J. Sci. Food Agric.* 25:931-938.
- Davey, C. L., and K. V. Gilbert. 1976. Thaw contracture and the disappearance of adenosinetriphosphate in frozen lamb. *J. Sci. Food Agric.* 27:1085.
- Davies, P. J. A. D. Wallack, M. C. Willingham, I. Pastan, M. Yamaguchi, and R. M. Robson. 1978. Filamin-actin interaction. Dissociation of binding from gelatin by Ca^{2+} -activated proteolysis. *J. Biol. Chem.* 253:4036-4042.

- Dayton, W. R., D. E. Goll, W. J. Reville, M. G. Zeece, M. H. Stromer, and R. M. Robson. 1974. Purification and some properties of a muscle enzyme that degrades myofibrils. *Fed. Proc.* 33:1580. (Abstr.)
- Dayton, W. R., D. E. Goll, M. H. Stromer, W. J. Reville, M. G. Zeece, and R. M. Robson. 1975. Cold Spring Harbor Conferences on Cell Proliferation. Vol. II. Proteases and Biological Control. Cold Spring Harbor, New York.
- Dayton, W. R., D. E. Goll, M. G. Zeece, R. M. Robson, and W. J. Reville. 1976a. A Ca^{2+} -activated protease possibly involved in myofibrillar protein turnover. Purification from porcine muscle. *Biochemistry* 15:2150-2158.
- Dayton, W. R., W. J. Reville, D. E. Goll, and M. H. Stromer. 1976b. A Ca^{2+} -activated protease possibly involved in myofibrillar protein turnover. Partial characterization of the purified enzyme. *Biochemistry* 15:2159-2167.
- Dayton, W. R., and J. V. Schollmeyer. 1981. Immunocytochemical localization of calcium-activated protease in skeletal muscle cells. *Exptl. Cell Res.* 136:423-433.
- Dayton, W. R., J. V. Schollmeyer, R. A. Lepley, and L. R. Cortes. 1981. A calcium-activated protease possibly involved in myofibrillar protein turnover. Isolation of a low-calcium-requiring form of the protease. *Biochim. Biophys. Acta* 659:48-61.
- Dean, R. T. 1980. Protein degradation in cell cultures: General considerations on mechanisms and regulation. *Fed. Proc.* 39:15-19.
- Decker, R. S., M. L. Decker, and A. R. Poole. 1980. The distribution of lysosomal cathepsin D in cardiac myocytes. *J. Histochem. Cytochem.* 28:231-237.
- Drummond, G. I., and L. Duncan. 1966. The action of calcium ion on cardiac phosphorylase b kinase. *J. Biol. Chem.* 241:3087-3103.
- Dutson, T. 1983. Relationship of pH and temperature to disruption of specific muscle proteins and activity of lysosomal proteases. *J. Food Biochem.* 7:223-245.
- Ebashi, S., and F. Ebashi. 1964. A new protein component participating in the superprecipitation of myosin B. *J. Biochem.* 55:604-613.
- Edmunds, T., W. C. Kleese, and D. E. Goll. 1984. Properties of high- Ca^{2+} and low- Ca^{2+} forms of the Ca^{2+} -dependent proteinase from bovine skeletal muscle. *Biophysical J.* 45:112a. (Abstr.)

- Etherington, D. J. 1981. Enzyme actions in the conditioning of meat. Pages 279-290 in V. Turk and Lj. Vitalo, eds. *Proteinases and their inhibitors: Structure, function, and applied aspects*. Pergamon Press, London, England.
- Evans, P., and D. J. Etherington. 1978. Characterization of cathepsin B and collagenolytic cathepsin from human placenta. *Eur. J. Biochem.* 83:87-97.
- Franzini-Armstrong, C. 1970. Details of the I-band structure as revealed by the localization of ferritin. *Tissue Cell.* 2:327-338.
- Franzini-Armstrong, C. 1973. Membranous systems in muscle. Pages 531-619 in G. H. Bourne, ed. *The structure and function of muscle*. Vol. II. 2nd Edition. Academic Press, Inc., New York.
- Franzini-Armstrong, C., and K. R. Porter. 1962. Sarcolemmal invaginations constituting the T system in fish muscle fibers. *J. Cell Biol.* 22:675-696.
- Goll, D. E. 1968. The resolution of rigor mortis. *Ann. Recip. Meat Conf. Proc.* 21:16-46.
- Goll, D. E., and R. M. Robson. 1967. Molecular properties of post-mortem muscle. I. Myofibrillar nucleosidetriphosphatase activity of bovine muscle. *J. Food Sci.* 32:323-329.
- Goll, D. E., D. W. Henderson, and E. A. Kline. 1964. Postmortem changes in physical and chemical properties of bovine muscle. *J. Food Sci.* 29:590-596.
- Goll, D. E., N. Arakawa, M. H. Stromer, W. A. Busch, and R. M. Robson. 1970. Chemistry of muscle proteins as a food. Pages 755-800 in E. J. Briskey, R. G. Cassens, and B. B. Marsh, eds. *The physiology and biochemistry of muscle as a food*. Vol. 2. Univ. of Wisconsin Press, Madison, Wisconsin.
- Goll, D. E., R. B. Young, and M. H. Stromer. 1974. Separation of subcellular organelles by differential and density gradient centrifugation. *Ann. Recip. Meat Conf. Proc.* 27:250-290.
- Goll, D. E., M. H. Stromer, and R. M. Robson. 1977. Skeletal muscle. Pages 504-530 in M. J. Swensen, ed. *Duke's physiology of domestic animals*. 9th Edition. Cornell University Press, Ithaca, New York.

- Goll, D. E., Y. Otsuka, P. A. Nagainis., J. D. Shannon, S. K. Sathe, and M. Muguruma. 1983a. Role of muscle proteinases in maintenance of muscle integrity and mass. *J. Food Biochem.* 7:137-177.
- Goll, D. E., J. D. Shannon, T. E. Edmunds, S. K. Sathe, W. C. Kleese, and P. A. Nagainis. 1983b. Properties and regulation of the Ca^{+2} -dependent proteinase. Pages 755-800 in B. de Bernard et al., eds. Elsevier/North Holland Biomedical Press, New York.
- Goll, D. E., R. M. Robson, and M. H. Stromer. 1984. Skeletal muscle. In M. J. Swensen, ed. *Duke's physiology of domestic animals*. 10th Edition. Chapter 36. Cornell University Press, Ithaca, New York. (In press.)
- Gould, R. P. 1973. The microanatomy of muscle. Pages 199-241 in G. H. Bourne, ed. *The structure and function of muscle*. Vol. II. 2nd Edition. Academic Press, Inc., New York.
- Greaser, M. L., and J. Gergely. 1973. Purification and properties of the components from troponin. *J. Biol. Chem.* 248:2125-2133.
- Greaser, M. L., S. M. Wang, and L. F. Lemanski. 1981. New myofibrillar proteins. *Ann. Recip. Meat Conf. Proc.* 34:13-15.
- Grove, B. K., and H. M. Eppenberger. 1983. Appearance of myomesin and M-protein during chicken muscle development. *J. Cell Biol.* 97:551a. (Abstr.)
- Hamm, R. 1982. Postmortem changes in muscle with regard to processing of hot-boned beef. *Food Technol.* 36:105-115.
- Hay, J. D., R. W. Currie, F. H. Wolfe, and E. J. Sanders. 1973. Effect of postmortem aging on chicken myofibrils. *J. Food Sci.* 38: 981-986.
- Henderson, D. W., D. E. Goll, and M. H. Stromer. 1970. A comparison of shortening and Z-line degradation in postmortem bovine, porcine, and rabbit muscle. *Am. J. Anat.* 128:117-136.
- Herring, H. K., R. G. Cassens, and E. J. Briskey. 1967. Factors affecting collagen solubility in bovine muscles. *J. Food Sci.* 32: 534-538.
- Hill, F. 1966. The solubility of intramuscular collagen in meat animals of various ages. *J. Food Sci.* 31:161-166.
- Huang, J. S., S. Huang, and J. Tang. 1979. Cathepsin D isozymes from porcine spleens. *J. Biol. Chem.* 254:11405-11417.

- Huiatt, T. W., R. M. Robson, N. Arakawa, and M. H. Stromer. 1980. Desmin from avian smooth muscle: Purification and partial characterization. *J. Biol. Chem.* 255:6981-6989.
- Huston, R. B., and E. G. Krebs. 1968. Activation of skeletal muscle phosphorylase kinase by Ca^{+2} . II. Identification of kinase activating factor as a proteolytic enzyme. *Biochemistry* 7:2116-2122.
- Huxley, H. E. 1968. Structural differences between resting and rigor muscle; evidence from intensity changes in the low-angle equatorial X-ray diagram. *J. Mol. Biol.* 37:507-520.
- Huxley, H. E. 1972. Molecular basis of contraction in cross-striated muscles. Pages 301-364 in G. H. Bourne, ed. *The structure and function of muscle*. Vol. I. 2nd Edition. Academic Press, New York.
- Huxley, H. E. 1983. Molecular basis of contraction in cross-striated muscles and relevance to motive mechanisms in other cells. Pages 2-104 in A. Stracher, ed. *Muscle and nonmuscle motility*. Vol. 1. Academic Press, Inc., New York.
- Huxley, H. E., and W. Brown. 1967. The low-angle X-ray diagram of vertebrate striated muscle and its behavior during contraction and rigor. *J. Mol. Biol.* 30:384-434.
- Ishiura, S., H. Murofushi, K. Suzuki, and K. Imahori. 1978. Studies of a calcium-activated neutral protease from chicken skeletal muscle. I. Purification and characterization. *J. Biochem.* 84:225-230.
- Ishiura, S., H. Sugita, I. Nonaka, and I. Imahori. 1980. Calcium-activated neutral protease. Its localization in the myofibril, especially at the Z-band. *J. Biochem.* 87:343-346.
- Kasang, L. E. 1983. Alterations in bovine skeletal muscle desmin and the cytoskeleton induced by temperature and electrical stimulation. Ph.D. Thesis. Iowa State University, Ames, Iowa.
- Kirschke, H., A. A. Kembhari, P. Bohley, and A. J. Barrett. 1982. Action of rat liver cathepsin L on collagen and other substrates. *Biochem. J.* 201:367-372.
- Kishimoto, A., N. Kajikawa, H. Tabuchi, M. Shiota, and Y. Nishizuka. 1981. Calcium-dependent neutral proteases, widespread occurrence of a species of protease active at lower concentration of calcium. *J. Biochem.* 90:889-892.
- Krebs, E. G., D. S. Love, G. E. Bratjold, K. A. Trayser, H. L. Meyer, and E. H. Fischer. 1964. Purification and properties of rabbit skeletal muscle phosphorylase b kinase. *Biochemistry* 3:1022-1033.

- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 227:680-685.
- Lah, T., and V. Turk. 1982. Autolysis studies of cathepsin D. *Hoppe-Seyler's Z. Physiol. Chem.* 363 S:247-254.
- Laki, K. 1971. Actin. Pages 97-133 in K. Laki, ed. *Contractile proteins and muscle*. Marcel Decker, Inc., New York.
- LaSalle, F., R. M. Robson, M. L. Lusby, F. C. Parrish, M. H. Stromer, and T. W. Huiatt. 1983. Localization of titin in bovine skeletal muscle by immunofluorescence and immunoelectron microscope labeling. *J. Cell Biol.* 97:258a. (Abstr.)
- Lawrie, R. A. 1980. The tenderizing effect of electrical stimulation of beef carcasses. *Meat Sci.* 4:51-68.
- Lawrie, R. A. 1983. Aspects of the biochemistry of meat. *Int. J. Biochem.* 3:233-242.
- Locker, R. H. 1960. Proteolysis in the storage of beef. *J. Sci. Food Agr.* 11:520-526.
- Locker, R. H., and N. G. Leet. 1976a. Histology of highly-stretched beef muscle II. Further evidence on the location and nature of gap filaments. *J. Ultrastruct. Res.* 55:157-172.
- Locker, R. H., and N. G. Leet. 1976b. Histology of highly-stretched beef muscle IV. Evidence for movement of gap filaments through the Z line, using the N₂-line and M-line as markers. *J. Ultrastruct. Res.* 56:31-38.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Lusby, M. L., J. F. Ridpath, F. C. Parrish, Jr., and R. M. Robson. 1983. Effect of postmortem storage on degradation of the recently discovered myofibrillar protein titin in bovine longissimus muscle. *J. Food Sci.* 48:1787-1790, 1795.
- MacBride, M. A., and F. C. Parrish, Jr. 1977. The 30,000-dalton component of tender bovine longissimus muscle. *J. Food Sci.* 42:1627-1629.
- McDonald, J. D., and S. Ellis. 1975. On the substrate specificity of cathepsins B1 and B2 including a new fluorogenic substrate for cathepsin B1. *Life Sci.* 17:1269-1276.

- Mak, A. S., L. B. Smillie, and G. R. Stewart. 1980. A comparison of the amino acid sequences of rabbit skeletal muscle α - and β -tropomyosins. *J. Biol. Chem.* 255:3647-3655.
- Marsh, B. B. 1977. Temperature and postmortem change: Energy use and meat quality. *Proc. Meat Ind. Res. Conf.* 30:13-23.
- Marsh, B. B., and W. A. Crase. 1974. Meat tenderness and the sliding filament hypothesis. *J. Food Technol.* 9:129-139.
- Marsh, B. B., and N. G. Leet. 1966. Studies in meat tenderness. *J. Food Sci.* 31:450-459.
- Martonosi, A. N. 1983. The regulation of cytoplasmic Ca^{2+} concentration in muscle and nonmuscle cells. Pages 234-335 in A. Stracher, ed. *Muscle and nonmuscle motility*. Vol. 1. Academic Press, Inc., New York.
- Maruyama, K., and S. Kimura. 1981. Muscle beta-actinin is not chicken serum albumin. *J. Biochem. (Tokoyo)* 90:563-566.
- Maruyama, K., S. Matsubara, R. Natori, Y. Nonomura, S. Kimura, K. Ohashi, F. Murakami, S. Handa, and G. Eguchi. 1977. Connectin, an elastic protein of muscle. *J. Biochem.* 82:317-337.
- Maruyama, K., M. Kimura, S. Kimura, K. Ohashi, K. Suzuki, and N. Katunuma. 1981a. Connectin, an elastic protein of muscle. Effects of proteolytic enzymes in situ. *J. Biochem.* 89:711-715.
- Maruyama, K., S. Kimura, K. Ohashi, and Y. Kuwano. 1981b. Connectin, an elastic protein of muscle. Identification of "titin" with connection. *J. Biochem.* 89:701-709.
- Matsukura, Y., A. Okitani, T. Nishimura, and H. Kato. 1981. Mode of degradation of myofibrillar proteins by an endogenous protease cathepsin L. *Biochim. Biophys. Acta* 662:41-47.
- Matsumoto, T., A. Okitani, Y. Kitamura, and H. Kato. 1983. Mode of degradation of myofibrillar proteins by rabbit muscle cathepsin D. *Biochim. Biophys. Acta* 755:76-80.
- Mellgren, R. L. 1980. Canine cardiac calcium-dependent proteases: Resolution of two forms with different requirements for calcium. *FEBS Letters* 109:129-133.
- Millward, D. J. 1980. Protein turnover in skeletal and cardiac muscle during normal growth and hypertrophy. Pages 161-196 in K. Widenthal, ed. *Degradative process in heart and skeletal muscle*. Monographs in cell and tissue physiology. Elsevier/North Holland Biomedical Press, New York.

- Moos, C. 1981. Fluorescence microscope study of the binding of added C protein to skeletal muscle myofibrils. *J. Cell Biol.* 90:25-31.
- Moos, C., L. M. Mason, J. M. Besterman, I.-N. M. Feng, and J. H. Dublin. 1978. The binding of skeletal muscle C-protein to F-actin, and its relationship to the interaction of actin with myosin sub-fragment-1. *J. Mol. Biol.* 124:571-586.
- Murachi, T., M. Hatanaka, Y. Yasumoto, N. Nakayama, and T. Kazuyoshi. 1981. A quantitative distribution study on calpain and calpastatin in rat tissues and cells. *Biochem. Int.* 6:651-656.
- Nagainis, P. A., S. K. Sathe, D. E. Goll, and T. Edmunds. 1983. Autolysis of high- Ca^{2+} and low- Ca^{2+} -forms of the Ca^{2+} -dependent proteinase from bovine skeletal muscle. *Fed. Proc.* 42:780. (Abstr.)
- Newbold, R. P. 1966. Changes associated with rigor mortis. Pages 213-224 in E. J. Briskey, R. G. Cassens, and J. C. Trautman, eds. *The physiology and biochemistry of muscle as a food.* University of Wisconsin Press, Madison, Wisconsin.
- Noda, T., K. Isogai, H. Hayashi, N. Katunuma. 1981. Susceptibilities of various myofibrillar proteins to cathepsin B and morphological alteration of isolated myofibrils by this enzyme. *J. Biochem.* 90:371-379.
- Ogunro, E. A., R. B. Lanman, J. R. Spencer, A. G. Ferguson, and M. Lesch. 1979. Degradation of canine cardiac myosin and actin by cathepsin D isolated from homologous tissue. *Cardiovascular Res.* 11:621-629.
- Ogunro, E. A., A. G. Ferguson, and M. Lesch. 1980. A kinetic study of the pH optimum of canine cardiac cathepsin D. *Cardiovascular Res.* 14:254-260.
- Ogunro, E. A., A. M. Samarel, A. G. Ferguson, and M. Lesch. 1982. Primary structure similarities in canine cardiac cathepsin D polypeptide chains. *J. Molec. and Cell. Cardiol.* 14:513-522.
- Okitani, A., D. E. Goll, M. H. Stromer, and R. M. Robson. 1976. Intracellular inhibitor of a Ca^{2+} -activated protease involved in myofibrillar protein turnover. *Fed. Proc.* 35:1746. (Abstr.)
- Okitani, A., U. Matsukara, H. Kato, and M. Fujimaki. 1980. Purification and some properties of a myofibrillar protein-degrading protease, cathepsin L, from rabbit skeletal muscle. *J. Biochem.* 87:1133-1143.

- Okitani, A., T. Matsumoto, Y. Kitamura, and H. Kato. 1981. Purification of cathepsin D from rabbit skeletal muscle and its action towards myofibrils. *Biochim. Biophys. Acta* 662:202-209.
- Olson, D. G., F. C. Parrish, Jr., and M. H. Stromer. 1976. Myofibril fragmentation and shear resistance of three bovine muscles during postmortem storage. *J. Food Sci.* 41:1036.
- Olson, D. G., and F. C. Parrish, Jr. 1977. Relationship of myofibril fragmentation index to measure of beef-steak tenderness. *J. Food Sci.* 42:506-509.
- Olson, D. G., F. C. Parrish, Jr., W. R. Dayton, and D. E. Goll. 1977. Effect of postmortem storage and calcium activated factor on the myofibrillar proteins of bovine skeletal muscle. *J. Food Sci.* 42: 117-124.
- O'Shea, J. M., R. M. Robson, T. W. Huiatt, M. K. Hartzler, and M. H. Stromer. 1979. Purified desmin from adult mammalian skeletal muscle: A peptide mapping comparison with desmin from adult mammalian and avian smooth muscle. *Biochem. Biophys. Res. Comm.* 89:972-980.
- O'Shea, J. M., R. M. Robson, M. K. Hartzler, T. W. Huiatt, W. E. Rathbun, and M. H. Stromer. 1981. Purification of desmin from adult mammalian skeletal muscle. *Biochem. J.* 195:345-356.
- Otsuka, Y., and D. E. Goll. 1980. Purification of the Ca²⁺-activated-protease-inhibitor from bovine cardiac muscle. *Fed. Proc.* 39:2044.
- Pagano, M., and R. Engler. 1982. Inhibition of human liver cathepsin L by α -thiol proteinase inhibitor. *FEBS Letters* 138:307-310.
- Page, S. A. 1968. Fine structure of tortoise skeletal muscle. *J. Physiol.* 197:709-715.
- Park, D. C., M. E. Parsons, and R. J. Pennington. 1973. Evidence for mast-cell origin of proteinase in skeletal-muscle homogenates. *Biochem. Soc. Trans.* 1:730-732.
- Parrish, F. C., Jr., D. E. Goll, W. J. Newcomb, II, B. O. Delumen, H. M. Chaudhry, and E. A. Kline. 1967. Molecular properties of postmortem muscle 7. Changes in nonprotein nitrogen and free amino acids of bovine muscle. *J. Food Sci.* 34:196-202.
- Parrish, F. C., Jr., R. B. Young, B. G. Miner, and L. D. Anderson. 1973. Effect of postmortem conditions on certain chemical, morphological and organoleptic properties of bovine muscle. *J. Food Sci.* 38: 690-695.

- Parrish, F. C., Jr., C. J. Selvig, R. D. Culler, and M. G. Zeece. 1981. CAF activity, calcium concentration, and the 30,000-dalton component of tough and tender bovine longissimus muscle. *J. Food Sci.* 46: 308-309, 311.
- Pemrick, S. M., S. Schneiderman, and A. Stracher. 1980. Ca^{2+} -specific degradation of the heavy chain of unphosphorylated skeletal myosin. *Fed. Proc.* 39:2043. (Abstr.)
- Penny, I. F. 1970. Conditioning of bovine muscle. II. Changes in the composition of extracts of myofibrils after conditioning. *J. Sci. Food Agric.* 21:303-306.
- Penny, I. F. 1980. The enzymology of conditioning. Pages 115-143 in R. A. Lawrie, ed. *Developments in Meat Science*. Vol. I. Applied Science Publishers, Barking, Essex, England.
- Phillips, D. R., and M. Jakabova. 1977. Ca^{2+} -dependent protease in human platelets. *J. Biol. Chem.* 252:5602-5605.
- Poole, A. R. 1977. Antibodies to enzymes and their uses with particular reference to lysosomal enzymes. Pages 245-312 in J. T. Dingle, ed. *Lysosomes: A laboratory handbook*. 2nd Edition. North Holland Publishing Co., Amsterdam, Netherlands.
- Puizdare, V., and V. Turk. 1981. Cathepsinogen D: Characterization and activation to cathepsin D and inhibitory peptides. *FEBS Letters* 132:299-304.
- Reville, W. J., D. E. Goll, M. H. Stromer, R. M. Robson, and W. R. Dayton. 1976. A Ca^{2+} -activated protease possibly involved in myofibrillar protein turnover. *J. Cell Biol.* 70:1-8.
- Richardson, F. L., M. H. Stromer, T. W. Huiatt, and R. M. Robson. 1981. Immunoelectron and immunofluorescence localization of desmin in mature avian muscle. *Eur. J. Cell Biol.* 26:91-101.
- Ridpath, J. F., R. M. Robson, T. W. Huiatt, A. H. Trenkle, and M. L. Lusby. 1982. Localization and rate of accumulation of rebulin in skeletal and cardiac muscle cell cultures. *J. Cell Biol.* 95:361a. (Abstr.)
- Robbins, F. M., J. E. Walker, S. H. Colten, S. Chatterjee. 1979. Action of proteolytic enzymes on bovine myofibrils. *J. Food Sci.* 44:1672-1677.
- Robson, R. M., and T. W. Huiatt. 1983. Roles of the cytoskeletal proteins desmin, titin and nebulin in muscle. *Ann. Recip. Meat Conf. Proc.* 36:116-124.

- Robson, R. M., and M. G. Zeece. 1973. Comparative studies of α -actinin from porcine cardiac and skeletal muscle. *Biochim. Biophys. Acta* 295:208-224.
- Robson, R. M., D. E. Goll, N. Arakawa, and M. H. Stromer. 1970. Purification and properties of α -actinin from rabbit skeletal muscle. *Biochim. Biophys. Acta* 200:296-318.
- Robson, R. M., M. H. Stromer, T. W. Huiatt, J. M. O'Shea, M. K. Hartzler, F. L. Richardson, and W. E. Rathbun. 1980. Biochemistry and structure of desmin and the recently discovered muscle cell cytoskeleton. Pages 22-25 in Proc. Eur. Meeting of Meat Res. Workers. Vol. I. American Meat Science Association, Chicago, Illinois.
- Robson, R. M., M. Yamaguchi, T. W. Huiatt, F. L. Richardson, J. M. O'Shea, M. K. Hartzler, W. E. Rathbun, P. J. Schreiner, L. E. Kasang, M. H. Stromer, S. Pang, R. R. Evans, and J. F. Ridpath. 1981. Biochemistry and molecular architecture of muscle cell 10-nm filaments and Z-line. Roles of desmin and α -actin. *Proc. Ann. Recip. Meat Conf.* 34:5-11.
- Robson, R. M., J. M. O'Shea, M. K. Hartzler, W. E. Rathbun, F. LaSalle, P. J. Schreiner, L. E. Kasang, M. H. Stromer, M. L. Lusby, J. F. Ridpath, Y.-Y. Pang, R. R. Evans, M. G. Zeece, F. C. Parrish, Jr., and T. W. Huiatt. 1984. Role of new cytoskeletal elements in maintenance of muscle integrity. *J. Food Biochem.* 8:1-24.
- Samarel, A. M., E. A. Ogunro, A. G. Ferguson, and M. Lesch. 1981. A double antibody radioimmunoassay for cardiac cathepsin D. *Cardiovascular Res.* 15:68-73.
- Schollmeyer, J. V., L. T. Furcht, D. E. Goll, R. M. Robson, and M. H. Stromer. 1976. Localization of contractile proteins in smooth muscle cells and in normal and transformed fibroblasts. *Cold Spring Harbor Conf. on Cell Prolif.* 3:361-388.
- Schwartz, W. N., and A. J. Barrett. 1980. Human cathepsin H. *Biochem. J.* 191:487-497.
- Schwartz, W. N., and J. W. C. Bird. 1977. Degradation of myofibrillar proteins by cathepsins B and D. *Biochem. J.* 167:811-820.
- Scott, P. G., and C. H. Pearson. 1978. Cathepsin D: Cleavage of soluble collagen and cross linked peptides. *FEBS Letters* 88:41-45.
- Seifter, S., and P. M. Gallop. 1966. The structure of proteins. Page 155 in H. Neurath, ed. *The proteins: Composition structure and function.* Vol. 4. Academic Press, New York.

- Shannon, J. D., and D. E. Goll. 1984. Properties of a protein that is purified from bovine skeletal muscle and that inhibits the Ca^{2+} -dependent proteinase. *Biophysical J.* 45:113a. (Abstr.)
- Starr, R., and G. Offer. 1978. The interaction of C-protein with heavy meromyosin and subfragment-2. *Biochem. J.* 171:813-816.
- Stracher, A. 1969. Evidence for the involvement of light chains in the biological functioning of myosin. *Biochem. Biophys. Res. Commun.* 35:519-525.
- Strechler, E. E., G. Pelloni, C. W. Heizmann, and H. M. Eppenberger. 1980. Biochemical and ultrastructural aspects of M_r 165,000 M-protein in cross-striated chicken muscle. *J. Cell Biol.* 86:775-783.
- Stromer, M. H., D. E. Goll, and L. E. Roth. 1967. Morphology of rigor shortened bovine muscle and the effect of trypsin on pre and postrigor myofibrils. *J. Cell Biol.* 34:431-445.
- Stromer, M. H., D. E. Goll, W. J. Reville, D. G. Olson, W. R. Dayton, and R. M. Robson. 1974. Structural changes in muscle during postmortem storage. *Proc. 4th International Congress Food Sci. Tech.*, Vol. I, pp. 401-418, Institute de Agroquimica y Tecnologia de Alimentos, Jaime Roig, 11, Valencia-10, Spain.
- Suzuki, A., and D. E. Goll. 1974. Quantitative assay for CASF (Ca^{2+} -activated sarcoplasmic factor) activity, and effect of CASF treatment on ATPase activities of rabbit myofibrils. *Agr. Biol. Chem.* 38:2167-2175.
- Suzuki, A., Y. Nonami, and D. E. Goll. 1975. Proteins released from myofibrils by CASF (Ca^{2+} -activated sarcoplasmic factor) and trypsin. *Agric. Biol. Chem.* 7:1461-1467.
- Suzuki, A., D. E. Goll, I. Singh, R. E. Allen, R. M. Robson, and M. H. Stromer. 1976. Some properties of purified skeletal muscle α -actinin. *J. Biol. Chem.* 251:6860-6870.
- Suzuki, A., M. Saito, H. Sato, and Y. Nonami. 1978a. Effect of materials released from myofibrils by CAF (Ca^{2+} -activated factor) on Z-disk reconstitution. *Agric. Biol. Chem.* 42:2111-2116.
- Suzuki, A., M. Saito, H. Iwai, and Y. Nonami. 1978b. Effect of individual fractions released from myofibrils by CAF (Ca^{2+} -activated factor) on Z-disk reconstitution. *Agric. Biol. Chem.* 42:2117-2122.

- Suzuki, K., S. Ishiura, S. Tsuji, T. Katamoto, H. Sugita, and K. Imahori. 1979. Calcium activated neutral protease from human skeletal muscle. *FEBS Letters* 104:355-358.
- Suzuki, K., S. Tsuji, S. Ishiura, Y. Kimura, S. Kubjta, and K. Imahori. 1981a. Autolysis of calcium-activated neutral protease of chicken skeletal muscle. *J. Biochem.* 90:1787-1793.
- Suzuki, K., T. Shuichi, and S. Ishiura. 1981b. Effect of Ca^{2+} on the inhibition of calcium-activated neutral protease by leupeptin, antipain and epoxysuccinate derivatives. *FEBS Letters* 136:119-122.
- Suzuki, A., H. Sasaki, and Y. Nonami. 1982. Electron microscopic observations on protein fractions released from CAF (Ca^{2+} -activated factor) - treated myofibrils. *Agric. Biol. Chem.* 46:813-815.
- Szpacenko, A. J. Kay, D. E. Goll, and Y. Otsuka. 1981. A different form of the Ca^{2+} -dependent proteinase activated by micromolar levels of Ca^{2+} . Pages 151-161 in V. Turk and L. Vitale, eds. *Proc. symp. proteinases and their inhibitors: Structure, function and applied aspects.* Pergamon Press, Oxford, England.
- Takahashi, K., T. Fukazawa, and T. Yasui. 1967. Formation of myofibrillar fragments and reversible contraction of sarcomeres in chicken pectoral muscle. *J. Food Sci.* 32:409-413.
- Toyo-Oka, T., T. Shimizu, and T. Masaki. 1978. The inhibition of proteolytic action of calcium activated neutral protease by leupeptin and antipain. *Biochem. Biophys. Res. Commun.* 82:484-491.
- Toyo-Oka, T., T. Masaki, J. Okamoto, and T. Tanaka. 1979. Calcium-activated neutral protease from bovine ventricular muscle: Isolation and some of its properties. *J. Molec. Cell. Cardiol.* 11:769-786.
- Trinick, J., and S. Lowey. 1977. M-protein from chicken pectoralis muscle: Isolation and characterization. *J. Mol. Biol.* 113:343-368.
- Walliman, T., G. Pelloni, D. C. Turner, and H. M. Eppenberger. 1978. Monovalent antibodies against M-creatine kinase remove the M-line from myofibrils. *Proc. Natl. Acad. Sci. U.S.A.* 75:4296-4300.
- Wang, K. 1981. Nebulin, a giant protein component of the N_2 -line of striated muscle. *J. Cell Biol.* 91:355a. (Abstr.)
- Wang, K., J. McClure, and A. Tu. 1979. Titin: Major myofibrillar components of striated muscle. *Proc. Natl. Acad. Sci. U.S.A.* 76:2698-3702.
- Wang, K., and C. Williamson. 1980. Identification of an N_2 line protein of striated muscle. *Proc. Natl. Acad. Sci. U.S.A.* 77:3254-3258.

- Wang, K., and R. Ramirez-Mitchell. 1983. A network of transverse and longitudinal intermediate filaments is associated with sarcomeres of adult vertebrate skeletal muscle. *J. Cell. Biol.* 96:562-570.
- Waxman, L. 1978. Characterization of two unique protease inhibitors and a calcium-activated protease from bovine cardiac muscle. Pages 363-377 in A. G. Segal and D. J. Doyle, eds. *Protein turnover and lysosome function*. Academic Press, Inc., New York.
- Waxman, L., and E. G. Krebs. 1978. Identification of two protease inhibitors from bovine cardiac muscle. *J. Biol. Chem.* 253:5888-5891.
- Weber, K., and M. Osborn. 1969. The reliability of molecular weight determination on dodecyl-sulphate polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244:4406-4412.
- Weeds, A. G., and S. Lowey. 1971. Structure of myosin molecule. II: The light chains of myosin. *J. Mol. Biol.* 61:701-725.
- Wheelock, M. J. 1982. Evidence for two structurally different forms of skeletal muscle Ca^{2+} -activated protease. *J. Biol. Chem.* 21:12471-12474.
- Whitaker, J. N. 1980. Comparative chemical, enzymatic and immunochemical features of brain cathepsin D. *Comp. Biochem. Physiol.* 68B:215-220.
- Wildenthal, K., and J. S. Crie. 1980. Lysosomes and cardiac protein metabolism. Pages 37-51 in K. Wildenthal, ed. *Degradative processes in heart and skeletal muscle*. Elsevier/North Holland Biomedical Press, New York.
- Woodbury, R. G., G. M. Gruzenski, and D. Lagunoff. 1978. Immunofluorescent localization of a serine protease in rat small intestine. *Proc. Natl. Acad. Sci. U.S.A.* 75:2785-2789.
- Yamaguchi, M., R. M. Robson, and M. H. Stromer. 1983. Evidence for actin involvement in cardiac Z-lines and Z-line analogues. *J. Cell. Biol.* 96:435-442.
- Yamamoto, K., K. Samejima, and T. Yasui. 1979. Changes produced in muscle proteins during incubation of muscle homogenates. *J. Food Sci.* 44:51-55.
- Yates, L. D., and M. L. Greaser. 1983. Quantitative determination of myosin and actin in rabbit skeletal muscle. *J. Mol. Biol.* 168:123-141.

Zeece, M. G., R. M. Robson, M. L. Lusby, and F. C. Parrish. 1983.
Proteolytic disassembly of myofibrils by calcium activated protease.
Institute of Food Technologists Ann. Meeting Program, New Orleans
38:153. (Abstr.)

VIII. ACKNOWLEDGEMENTS

I thank my major professor, Dr. R. M. Robson, for the counsel and excellent training received under his direction during the many years we have worked together, both before and during my doctoral studies, and for the assistance in writing this dissertation.

I am also grateful to Dr. M. H. Stromer for providing microscopy and dark room facilities and to Dr. F. C. Parrish, Jr. and Dr. A. A. Kraft for their continued guidance and support. Appreciation is extended to Dr. A. H. Trenkle for serving on my committee and to Dr. D. E. Goll of the University of Arizona for his assistance in providing his unpublished information on the chromatographic procedures for CAF.

Special thanks are extended to Mary Bremner, Jo Sprague, Lisa Nyre, and Teresa Anderson for their expert technical assistance in the laboratory.

Finally, my deepest appreciation goes to my wife, Pauline, and my children, Michael, Eric, and Megan, for their encouragement, patience, and understanding.