AN ABSTRACT OF THE THESIS OF

JAMES EDWARD DEWEY (Name) for the DOCTOR OF PHILOSOPHY (Degree)

in FOOD SCIENCE (Major) presented on April 8, 1970 (Date)

Title: INFLUENCE OF POST-MORTEM AGING UPON SOME CHEMICAL CHARACTERISTICS OF TROPOMYOSIN PREPARATIONS FROM BOVINE SKELETAL MUSCLE

Abstract approved: Allen F. Anglemier

Investigations were carried out to determine whether the bovine skeletal muscle protein, tropomyosin, was hydrolyzed during post-mortem aging. Two procedures were used for the preparation of tropomyosin from beef psoas major muscle; one was a direct extraction from muscle homogenates while the second method involved the initial isolation of myofibrils from which tropomyosin was then extracted. These extractions were performed on excised muscles shortly after slaughter, two days, and nine days of aging at 4°C. N-Terminal amino acid analysis, peptide mapping of tryptic digests, polyacrylamide-gel electrophoresis and viscometry were the analytical methods employed to detect hydrolytic changes in the various tropomyosin preparations. Estimation of actomyosin and nucleotide contamination in the different tropomyosin preparations was also made.

N-Terminal amino acid analysis of the various tropomyosin preparations examined during the nine-day aging period did not show the formation of new N-terminal amino acids which would have been indicative of hydrolysis. No significant differences were noted when comparing the peptide maps of tropomyosin preparations developed at the time of initial sampling with those prepared after two and nine days of aging. Polyacrylamide-gel electrophoretic patterns of all tropomyosin preparations obtained during the nine-day aging period were quite similar, which would indicate that little, if any, hydrolysis had occurred.

Interpretation of the viscometry data was complicated by the aggregation tendency of tropomyosin in the solvents employed. No differences in the viscometric results were observed between the tropomyosin extracted from myofibrils at the initial sampling time and that extracted in the same manner after two and nine days of aging. Conversely, a reduction in the limiting viscosity number of tropomyosin extracted directly from muscle was noted as aging time increased. This reduction appeared to be related to a decreased aggregation tendency rather than to a lower average molecular weight of tropomyosin in these preparations. Moreover, actomyosin contamination of the tropomyosin preparations extracted directly from muscle appeared to increase with aging time. This increase, however, was accompanied by a decrease in nucleotide levels.

It was concluded from the evidence obtained in this study that tropomyosin was not hydrolyzed during an aging period of nine days. The reduced aggregation observed in the tropomyosin preparations obtained from the direct extraction of muscle as aging time progressed might be associated, in an unknown manner, with the increase in actomyosin contamination and the decrease in nucleotide content.

Influence of Post-Mortem Aging upon Some Chemical Characteristics of Tropomyosin Preparations from Bovine Skeletal Muscle

by

James Edward Dewey

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1970

APPROVED:

Associate Professor of Food Science
in charge of major

Head of Department of Food Science and Technology

Dean of Graduate School

Date thesis is presented April 8, 1970

Typed by Mary Jo Stratton for James Edward Dewey

ACKNOWLEDGEMENT

The author wishes to express appreciation and gratitude to Dr. Allen F. Anglemier for his support and encouragement throughout the course of this study and in the preparation of this thesis.

Gratitude is expressed to Dr. Morris W. Montgomery for many guiding opinions and suggestions and to Dr. William D. Davidson for help in obtaining meat samples and for helpful ideas.

Appreciation is expressed to D. E. Nebergall Meat Company, Steen Brothers Meat Company, and Valley Meat Company for their help in obtaining beef samples soon after slaughter.

Sincere thanks and deepest appreciation are due to my wife, Paula, whose constant encouragement and many sacrifices greatly contributed to completion of this work.

I would also like to acknowledge the NDEA Fellowship Program for financial support during the last year of this study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

INFLUENCE OF POST-MORTEM AGING UPON SOME CHEMICAL CHARACTERISTICS OF TROPOMYOSIN PREPARATIONS FROM BOVINE SKELETAL MUSCLE

INTRODUCTION

The post-rigor tenderization of beef stored at chill temperatures for 7-14 days has long been used to develop the optimum organoleptic qualities of the meat. The holding process, known as aging or conditioning, gradually reverses the tenderness decrease which takes place as meat goes into rigor.

Many theories of post-rigor tenderization of meat have been advanced but none adequately describe the precise biochemical events involved therein. Elucidation of these events could be of primary value to the meat industry and could have fundamental significance for the muscle chemist. From a practical standpoint, such information could enable the meat industry to produce higher quality meat in shorter time, resulting in a reduction of capital investment in animal carcasses in storage and reducing storage costs and losses. More basic, clarification of the biochemistry of aging could provide more complete knowledge of muscle function, disease and abnormalities. Research on the biochemical mechanics of rigor and the subsequent tenderization of meat during aging has gained impetus since the determination of the micro-structure of muscle and the intricacies of muscle contraction by Huxley (1965) using X-ray diffraction and electron microscopy. His

sliding filament theory of muscle contraction is now widely accepted and involves the interdigitation of filaments of two structural proteins, actin and myosin. During contraction these filaments slide together, reducing the length of the muscle cell and the proteins complex to form actomyosin. During relaxation the actomyosin complex dissociates, the filaments slide apart, and the muscle cell returns to rest length (Huxley, 1965). Rigor results when post-mortem muscle goes into a state of continuous contraction and remains firmly bound in this condition (Lawrie, 1966).

The resolution of rigor and subsequent tenderization of meat during aging do not parallel the relaxation process, i.e., the actomyosin complex does not dissociate (Lawrie, 1966). A recent study indicates that a structural anchor point for the actin filaments, the Z-line, disintegrates during aging (Davey and Gilbert, 1969). A third structural protein of muscle, tropomyosin, appears to play a key role in anchoring the actin filaments to the Z-line (Huxley, 1963).

Proteolytic enzymes are known to be elaborated by living muscle (Lawrie, 1966). It has been suggested by several investigators that proteolytic hydrolysis of tropomyosin may influence tenderization of meat during aging (Davey and Gilbert, 1969; Khan, 1968; Penny, 1968).

The purpose of this work was to investigate the possibility that tropomyosin undergoes specific hydrolysis during the aging of meat.

REVIEW OF LITERATURE

Muscle proteins have classically been divided into three types: stroma, sarcoplasmic, and myofibrillar. The stroma proteins are the constituents of connective tissue within the muscle; the sarcoplasmic proteins consist of the glycolytic enzymes and pigments; and the myofibrillar proteins constitute the contractile elements of the muscle fiber (Briskey, 1967). These proteins all have an essential role in the function of muscle in vivo. However, the stroma and sarcoplasmic proteins apparently do not play a significant role in the post-rigor tenderization of meat (Lawrie, 1966) and therefore, will not be considered in this review.

The purpose of this review is to place the various myofibrillar proteins in proper perspective on the basis of their involvement in muscle contraction and their possible significance in post-rigor tenderization. Particular emphasis is given to tropomyosin in light of its possible key function in the aging process (Davey and Gilbert, 1969; Khan, 1968; Penny, 1968).

Structure and Function of the Myofibril

The structure and function of the myofibril and the relationship of myofibrillar proteins within the myofibrillar structure have been reviewed by Bendall (1964), Lawrie (1966), and Briskey (1967). Skeletal muscle is composed of long muscle fibers running its length

which are further subdivided into long, unbranching threads of protein known as myofibrils (Lawrie, 1966; Briskey, 1967). Myofibrils exhibit alternate dark and light bands when viewed through the light microscope and adjacent myofibrils lie with their dark and light bands in register, conferring a striated appearance upon the muscle fiber. The dark or A(anisotropic)-band has a clear central area (the H-zone) and the light or I(isotropic)-band has a dark central division designated as the Z-line (Bendall, 1964; Lawrie, 1966). The distance between two adjacent Z-lines is known as the sarcomere, which is the functional unit of the myofibril (Lawrie, 1966). The myofibril is thus composed of an end-to-end progression of sarcomeres.

The ultrastructure of the myofibril, as revealed by the electron microscope, is composed of two sets of filaments; thick filaments extending from edge to edge of the A-band, and thin filaments extending from the Z-line to the edge of the H-zone (Bendall, 1964). The thick and thin filaments thus overlap within the sarcomere and when viewed in cross-section six thin filaments lie in hexagonal array around each thick filament (Huxley, 1965). The thick and thin filaments consist primarily of the myofibrillar proteins myosin and actin, respectively (Huxley, 1965). A third myofibrillar protein, tropomyosin, is known to exist in the thin filaments and possibly within the Z-line (Briskey, 1967). Other more recently discovered myofibrillar proteins include troponin, α-actin, and β-actinin (Briskey, 1967).

The protein composition of the myofibril has been determined as 55% myosin, 20% actin, 7% tropomyosin, 2% troponin, 10% α-actinin, 2% β-actinin, and 4% other proteins as yet unknown according to Ebashi (as cited by Rampton, 1969) and Briskey (1967).

The myosin molecule is known to be a long rod-like molecule with two globular protein heads on one end (Slayter and Lowey, 1967). It is also known to have calcium activated enzyme activity as an adenosine triphosphatase (ATPase) in the globular portions of the molecule (Bendall, 1964). The rod-like portions of the myosin molecules are aligned in the thick filaments and the globular heads form flexible cross-bridges to the actin of the thin filaments, forming the protein complex, actomyosin (Lawrie, 1966). The thin filaments are composed of spherical G(globular)-actin monomers polymerized to form a F (fibrous)-actin double helix (Briskey, 1967). The thick and thin filaments are known to slide past one another during contraction-relaxation in muscle (Huxley, 1965).

The probable sequence of events in contraction has been outlined by Lawrie (1966). A stimulus from the nervous system results in a release of calcium ions which in turn release ATP from an inert complex with magnesium ions. The calcium ions also stimulate myosin ATPase which hydrolyzes ATP to ADP providing energy for contraction. Actin filaments are pulled into the H-zone, passing between the myosin filaments. Actin and myosin unite through the cross-bridges

to form actomyosin. The sarcomere maintains this contracted state until the calcium ions are pumped out, inhibiting the myosin ATPase. New ATP floods the system as magnesium-ATP, breaks the actomyosin complex and the filaments slide apart to the relaxed state (Lawrie, 1966).

Actin and myosin are generally considered to be the active participants in the contraction process (Huxley, 1965) although this has been questioned (Ullrick, 1967). Nevertheless, the importance of the Z-line should not be overlooked. The Z-line serves as an anchor point for the thin filaments (Knappeis and Carlsen, 1962) and allows the development of tension by keeping adjacent sarcomeres securely linked.

Development and Resolution of Rigor

The probable sequence of events in the rigor process has also been outlined by Lawrie (1966). After slaughter of an animal glycolysis continues but oxidative phosphorylation through the electron transport chain is terminated by lack of oxygen after blood circulation ceases. Thus, anaerobic metabolism continues to produce ATP until the energy reserves of the muscle are depleted or lactic acid accumulation inhibits glycolytic enzymes. Calcium ions are released by the shock to the nervous system and myosin ATPase is able to function continuously. The muscle then goes into continuous contraction and fails to relax since calcium ions are not pumped away. Myosin

ATPase ultimately depletes the ATP supply and the muscle is bound in the contracted state. Muscle in this condition is inextensible and tough. Allowed sufficient time, however, the muscle becomes soft and pliable again. This time period and softening effect are generally referred to as aging time and tenderization, respectively.

Evidence is available that the resolution of rigor is not due to dissociation of actomyosin (Lawrie, 1966). Marsh (1954) concluded that the inextensibility of muscle achieved during maximum rigor was not reversed with time since the high elastic modulus observed for muscle in rigor was maintained post-rigor. Davey (as cited by Lawrie, 1966) reported that high ionic strength solvents removed mainly actomyosin from post-rigor muscle and suggested that the actin filaments had been detached from the Z-line. Davey and Gilbert (1969), using the light microscope, studied the breakdown in myofibrillar structure during controlled homogenization of fresh and aged muscle fibers. They concluded that tenderization during aging is due to disruption and possible dissolution of Z-line material, leading to a weakening of intermyofibrillar linkages. These changes could be prevented by adding ethylenediaminetetraacetate (EDTA) to the system, suggesting that calcium ions may be involved in the tenderization process (Davey and Gilbert, 1969).

In vivo muscle is known to elaborate proteolytic enzymes (termed cathepsins) in an inactive form within particles known as lysosomes

(Lawrie, 1966). These enzymes are liberated and activated when the lysosome membrane is weakened by decreasing pH. Differing cathepsins having pH optima of 4, 5, 8-9, and 10 have been isolated (Lawrie, 1966). The probable proteolysis of myofibrillar proteins by catheptic enzymes during aging has been examined extensively (Locker, 1960; Khan, 1968; Penny, 1968; Davey and Gilbert, 1966). Locker (1960) determined non-protein nitrogen, free amino acids, and N-terminal amino acids during aging of beef. He reported a definite but small increase in non-protein nitrogen and free amino acids but observed no change in the N-terminal amino acids of extractable myofibrillar proteins during aging of beef. He concluded that proteolysis is not a significant factor in the tenderizing of meat during aging. Bodwell and Pearson (1964) found no detectable activity of catheptic enzymes on actin, myosin, and actomyosin. They reported that the water-soluble sarcoplasmic proteins of muscle were readily hydrolyzed by cathepsins. They also suggested that peptide synthesis or transpeptidation may result from the action of some catheptic enzymes. Davey and Gilbert (1966) reported an increase in non-protein nitrogen in meat during aging representing 2.3% of the meat protein. These workers found that proteolysis was not paralleled by tenderization and concluded that proteolysis was not responsible for tenderization and only sarcoplasmic proteins had been hydrolyzed. Khan (1968) examined the extractibility of actomyosin during aging and proposed that specific proteolysis may

weaken or break bonds that are formed between actomyosin and the matrix of the muscle. Martins and Whitaker (1968) reported that purified cathepsin D had no activity on actomyosin. This does not rule out the possibility of other cathepsins hydrolyzing actomyosin.

Haga et al. (1966) have shown that the presence of 1-10 mM calcium ions during the extraction of protein from myofibrils may effect the detachment of actin from the Z-line. Davey and Gilbert (1969) then suggested that the post-mortem release of calcium ions in muscle may induce fragility of the Z-lines. Penny (1968) extracted myofibrils from aged muscle with pyrophosphate-containing buffer. He observed that all of the myosin was extracted from myofibrils from pre-rigor muscle and that as aging progressed actin and tropomyosin were extracted also. He suggested that these changes may be due to disruption of the Z-line structure, perhaps due to tropomyosin hydrolysis. A basis for the suggested tropomyosin hydrolysis is described in the following section.

Okitani et al. (1968) found that the tropomyosin content in actomyosin preparations was unchanged by meat aging and therefore, discounted an involvement of tropomyosin in the tenderization process. Davey and Gilbert (1968b) reported a slight increase in tropomyosin extractibility after aging of myofibrils. Actin was extracted in increasing amounts as aging progressed. These investigators then postulated that the loss in tensile strength of myofibrils during meat

aging is due to weakening of the Z-structure (Davey and Gilbert, 1968b).

Totally, the results of the previously mentioned investigations suggest that weakening or dissolution of the Z-structure is of primary importance in the post-rigor tenderization of meat. Detachment of actin filaments from the Z-line would allow the sarcomere to return to rest length without the actomyosin complex first dissociating. Proteolytic hydrolysis of the Z-line by catheptic enzymes with a possible involvement of calcium ions appears a logical hypothesis for the tenderization process. Before evidence of such hydrolysis can be obtained an understanding of the Z-structure is essential.

Structure of the Z-Band

Knappeis and Carlsen (1962) presented a detailed description of the Z-band structure based on electron microscope studies. They first reported that the actin (thin) filaments are not continuous through the Z-line and do not extend directly from one sarcomere to the next. Their model of the Z-structure shows four filamentous projections proceeding from the end of each actin filament into the tetragonal, zig-zag configuration of the Z-line. Reedy (1964) confirmed the evidence for this model.

Huxley (1963) reported that the actin filaments have a definite polarity, those on one side of the Z-line being pointed in the opposite

direction of those on the other side. He also reported that tropomyosin exhibits a lattice structure similar to that seen in the Z-bands and proposed that as the thin filaments terminate at the Z-line, the tropomyosin polymer continues into the Z-structure. Huxley (1957) suggested that two strands of tropomyosin (each a coiled-coil of two peptide chains) lie in the grooves of the F-actin double helix. At the thin filament-Z-line junction the actin helix stops but the tropomyosin continues, uncoiled, into the Z-line and to the other side to connect with another thin filament. Huxley's proposal was the basis for Penny's suggested tropomyosin hydrolysis during post-rigor tenderization (1968).

Hanson and Lowy (1963) suggested that the long, thin tropomyosin molecule could easily be accommodated in the grooves of the beaded actin double helix or at the Z-line. Location in the thin filament would explain the observed difficulty of preparing actin free of tropomyosin (Laki et al., 1962; Drabikowski and Gergely, 1962). The presence of tropomyosin in the thin filaments could also account for the apparent discrepancy between the optical protein estimate by interference microscopy and the amount of extractable actin (Huxley and Hanson, 1957).

Tropomyosin is now known to be a nearly 100% α-helical, double stranded molecule (Tsao et al., 1951; Cohen and Holmes, 1963; McCubbin and Kay, 1969; Woods, 1967). It is also known to possess

asymmetry in excess of 20 and to polymerize head-to-tail (Caspar et al., 1969; Hanson and Lowy, 1963). Endo (1966) has reported that tropomyosin exists along the entire length of the thin filaments and in the Z-band from fluorescent protein and fluorescent antibody studies. Pepe (1966) confirmed the presence of tropomyosin in the thin filaments but failed to find evidence of tropomyosin in the Z-band. He stated that the failure of anti-tropomyosin antibodies to bind at the Z-line does not prove the absence of tropomyosin since the protein may be masked and unavailable to antibodies. The thin filaments exhibit a 400 Å axial period which may originate in the 400 Å monomer length of tropomyosin since this spacing is not explicable on the basis of the actin structure (Caspar et al., 1969; Hanson and Lowy, 1963). Tropomyosin is known to complex with F-actin (Laki et al., 1962).

Caspar et al. (1969) characterized tropomyosin crystals by X-ray diffraction and electron microscopy. These investigators reported that the symmetry observed was not compatible with the symmetry of the Z-line structure as suggested by Huxley (1963). They stated, however, that the Z-line may contain tropomyosin bonded in an altered polymorphic pattern.

α-Actinin has been shown by the fluorescent antibody technique to be a major component of the Z-band (Masaki et al., 1967). However, this component does not exceed 3% of the total myofibrillar protein and since the amount of Z-band material is estimated at 7-8%

of the myofibrillar protein (Huxley and Hanson, 1957) other protein must be present (Masaki et al., 1967).

β-Actinin has been shown to complex with F-actin and was thought to regulate the length of the F-actin polymer at 1-2μ (Maruyama, 1966). This function has also been proposed for tropomyosin in the thin filament (Caspar et al., 1969).

Troponin is known to be distributed along the length of the thin filaments and also to exhibit a 400 Å periodicity (Ohtsuki et al., 1967). Troponin has been shown to bind specifically to tropomyosin (Hartshorne and Mueller, 1967; Ebashi and Kodama, 1965; Ebashi and Kodama, 1966) which likely explains the observed periodicity of troponin in the thin filament (Caspar et al., 1969). Both troponin and tropomyosin are required for the calcium regulation of the enzyme activity (ATPase) of actomyosin (Ebashi and Kodama, 1965; Ebashi and Kodama, 1966). Ebashi and Nonomura (as cited by Caspar et al., 1969) reported that the calcium regulation depends on specific binding to troponin. In view of these reports Caspar et al. (1969) proposed as a regulation mechanism that the troponin-tropomyosin complex with actin inhibits the enzyme activity of myosin in the absence of calcium ions. They further postulated that tropomyosin is a structural link between actin and troponin.

In recent work on reconstitution of Z-lines in myofibrils which had the Z-lines removed by 10-day extraction with low ionic strength

buffer it was shown that the dilute 10-day extract would reconstitute the Z-line (Masaki et al., 1967). α-Actinin or tropomyosin alone would not reconstitute the Z-line. No attempt was made to reconstitute the Z-line from a mixture of α-actinin and tropomyosin.

The presence of tropomyosin in the Z-line as suggested by Huxley (1963) is hypothetical. In spite of circumstantial evidence that tropomyosin is not a Z-line component (Caspar et al., 1967; Endo et al., 1966; Pepe, 1966; Stromer et al., 1969) researchers seem reluctant to discard Huxley's proposal. The trypsin sensitivity of both tropomyosin and the Z-line (Stromer et al., 1967) is not compelling evidence for tropomyosin presence in the Z-structure. Caspar et al. (1969) pointed out that if tropomyosin is a Z-line component it must exhibit a unique polymorphism for which there is no known precedent.

The involvement of tropomyosin in the thin filament, however, cannot be disputed (Caspar et al., 1967; Endo et al., 1966; Pepe, 1966). In complexing to F-actin it may serve to anchor the thin filament to the Z-line. Proteolysis of tropomyosin could then be responsible for the tenderization of muscle during aging.

EXPERIMENTAL PROCEDURE

Design of Experiments

To investigate the possible specific hydrolysis of tropomyosin during meat aging, tropomyosin was prepared from pre-rigor meat, meat in rigor, and post-rigor meat. Two methods of tropomyosin preparation were employed; one a direct extraction from meat (Rampton, 1969), the other an extraction of initially prepared myofibrils (Hartshorne and Mueller, 1969). The purification scheme employed (Hartshorne and Mueller, 1969) was not carried to completion in order to reduce the possibility of discarding "non-native' or hydrolyzed tropomyosin.

Four analytical methods were employed to detect specific hydrolysis of tropomyosin during meat aging; N-terminal amino acid analysis, peptide maps of tryptic digests, polyacrylamide-gel electrophoresis, and viscometric analysis. Detection of a new N-terminal amino acid in tropomyosin from aged meat would be evidence of cleavage of the primary structure of the molecule. Loss of a spot or detection of a new spot on peptide maps of tryptic digests of tropomyosin from aged meat would be evidence of change in the net charge and/or size of the peptides involved. Loss of a band or detection of a new band on polyacrylamide-gel electrophoresis patterns of tropomyosin from aged meat would be evidence of change in the net charge and/or

size of the tropomyosin molecule. Reduction in the limiting viscosity number for tropomyosin from aged meat would indicate a reduction in average molecular weight. Change in size (molecular weight) or net charge could be evidence of hydrolysis of the tropomyosin molecule.

It is possible that hydrolysis of a limited, small number of the tropomyosin molecules could result in the tenderization of meat. Detection of changes in a small percentage of the tropomyosin molecules was of concern throughout the analytical procedures.

Sampling Procedure

Meat samples were taken from two Hereford cows at a local slaughter plant within 30 minutes of stunning the animals. The psoas major muscle was excised from one side of each carcass, wrapped in a plastic bag, and chilled 30 minutes in ice. After chilling, a portion of each muscle was removed, trimmed of fat, and diced for homogenization. The remainder of each muscle was sprayed with a solution containing 100 ppm aureomycin and 100 ppm chloramphenicol, wrapped in a plastic bag, and stored at $4^{\circ}C$. Aging times were two and nine days. At two days and nine days a portion of each muscle was removed, trimmed of fat, surface trimmed to remove any existing microbial population, and diced for homogenization. After the two-day sampling the remaining portions were sprayed and stored as before.

pH Determination

The pH of meat samples was determined at the time of initial homogenization with a Beckman Zeromatic pH Meter on homogenates of 3 gm of meat in 15 ml of 2 mM sodium iodoacetate, pH 7 (Marsh and Snow, 1950).

Preparation of Myofibrils and Fiber Pieces

Washed myofibrils and fiber pieces were prepared by a method adapted from Davey and Gilbert (1968a; 1969) and Perry and Grey (1956). All manipulations were carried out at 4°C. Chilled, diced, lean meat (200 gm) was homogenized one minute with one liter of KCl-borate buffer (0.1 M KCl-0.039 M boric acid-2 mM β-mercaptoethanol, pH 7.1, μ =0.1) in a Waring Blendor, and the homogenate centrifuged 15 minutes at 600xG. The residue, resuspended in one liter of KCl-borate buffer, was homogenized two minutes and centrifuged 15 minutes at 600xG. This residue was resuspended in one liter of KCl-borate buffer and long strands of connective tissue were removed with a stirring rod. The suspension was passed through nylon mesh (1.2 mm mesh diameter) to further remove connective tissue and centrifuged 15 minutes at 600xG. Again the residue was resuspended in one liter of KCl-borate buffer and centrifuged 30 minutes at 1,500xG. This residue was resuspended in approximately 100 ml of 0.16 M KCl-2 mM β-mercaptoethanol, pH 7.0, and centrifuged 30 minutes at

2,000x G. The residue from this centrifugation, containing myofibrils and fiber pieces as described by Davey and Gilbert (1969), was then used for tropomyosin preparation.

The supernatants, containing soluble proteins and lipid, were discarded after each centrifugation.

Extraction of Tropomyosin from Myofibrils and Fiber Pieces

The extraction of tropomyosin from myofibrils and fiber pieces was adapted from the method of Hartshorne and Mueller (1969). Myofibrils and fiber pieces prepared from 200 gm lean meat as described above were extracted with 200 ml of pyrophosphate buffer (0.9 M KCl-0.01 M KH_2PO_4-0.01 M K_2HPO_4-1 mM $MgCl_2$-0.01 M $K_4P_2O_7$-2 mM β-mercaptoethanol, pH 7.0, μ=0.98) (Davey and Gilbert, 1968b). The pyrophosphate buffer was added slowly with stirring to avoid formation of lumps. After a 24 hour extraction period the solution was centrifuged 20 minutes at 10,000 x G. The supernatant was diluted with 2 mM β-mercaptoethanol, pH 7.0, to μ=0.043 with stirring and the "natural actomyosin" precipitate (Perry and Corsi, 1958) was allowed to settle 24 hours. The clear supernatant was siphoned off and the remaining suspension was centrifuged in 500 ml portions for 20 minutes at 1,500 x G until the precipitate was compacted into a volume less than 200 ml. A final centrifugation was carried out for 30 minutes at 1,500 x G. The residue was washed twice with 500 ml of diethyl ether-

2 mM dithiothreitol. Each washing was followed by a ten minute centrifugation at 1,500xG. The residue from the second ether washing was evaporated to near dryness in the hood. Large lumps were broken up with a spatula and the finely chopped residue was extracted 24 hours with 300 ml of 1 M KCl-2 mM β-mercaptoethanol, pH 7.0. The extraction mixture was then centrifuged one hour at 30,000xG. The supernatant was adjusted to pH 4.3 with 1 N HCl and allowed to stand one hour, then centrifuged ten minutes at 10,000xG. The precipitate was dispersed in 250 ml of 2 mM β-merceptoethanol, pH 7.0, and the pH was adjusted to 7.0 with 1 N HCl. Ammonium sulfate was added to 40% saturation and the pH was again adjusted to 7.0 with 1 N HCl. The solution was allowed to stand one hour and then centrifuged ten minutes at 10,000xG. Ammonium sulfate was added to the supernatant to 60% saturation, the pH adjusted to 7.0 with 1 N HCl, and the solution allowed to stand one hour. The solution was centrifuged 15 minutes at 15,000xG. The precipitate was dispersed in approximately 5 ml water and dialyzed against three changes of three liters of deionized water over 24 hours. The protein content of the dialyzed solution was determined by the biuret reaction, after which the solution was divided into 5 ml portions, frozen in liquid nitrogen, and stored at -38°C for later analyses.

Extraction of Tropomyosin from Meat

The direct extraction of tropomyosin from meat was adapted from

the method of Rampton (1969) who modified the original procedure of Bailey (1948). The final workup, beginning with the isoelectric precipitation at pH 4.3, was adapted from the method of Hartshorne and Mueller (1969). All manipulations were carried out at 4°C.

Chilled, diced, lean meat (150 gm) was homogenized with 300 ml of 2 mM β-mercaptoethanol, pH 7.0, for two minutes in a Waring Blendor. The homogenate was allowed to stand 30 minutes and then centrifuged 15 minutes at 1,500xG. The residue was washed with the following sequence of organic solvents: 150 ml of 95% ethanol-2 mM β-mercaptoethanol, 600 ml of 95% ethanol:water (1:1 v/v)-2 mM β-mercaptoethanol, twice with 150 ml of 95% ethanol-2 mM β-mercaptoethanol, and twice with 150 ml of diethyl ether-2 mM dithiothreitol. Each washing was followed by a ten minute centrifugation at 1,500xG. The final residue was evaporated to near dryness in the hood. Large pieces were broken up with a spatula and the finely chopped, fibrous residue was extracted 24 hours with 1,500 ml of 1 M KCl-2 mM β-mercaptoethanol, pH 7.0. The extraction mixture was then centrifuged 20 minutes at 1,500xG. The supernatant was filtered through glass wool to remove floating particles and the pH was adjusted to 4.3 with 1 N HCl. The solution was allowed to stand one hour and centrifuged ten minutes at 1,500xG. The residue was dispersed in 250 ml of 2 mM β-mercaptoethanol, pH 7.0, and the pH was adjusted to 7.0 with 1 N HCl. Ammonium sulfate was added to 40% saturation and the

pH again adjusted to 7.0 with 1 N HCl. The solution was allowed to stand for one hour and centrifuged ten minutes at 10,000xG. Ammonium sulfate was added to the supernatant to 60% saturation, the pH was adjusted to 7.0 with 1 N HCl, and the solution was allowed to stand for one hour. The solution was then centrifuged 15 minutes at 15,000xG. The residue was dispersed in approximately 10 ml water and the suspension was dialyzed against three changes of three liters of deionized water over 24 hours. The protein content of the dialyzed solution was determined by the biuret reaction, after which the solution was divided into 5 ml portions, frozen in liquid nitrogen, and stored at -38°C for later analyses.

Coding of Tropomyosin Preparations

Twelve tropomyosin preparations resulted from the application of two extraction methods to six meat samples (three aging times for each of two animals sampled). The preparations were coded for the extraction procedure, the meat aging time, and the animal from which the meat was taken. Preparations by the extraction method adapted from Rampton (1969) were coded R. Preparations by the extraction of myofibrils with pyrophosphate buffer were coded P. A number -- 0, 2, or 9 -- indicating aging time in days follows the letter designation. A lower number -- 1 or 2 -- indicates the animal from which the meat sample was obtained. A tropomyosin preparation coded

R-0/1 indicates that the preparation was derived from the modified Rampton extraction method on a 0-days aged sample from cow No. 1.

Special Chemicals

All chemicals used were reagent grade or appropriate high quality. Chemicals which were specially ordered are listed hereafter.

Phenylisothiocyanate and β-mercaptoethanol were obtained from Eastman Organic Chemicals. Dithiothreitol, Cleland's reagent, and p-chloromercuribenzoate, sodium salt, were acquired from Calbiochem. Dowex 50X2 (100 mesh) and bovine trypsin, crystallized, dialyzed, and lyophilized, were purchased from Sigma Chemicals Company. Ninhydrin was from Pierce Chemical Company. Cyanogum-41 (composed of 95% acrylamide and 5% N, N'-methylenebisacrylamide), TMED (N, N, N', N'-tetramethylene diamine), and ammonium persulfate were obtained from E-C Apparatus Corporation, Philadelphia, Pennsylvania.

Phenylthiohydantoin (PTH) derivatives of 24 amino acids were purchased from Nutritional Biochemicals Corporation. The 24 PTH-amino acids were: PTH-DL-alanine, PTH-DL-arginine, PTH-DL-asparagine, PTH-DL-aspartic acid, PTH-DL-cysteic acid, PTH-DL-cystine, PTH-DL-glutamic acid, PTH-DL-glutamine, PTH-DL-glycine, PTH-DL-histidine, PTH-hydroxy-DL-proline, PTH-DL-isoleucine, PTH-DL-leucine, PTH-DL-methionine, PTH-DL-

methionine sulfoxide, PTH-DL-phenylalanine, PTH-ε-phenylthiocarbamyl-DL-lysine, PTH-DL-proline, PTH-DL-serine, PTH-DL-tryptophan, PTH-DL-tyrosine, PTH-DL-valine, PTH-DL-ornithine, and PTH-DL-citrulline.

Protein Determination

Protein determinations were carried out by the biuret method of Layne (1957) using bovine serum albumin to produce a standard curve.

Estimation of Impurities

To test for the presence of actomyosin in the tropomyosin preparations an adaptation of the method of Carstens (1968) was used. Tropomyosin preparations were diluted with deionized water to a protein concentration of 5 mgm/ml. A 1.8 ml aliquot of each diluted preparation was treated with 0.2 ml of 1 M phosphate, pH 7.0. Solutions were heated in a water bath at 60°C for five minutes and cooled briefly in an ice bath. Absorbance of the solutions before and after heating was determined at 660 nm and any increase in absorbance was assumed to be due to light scattering by precipitated actomyosin (Hamoir and Laszt, 1962; Parsons et al., 1969).

To test for the presence of nucleoprotein in the tropomyosin preparations the ultraviolet absorption spectra of the preparations were determined with a Beckman Model DB recording

spectrophotometer. The spectra showed maxima at 276-265 nm and minima at 250-247 nm. Nucleoprotein contamination was determined from the absorbance ratios between maximum and minimum wavelengths (Ooi, 1967; Kominz et al., 1957). This ratio has been reported to be approximately three for purified tropomyosin and to decrease when nucleotide is present (Ooi, 1967).

N-Terminal Amino Acid Analysis

N-Terminal amino acid analyses of the tropomyosin preparations were done by the Edman method as modified by Schroeder (1967). Briefly, the method proceeds by reaction of phenylisothiocyanate with the N-terminal amino group of the protein to produce a phenylthiocarbamyl derivative. This product is hydrolyzed in an acidic atmosphere to produce a phenylthiohydantion (PTH) derivative of the N-terminal amino acid of the protein. The amino acid is then determined from the R_f of the PTH-derivative on paper chromatography as compared with R_f's of commercially available PTH-amino acids. One dimensional paper chromatography was run using solvents A, D, and E of Schroeder (1967). Solvent A was a 7:3 (v/v) mixture of heptane and pyridine. Solvent D was xylene. Solvent E was prepared in three steps. First, n-butyl acetate (970 ml) was thoroughly mixed with water and the two phases were allowed to equilibrate overnight. Propionic acid was then added to the water-saturated n-butyl acetate to

a concentration of 3%. Finally, this mixture was saturated with formamide (40 ml was sufficient).

Peptide Mapping

Peptide maps of tryptic digests of the tropomyosin preparations were prepared by the method of Katz and Carsten (1963) and Carsten (1968). Samples of the tropomyosin preparations (10 ml at a concentration of 5 mgm/ml) were allowed to react 12 hours at room temperature with an excess of p-chloromercuribenzoate (10 ml, 0.56 mM) in 0.02 M Tris-NO_3 buffer, pH 7.6. The buffer and excess mercurial were removed by 24 hour dialysis against deionized water at 4°C. Crystalline urea was added to a final concentration of 6 M and the solution was allowed to stand 30 minutes at room temperature. Two volumes of water were added to reduce the urea concentration to 2 M and two drops of phenol red indicator were added. The solution was adjusted to pH 7.6 with 0.1 N NaOH and trypsin (1 ml at a concentration of 1 mgm/ml) was added. The digestion was allowed to proceed for 18 hours at room temperature, the pH being maintained at 7.6 by titration with 0.1 N NaOH. The digestion was stopped by placing the reaction mixture on a column of Dowex 50X2 (100 mesh) in the H^+ form (4-5 ml bed volume). The column was washed exhaustively with deionized water (80 ml) to remove urea, then eluted with 20 ml of 4 M NH_4OH. The eluate was lyophilized and the dry peptides weighed.

Peptide maps were prepared using 3 mgm of peptides in 50 µl deionized water applied to Whatman 3 MM filter paper (46 x 57 cm sheets). Descending chromatography in n-butanol-acetic acid-water (4:1:5 v/v/v), upper phase from a mixture equilibrated overnight, was run 24 hours; which allowed the front to reach the edge of the paper. Electrophoresis (High Voltage Electrophorator, Model D, Gilson Medical Electronics, Middleton, Wisconsin) in pyridine-acetic acid-water (10:100:2890 v/v/v), pH 3.7, was then run at 3,000 V for one hour. The papers were sprayed with a solution made up of cadmium acetate (100 mgm), water (10 ml), acetic acid (5 ml), acetone (100 ml), and ninhydrin (1 gm) (Bailey, 1962), dried in the hood, and developed 3-5 minutes in a 100°C oven.

Polyacrylamide-Gel Electrophoresis

A vertical-gel electrophoresis apparatus (Model EC-470, E-C Apparatus Corporation, Philadelphia, Pennsylvania) and companion power supply (Model EC-454) were used for discontinuous polyacrylamide-gel electrophoresis of the tropomyosin preparations. The discontinuous method is described in detail by Petropakis (1968) and by E-C Apparatus Corporation (1966). Two electrophoretic runs were carried out for each tropomyosin preparation under conditions that were identical except for the addition or non-addition of β-mercaptoethanol (2 mM) to the system.

Plug and running gel solutions were prepared by dissolving 10 gm and 6 gm, respectively, of Cyanogum-41 and 30 gm of urea in 50 ml of 0.76 M Tris-HCl, pH 9.0, and diluting these solutions to 100 ml with deionized water to produce 10% and 6% gel solutions, each containing 5 M urea-0.38 M Tris-HCl. Final pH was 8.9. Spacer gel solution was prepared by dissolving 4 gm of Cyanogum-41 and 30 gm of urea in 10 ml of 0.62 M Tris-HCl, pH 6.7, plus 40 ml deionized water and diluting this solution to 100 ml with deionized water to produce a 4% gel solution containing 5 M urea-0.062 M Tris-HCl. Final pH was 7.0.

To each of these 100 ml solutions was added 0.1 ml of TMED, 0.02 ml of Tween 80, and 0.014 ml β-mercaptoethanol if it was to be used. The solutions were filtered through Whatman No. 1 filter paper. Polymerization of the gel solutions was catalyzed by addition of 0.1 gm of ammonium persulfate.

Buffer solutions were prepared in liter quantities for later use. However, Cyanogum-41 and urea were added just prior to use in order to minimize decomposition (Ritchie et al., 1966) and hydrolysis (Morrison and Boyd, 1959).

Electrode buffer was prepared by dissolving 600 gm of urea in one liter of 0.033 M Tris-0.078 M glycine, pH 8.65, and diluting this solution to 2 l with deionized water. β-Mercaptoethanol (0.28 ml) was added if it was to be used. This produced an electrode buffer containing 5 M urea-0.0165 M Tris-0.039 M glycine (-2 mM β-mercaptoethanol),

pH 8.65. This buffer was then held at 4°C.

Sample mixtures were prepared for electrophoresis by diluting the tropomyosin preparations with deionized water to a protein concentration of 6 mgm/ml. To a 1 ml aliquot of each diluted preparation was added 0.1 ml of 0.62 M Tris-HCl, pH 6.7, 0.1 gm of sucrose, 0.3 gm of urea, and 1 drop of a water solution of bromphenol blue of sufficient concentration to color the sample mixtures blue. β-Mercaptoethanol (0.05 ml) was added if it was to be used. The mixtures were stirred with a glass rod until all urea and sucrose had dissolved and clear blue solutions resulted.

Before assembling the electrophoretic cell, the rubber gaskets were coated with a heated, 1% agarose solution to assure a good seal. The cell was then assembled and the gels polymerized in the cell at room temperature as described by Petropakis (1968). Upon completion of gel polymerization the cell was placed in the cold room (4°C) and the upper chamber was filled with electrode buffer, allowing buffer to overflow until the lower chamber electrode was completely covered. Coolant water was circulated through the cell for one hour to cool the gel to 4°C. Excess gel above the slot form was then removed and the slot form carefully taken out. Sample slots were then partially filled with 0.05 ml of each sample mixture and an electric potential of 200 V was applied until the sample was completely stacked in the spacer gel. When the sharp sample layer had moved to the surface of the

running gel, the potential was increased to 400 V and circulation of the electrode buffer was begun. The electrophoretic run was terminated when the blue dye reached the gel plug. A slight warming of the cell occurred during the latter part of the run. The time required for sample stacking was one hour. The remainder of the run required three hours.

After electrophoresis was completed, gel slabs were stained for 20 minutes in a dye solution prepared by dissolving 4 gm of amido black-10B in a solvent consisting of 1000 ml of methanol, 1000 ml of distilled water plus 200 ml of acetic acid. After the proteins were fixed and stained, excess dye was removed with an electrophoretic destainer (Model EC-479) as described by Petropakis (1968). The destaining solution was composed of methanol, distilled water, and acetic acid (8:8:1 v/v/v).

Viscometric Analysis

Measurements of viscosities of a series of dilutions of the tropomyosin preparations were carried out at 25°C in two different media. The first was 0.039 M borate, pH 7.1, μ=0.003 (Davey and Gilbert, 1968b). The second was the electrode buffer containing β-mercaptoethanol used for polyacrylamide gel electrophoresis (5 M urea-0.0165 M Tris-0.039 M glycine-2 mM β-mercaptoethanol, pH 8.65, μ=0.0135). Ostwald viscometers were employed for the viscosity

determinations.

In carrying out the viscosity determinations in borate, 5.0 ml aliquots were used in two Ostwald viscometers which had water flow-through times of 80.8 seconds and 72.9 seconds. Protein was determined with the biuret method for each dilution of tropomyosin preparations. The 1 ml aliquots taken for the biuret assay were carefully weighed since accurate delivery of 1.0 ml of the very viscous samples from a pipette was impossible. The protein concentration determined was then corrected for the weighed amount of sample used assuming the density to be the density of water. The density of the tropomyosin preparations was previously empirically found not to differ significantly from the density of water over the range of protein concentrations employed in these experiments.

In carrying out the viscosity determinations in 5 M urea, 4.0 ml aliquots were used in three Ostwald viscometers which had water flow-through times of 78.9 seconds, 71.6 seconds, and 64.4 seconds. Portions (5 ml) of all tropomyosin preparations were initially dialyzed 54 hours versus 2 l of the electrode buffer at room temperature. This buffer solution was then used for a series of dilutions of tropomyosin preparations for viscosity determinations.

Direct determination of the protein concentration of the dialyzed preparations was not possible by the biuret method due to the presence of the 5 M urea in the electrode buffer. Therefore a pre-dialysis

absorptivity in the range of 267 to 276 nm (λmax. of each preparation) was obtained for each preparation based upon a biuret protein determination for each preparation. Using this absorptivity the post-dialysis protein concentration of each of the tropomyosin preparations was obtained from the absorbance readings. Precise volumetric dilution then enabled calculation of the protein concentration for each dilution in the series of dilutions made for viscosity determination.

RESULTS AND DISCUSSION

pH Determination

The pH of meat samples was taken at the time of the initial homogenization in the workup procedure. These results are shown in Table 1. The pH measurements were taken in order to estimate the status of post-mortem glycolysis. It was hoped that the sampling procedure would be fast enough that the physiological condition of the 0-day sample would be similar to that of muscle in the living animal. If this was the case then the pH of the 0-day samples should have been 6.8-7.0 since post-mortem anaerobic glycolysis should not yet have caused a marked reduction in pH due to lactic acid increase (Lawrie, 1966; Bodwell et al., 1965; Disney et al., 1967). Since the pH of the 0-day sample from cow No. 1 had decreased to 6.1 by the time of the initial homogenization (Table 1), the sampling procedure was not fast enough to "catch" this sample in the most desirable post-mortem state. A better result was achieved with cow No. 2. The pH difference between the two samples might be attributed to inherent differences that are known to occur between animals in the rate of post-mortem glycolysis (Lawrie, 1966).

Table 1. pH of meat samples at the time of initial homogenization.

	Aging time (days)		
	0	2	9
Cow No. 1	6.1	5.5	5.6
Cow No. 2	6.6	5.8	5.5

The 30 minute delay between stunning of animals and excision of meat samples could not be reduced under the existing operating conditions of the slaughter facility. The 30 minute chill time before homogenization of the sample was found essential to prevent solidification of fat upon the muscle fibers during homogenization in the cold solvent medium. Fat deposition upon the muscle fibers at this point made extraction of protein virtually impossible as observed by extremely low yields in preliminary experiments. Thus the one hour delay between stunning of the animals and the initial homogenization of the 0-day samples could not be reduced.

Aging times of zero, two, and nine days were selected in order to have samples from fresh pre-rigor meat, meat well into rigor, and post-rigor meat, respectively. The physical condition of the meat at these sampling times indicated that this had been achieved. The meat was very soft at zero days, very stiff at two days, and soft again at nine days of aging. While the pH data in Table 1 cannot be taken as definitive proof that the desired rigor conditions were attained by the sampling times, they do indicate normal post-mortem glycolysis (Lawrie, 1966; Bodwell et al., 1965; Disney et al., 1967). Therefore, it was assumed that samples were taken pre-rigor, during rigor, and post-rigor.

Development of Extraction Procedures

Since the isolation of tropomyosin by Bailey in 1948 subsequent procedures used for its preparation have been based on his original work. Mueller (1966) modified the procedure to include dithiothreitol in all solutions used in the extraction to prevent oxidation of sulfhydryl groups. Woods (1967) confirmed the importance of including a sulfydryl-protecting agent and also added 0.01 M ethylenediaminetetra-acetic acid (EDTA) to bind heavy metal ions. Rampton (1969) characterized tropomyosins prepared by four different but similar methods. His results indicated little difference between the preparations provided care was taken to prevent oxidation of the sulfhydryl groups.

Perry and Corsi (1958) were able to extract tropomyosin from myofibrils in yields of 10-12% of total nitrogen content of myofibrils with low ionic strength buffers (μ=0.025-0.1). Davey and Gilbert (1968b) were able to extract tropomyosin from myofibrils in good yield (8.7-8.9% of total myofibrillar protein) only by employing a buffer of high ionic strength (μ=0.98) containing 10 mM pyrophosphate. Their yields increased very slightly (9.1-9.2% of total myofibrillar protein) after 21-22 days aging of well-washed myofibrils. Hartshorne and Mueller (1969) developed a purification scheme to prepare tropomyosin free of troponin. Their method proceeds via the initial preparation of myofibrils but is also applicable to purification of tropomyosin

prepared by direct extraction of meat.

In the present work tropomyosin was prepared according to two methods; one a direct extraction of meat, the other an extraction of myofibrils. The direct extraction of meat was adapted from the method of Rampton (1969) who interpreted Bailey's original work (1948). Bailey's purification cycle is somewhat ambiguous according to Mueller (1966). β-Mercaptoethanol (2 mM) was included in all extraction solutions making the method similar to that of Mueller (1966) who used dithiothreitol to prevent sulfhydryl oxidation. Dithiothreitol would have been the reagent of choice but was impractical to use in the large volume solutions employed in the procedure owing to its high cost. Dithiothreitol was used in the diethyl ether wash of homogenized meat residue since final traces of ether were removed by air evaporation.

The preparation of myofibrils was adapted from the method of Davey and Gilbert (1968a). The extraction of tropomyosin from myofibrils was adapted from the method of Hartshorne and Mueller (1969) but employed the pyrophosphate buffer of Davey and Gilbert (1968b). β-Mercaptoethanol (2 mM) was used throughout except for the diethyl ether wash of "natural actomyosin" residue. Dithiothreitol was used in the ether for the same reason as mentioned above.

In order to have some degree of consistency in the relative purity of preparations from the two procedures used in this work, the same purification scheme from the point of isoelectric precipitation at

pH 4.3 was used for both (Hartshorne and Mueller, 1969). The extraction methods and purification cycle were developed to isolate native tropomyosin. It is possible that structurally altered, hydrolyzed, or fragmented "non-native" tropomyosin would not be isolated by these procedures. To reduce the possibility of discarding "non-native" tropomyosin in the preparatory procedure the purification scheme of Hartshorne and Mueller (1969) was not carried to completion. Highly purified tropomyosin preparations were not used in this work.

Some hope that "non-native" tropomyosin would be isolated by these procedures comes from the highly-ordered nature of the native molecule. Tropomyosin is reported to consist of two α-helical chains, side-to-side, twisted about one another (McCubbin et al., 1967; Woods, 1967). Its resistance to the denaturing effects of high ionic strength (μ=0.1-1.1) solutions (McCubbin and Kay, 1969; Tsao et al., 1951) attests to the stability of the native structure. Therefore, it may be possible that limited proteolysis, which might occur during meat aging, would not alter the structure to such an extent that it would not be isolated by these procedures.

Estimation of Impurities

Estimation of the amount of actomyosin contamination in tropomyosin preparations was made by heat precipitation of the actomyosin (Carsten, 1968). The absorbance at 660 nm of each preparation was

recorded before and after heating five minutes at 60°C. The increase in absorbance was assumed to be due to light scattering by precipitated actomyosin. The absorbance increases are shown in Table 2. This assay has not previously been used to obtain a quantitative estimate of actomyosin contamination in tropomyosin preparations. Rather it has been used to obtain a simple yes-no result for the presence or absence of actomyosin and precipitation has been determined visually (Carsten, 1968). Therefore, no standard is available for comparison. While the actomyosin content of the preparations cannot be quantitated from these data, relative actomyosin contamination in the preparations can be estimated.

Table 2. Increases in absorbance at 660 nm of tropomyosin preparations heated five minutes at 60°C.

Preparation	Absorbance increase	Preparation	Absorbance increase
R-0/1	.106	P-0/1	.116
R-2/1	.194	P-2/1	.132
R-9/1	.202	P-9/1	.107
R-0/2	.139	P-0/2	.257
R-2/2	.184	P-2/2	.262
R-9/2	.222	P-9/2	.184

The R-preparations show a definite increase in actomyosin content as aging progresses. The P-preparations, on the other hand, exhibit an increase in actomyosin content in preparations from meat in rigor (2-day preparations) then a marked decrease in actomyosin content in preparations from post-rigor meat (9-day preparations). These

results may reflect changes in the ease of dissociating the known F-actin-tropomyosin complex as aging progresses (Laki et al., 1962).

As a measure of nucleotide contamination the ultraviolet absorbance spectra of the tropomyosin preparations were determined.

The absorbance ratio between maximum and minimum wavelengths was calculated. These data are given in Table 3. Ooi (1967) reported a value of 3 for the ratio of absorbance at 277 nm (λ max.) to absorbance at 252 nm (λ min.). This value was for purified tropomyosin and decreased with nucleotide contamination. Kominz et al. (1957) reported the inverse ratio, i.e., the ratio of absorbance at 260 nm to absorbance at 280 nm. No mention was made of choosing the wavelengths of maximum and minimum absorbance. Inverting the ratios of Kominz et al. (1957), so that they correspond to the data of Ooi (1967) and the data in Table 3, a ratio of 1.82 is calculated for pure tropomyosin and 0.5 for nucleotropomyosin.

Ribosomes and ribonucleic acids are known to be closely associated with myofibrils (Zak et al., 1967). Tropomyosin from smooth muscle is often extracted as nucleotropomyosin (Hamoir, 1951; Needham and Williams, 1963). RNA, but not DNA, has been detected in further extracts of nucleotropomyosin (Zak et al., 1967).

The data in Table 3 do not give a quantitative measure of nucleoprotein contamination in the tropomyosin preparations since no standard curve of absorbance ratio versus nucleoprotein concentration is

Table 3. Maximum and minimum ultraviolet absorbance wavelengths of tropomyosin preparations and the ratio of absorbance at the maximum wavelength to absorbance at the minimum wavelength.

Preparation	Max. (nm)	Min. (nm)	Amax.: Amin.
R-0/1	274	250	1.50
R-2/1	273	250	1.35
R-9/1	267	247	1.29
R-0/2	270	249	1.29
R-2/2	270	249	1.29
R-9/2	265	247	1.23
P-0/1	276	250	1.98
P-2/1	276	250	2.43
P-9/1	276	250	1.61
P-0/2	275	250	1.79
P-2/2	275	250	1.90
P-9/2	275	250	1.65

available. However these data do allow a comparison between preparations in the table and a comparison to the previously reported values (Ooi, 1967; Kominz et al., 1957; Hamoir, 1951; Needham and Williams, 1963).

The data in Table 3 show that the P-preparations had less nucleotide contamination than the R-preparations, that nucleotide contamination in P-preparations from meat in rigor (2-day preparations) decreased markedly and increased again in post-rigor preparations (9-day preparations), and that the nucleotide contamination in R-preparations increased slightly as aging progressed. Also an observed "blue shift" in the wavelengths of maximum and minimum absorbance in R-preparations as aging progressed was apparently correlated with the amount of nucleotide contamination in the R-preparations.

Nucleotide contamination in the preparation correlates inversely with the observed actomyosin contamination (Tables 2, 3) suggesting that an increase in actomyosin content, perhaps due to formation of an actomyosin-tropomyosin complex (Laki et al., 1962), results in reduced nucleotide binding to the tropomyosin.

N-Terminal Amino Acid Analyses

Chromatograms from the N-terminal amino acid analyses of the tropomyosin preparations by the Edman degradation are shown in Figures 1 and 2. All tropomyosin preparations consistently showed two well defined spots on the chromatograms which had migration rates which corresponded to those of the phenylthiohydantoin (PTH) derivatives of methionine and methionine sulfoxide. A third less pronounced spot which remained on the origin in all solvent systems corresponded to either PTH-cysteic acid or PTH-serine. Several other faint spots can be seen in the chromatograms indicating trace amounts of other PTH-amino acids. No attempt was made to identify these as their origin was most likely from contaminating proteins in the tropomyosin preparations.

No apparent differences between tropomyosin preparations were seen in these experiments. The appearance of a new PTH-amino acid in tropomyosin preparations from aged meat which was not present in tropomyosin preparations at 0-days aging would have indicated a

Figure 1. Chromatograms from N-terminal amino acid analysis of R-tropomyosin preparations. PTH-Amino acid standards were run with tropomyosin preparations for comparison. Amino acids whose PTH derivatives were used are abbreviated as: ser, serine; arg, arginine; met, methionine; met sulf, methionine sulfoxide; cysteic, cysteic acid; cys, cystine; lys, lysine; orn, ornithine; pro-OH, hydroxy proline; pro, proline; gly, glycine; asp, aspartic acid; glN, glutamine; glu, glutamic acid, and his, histidine.

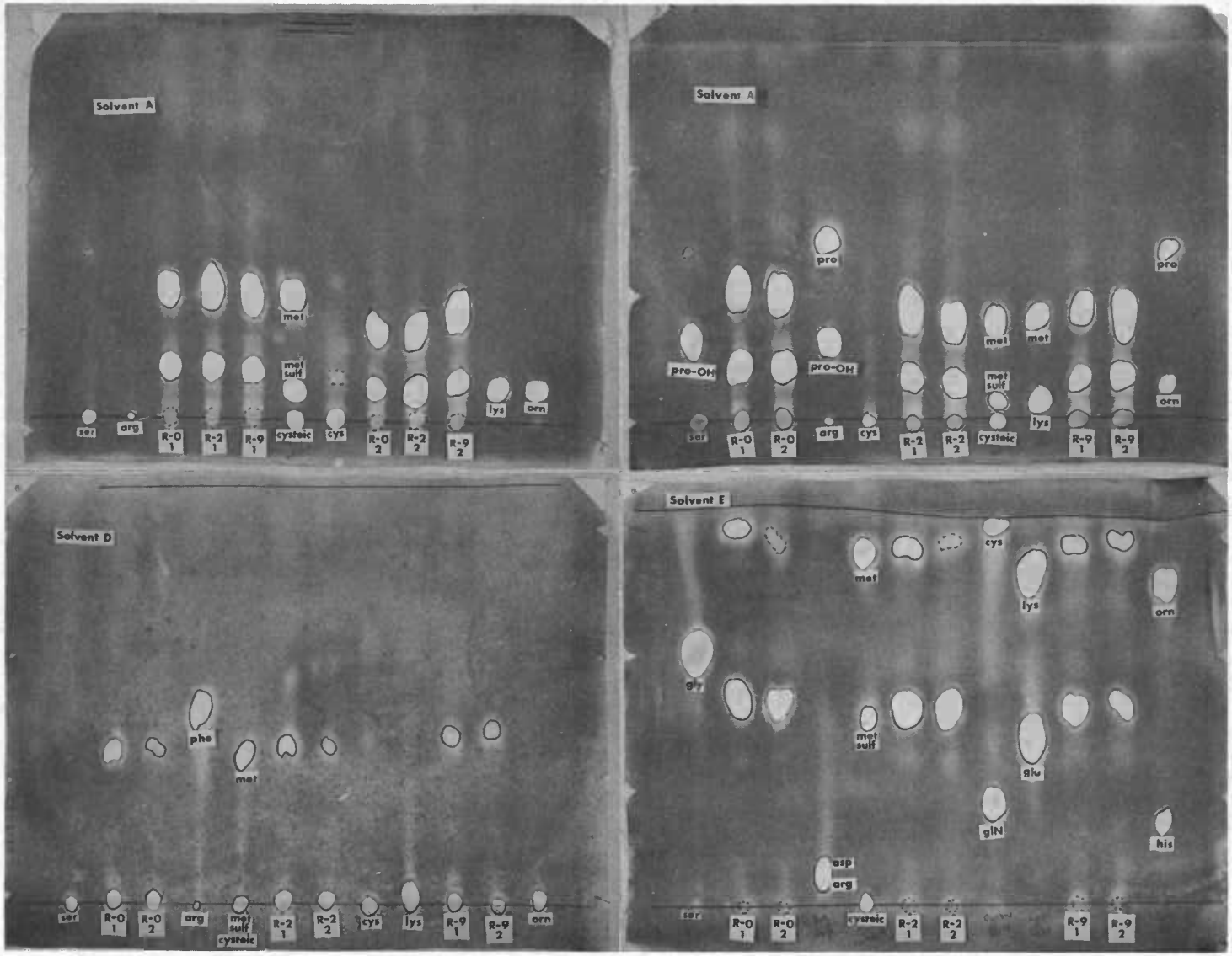
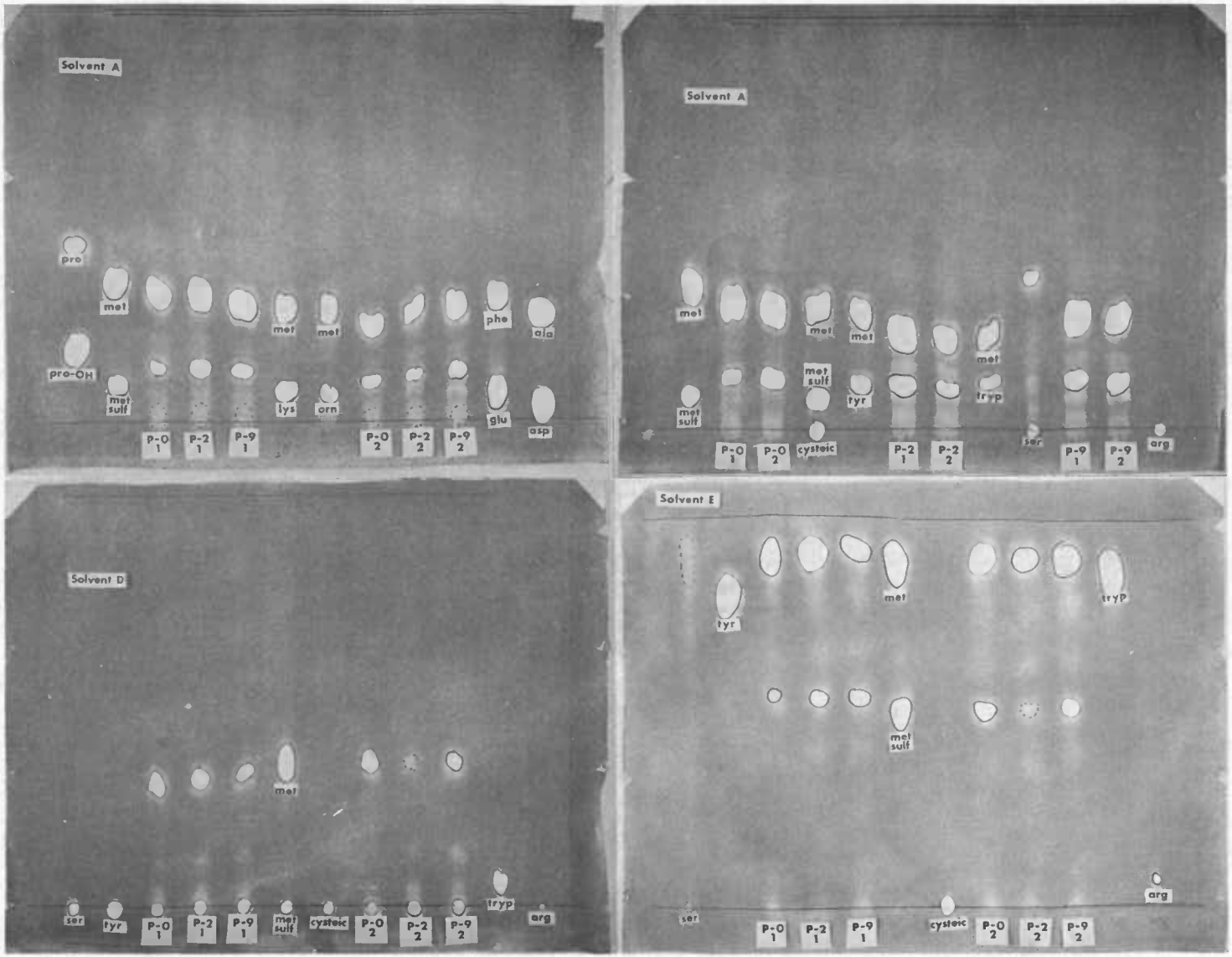

Solvent A
ser
arg
R-0 1
R-2 1
R-9 1
met
met sulf
cysteic
cys
R-0 2
R-2 2
R-9 2
lys
orn
Solvent A
pro
pro
pro-OH
pro-OH
met
met
met sulf
cysteic
lys
orn
ser
R-0 1
R-0 2
arg
cys
R-2 1
R-2 2
R-9 1
R-9 2
Solvent D
phe
met
ser
R-0 1
R-0 2
arg
met sulf
cysteic
R-2 1
R-2 2
cys
lys
R-9 1
R-9 2
orn
Solvent E
met
cys
lys
orn
gly
met sulf
glu
glN
his
asp
arg
ser
R-0 1
R-0 2
cysteic
R-2 1
R-2 2
R-9 1
R-9 2

Figure 2. Chromatograms from N-terminal amino acid analysis of P-tropomyosin preparations. PTH-Amino acid standards were run with tropomyosin preparations for comparison. Amino acids whose PTH derivatives were used are abbreviated as: ser, serine; tyr, tyrosine; met, methionine; met sulf, methionine sulfoxide; cysteic, cysteic acid; tryp, tryptophan; arg, arginine; pro, proline; pro-OH, hydroxy proline; lys, lysine; orn, ornithine; phe, phenylalanine; glu, glutamic acid; ala, alanine, and asp, aspartic acid.

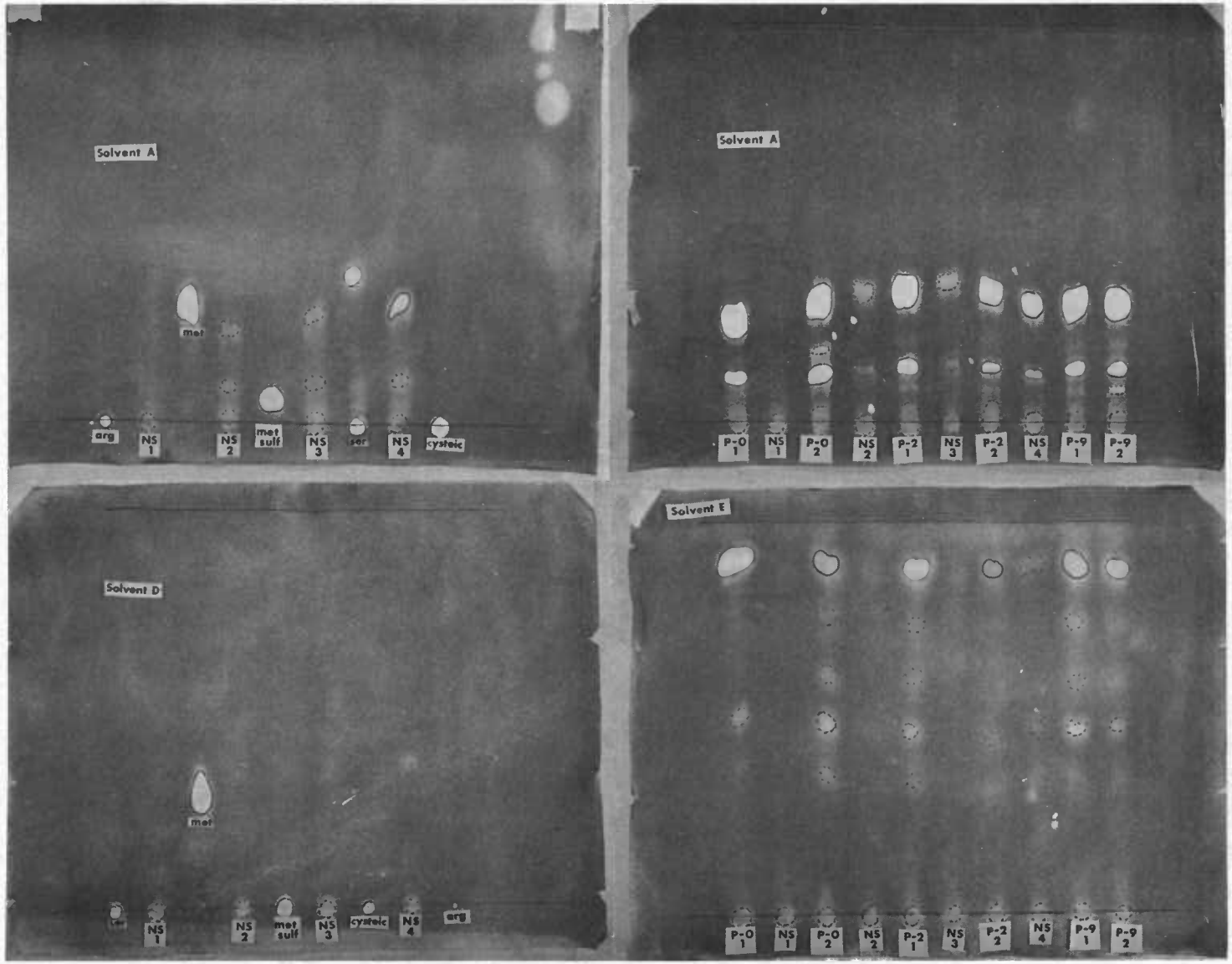

Solvent A
pro
met
met
met
phe
ala
pro-OH
met sulf
lys
orn
glu
asp
P-0 1
P-2 1
P-9 1
P-0 2
P-2 2
P-9 2
Solvent A
met
met
met
met sulf
met sulf
tyr
met
tryp
ser
cysteic
arg
Solvent D
met
ser
tyr
met sulf
cysteic
tryp
arg
Solvent E
tyr
met
tryp
met sulf
ser
cysteic
arg

new N-terminal amino acid in the tropomyosin preparations from aged meat. This would have been indicative of hydrolysis of tropomyosin during aging of meat. No evidence of this nature was observed in these experiments.

The evidence for the presence of N-terminal methionine, methionine sulfoxide, and cysteic acid or serine is doubtful at best. These spots on the chromatograms are most likely due to experimental artifact. There is in the Edman procedure a critical point at which the paper strips carrying the protein and saturated with a 20% solution of phenylisothiocyanate in dioxane are removed from the reaction vessel, dried briefly, and dropped into benzene to extract excess reactants. The strips are to be dried only to the degree that they lose translucency before being dropped into the benzene. If they are not dried sufficiently some phenylthiocarbamyl-protein may be extracted by the benzene. If they are dried too thoroughly the benzene does not extract diphenyl-thiourea, a product of a side reaction (Schroeder, 1967).

An experiment was run to determine if diphenylthiourea could be detected in this system. Blank paper strips, carrying no protein or peptide, were run through the complete Edman degradation scheme. At the critical drying point the strips were dried varying times up to two minutes. Chromatograms from this experiment are shown in Figure 3. Strips carrying no protein were coded NS (no sample). Drying times before benzene extraction were 0, 30, 60, and 120 seconds for

Figure 3. Chromatograms from N-terminal amino acid analysis performed on paper strips containing no protein or peptide. The strips were dried varying times before benzene extraction. PTH-Amino acid standards and P-tropomyosin preparations were run for comparison. NS indicates "no sample." '1', '2', '3', '4' indicate drying times of 0, 30, 60, 120 seconds, respectively, before benzene extraction. Amino acids whose PTH derivatives were used are abbreviated as: ser, serine; met, methionine; met sulf, methionine sulfoxide; cysteic, cysteic acid, and arg, arginine.


Solvent A
met
arg
NS 1
NS 2
met sulf
NS 3
ser
NS 4
cysteic
Solvent A
P-0 1
NS 1
P-0 2
NS 2
P-2 1
NS 3
P-2 2
NS 4
P-9 1
P-9 2
Solvent D
met
NS 1
NS 2
met sulf
NS 3
cysteic
NS 4
arg
Solvent E
P-0 1
NS 1
P-0 2
NS 2
P-2 1
NS 3
P-2 2
NS 4
P-9 1
P-9 2

NS/1, NS/2, NS/3, and NS/4 respectively. The NS/4 preparation exhibits three primary spots as seen for all tropomyosin preparations. This is best observed in solvent A.

This evidence of experimental artifacts then raises serious doubts as to the tropomyosin preparations containing any N-terminal amino acids in more than trace amounts. While compounds responsible for the spots corresponding to PTH-methionine sulfoxide and PTH-cysteic acid or PTH-serine can only be speculated upon, the presence of these spots and the spot corresponding to PTH-methionine (most likely due to diphenylthiourea) in preparation NS/4 makes the similar spots meaningless in the results for tropomyosin preparations. It also raises the possibility that if methionine, methionine sulfoxide, cysteic acid, or serine were present as N-terminal amino acids in the tropomyosin preparations, they would not be detected due to the masking effect of spots produced by experimental artifact.

A further control experiment was carried out in which the critical drying time was eliminated for samples of the tropomyosin preparations. Only R-preparations were used and a pair of samples from each preparation was run. The first sample strip of each pair was given 60 seconds drying time at the point in question. The second sample strip was placed directly into benzene with no drying time allowed. Results are shown in Figure 4. Elimination of the drying time prior to benzene extraction failed to remove the spot which migrates as PTH-

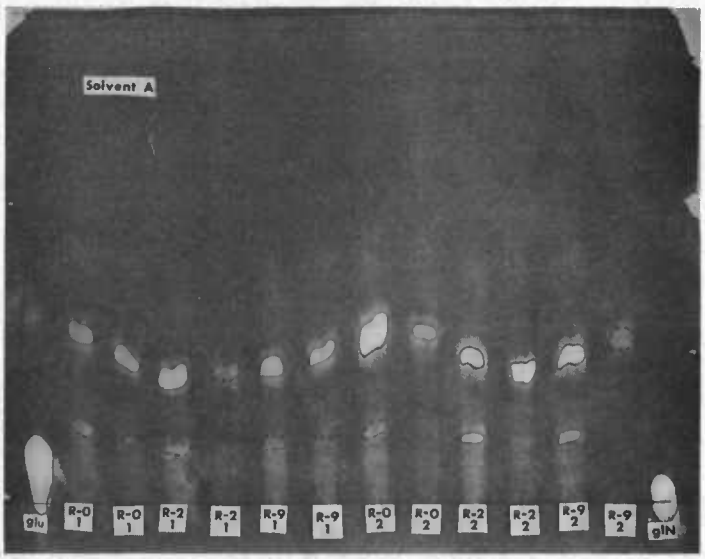


Figure 4. Chromatogram from N-terminal amino acid analysis of paired samples of R-tropomyosin preparations. Drying times before benzene extraction were 60 and 0 seconds, left and right sample respectively, for each pair shown. PTH-DL-Glutamic acid, glu, and PTH-DL-glutamine, glN, were run for comparison.

methionine or diphenylthiourea. Differences in preparations from aged meat are still not seen.

Bailey in 1951 concluded that tropomyosin is a cyclic protein since he was unable to detect any N-terminal amino acids by the method of Sanger (1945) using fluorodinitrobenzene. Since that time other workers have looked for N-terminal and C-terminal amino acids in tropomyosin and conflicting reports exist (Saad and Kominz, 1961; Jen et al., 1965; Locker, 1954; Chibnall and Spahr, 1958; Jen and Tsao, 1957; Bailer, 1951). Chibnall and Spahr (1958) confirmed Bailey's result using methoxycarbonyl chloride to label N-terminal amino acids on rabbit tropomyosin. Jen and Tsao (1957) employed fluorodinitrobenzene to look for N-terminal groups in tropomyosin from rabbit skeletal muscle, sepia mantle, duck gizzard, pig heart, and prawn body muscle. They found no N-terminal amino acids.

Locker (1954) reported isoleucine to be a C-terminal amino acid in tropomyosin as determined with carboxypeptidase. He concluded that tropomyosin is not cyclic and that the N-terminal amino acid must be buried internally, sterically hindered from reacting with fluorodinitrobenzene in view of Bailey's result (1951). Saad and Kominz (1961) reacted fluorodinitrobenzene with rabbit tropomyosin in 6 M urea and reported that glutamic acid or glutamine is N-terminal in the protein.

In the most recent work Jen et al. (1965) ran N-terminal amino

acid determinations on rabbit tropomyosin with phenylisothiocyanate -- as in the present work, fluorodinitrobenzene, and fluorodinitrobenzene with 6 M urea -- as done by Saad and Kominz (1961). No N-terminal amino acids were found (Jen et al., 1965).

Kominz et al. (1957) reported two C-terminal amino acids per molecule of tropomyosin as determined with carboxypeptidase and assuming a molecular weight of 100,000 for tropomyosin. The two C-terminal amino acids were isoleucine and serine in striated and bladder muscle and asparagine and leucine in uterus muscle.

Although the question of the cyclic or non-cyclic nature of tropomyosin is not resolved, it would appear from these reports that the expected result of the N-terminal amino acid analysis of tropomyosin preparations from 0-days aged meat would be no spots. The spots observed in this work are most likely due to experimental artifact. The important point is that no differences with aging of meat were observed.

Although the problem of experimental artifacts may have been reduced by shortening the drying time before benzene extraction, the possible loss of protein which may have contained a new N-terminal amino acid and which may have been present in low concentration at the outset dictated that the drying time could not be reduced.

Peptide Maps

Peptide maps of tryptic digests of all tropomyosin preparations are shown in Figures 5-8. These figures indicate that the reproducibility of the method was excellent. There appear to be no significant differences between tropomyosin preparations from the same extraction method and only minor differences between tropomyosin preparations from different extraction methods. Although a few of the very faint spots on some of the peptide maps were not detectable on others, no consistent differences could be attributed to the aging of meat. No peptides were lost or new peptides detected in the tryptic digests of tropomyosin preparations from meat aged two or nine days. Thus no evidence of hydrolysis of tropomyosin during meat aging was observed on the peptide maps.

Generally the peptide maps were quite similar to those reported by Carsten (1968) for human uterus, sheep uterus, rabbit skeletal, and sheep skeletal tropomyosins and to those reported by Katz and Converse (1964) for rabbit skeletal and rabbit cardiac tropomyosins. Carsten reported 35 peptides from skeletal tropomyosins while Katz and Converse reported 42. In the present work 54-60 spots were observed. The area of spots A through G was quite smeared and the designation of these spots was somewhat arbitrary. All other spots were distinct and were outlined prior to photography in order to be more easily discernible. Spots which appeared faint were outlined

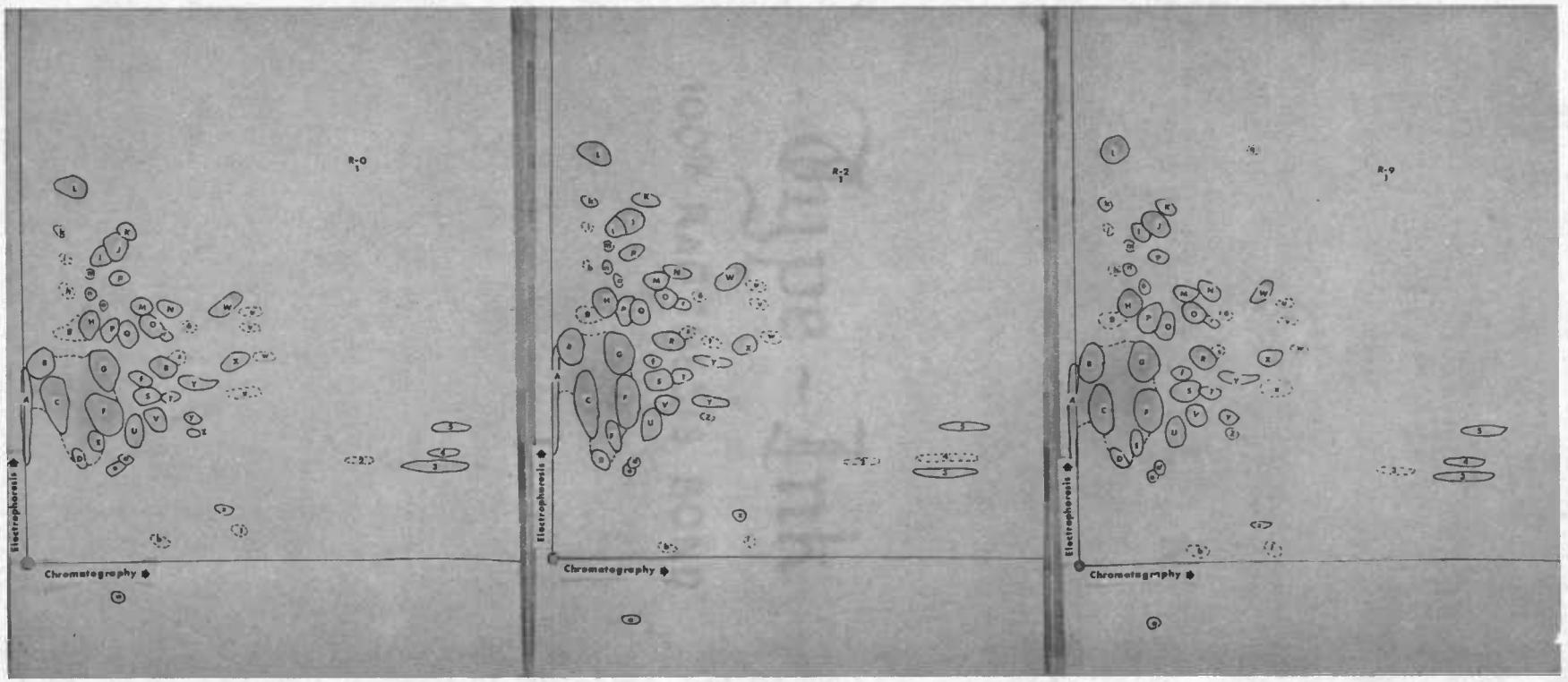


Figure 5. Peptide maps of tryptic digests of R-preparations from cow No. 1.

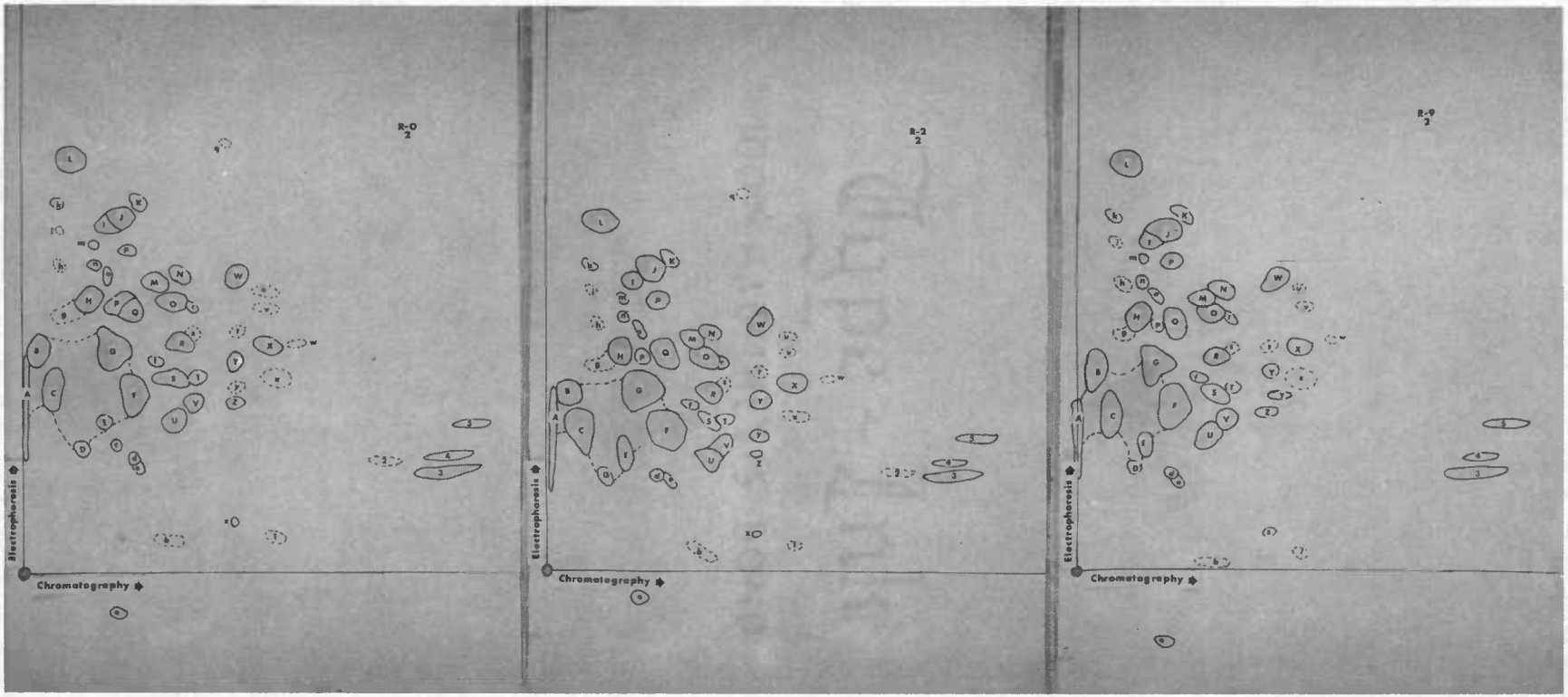


Figure 6. Peptide maps of tryptic digests of R-preparations from cow No. 2.

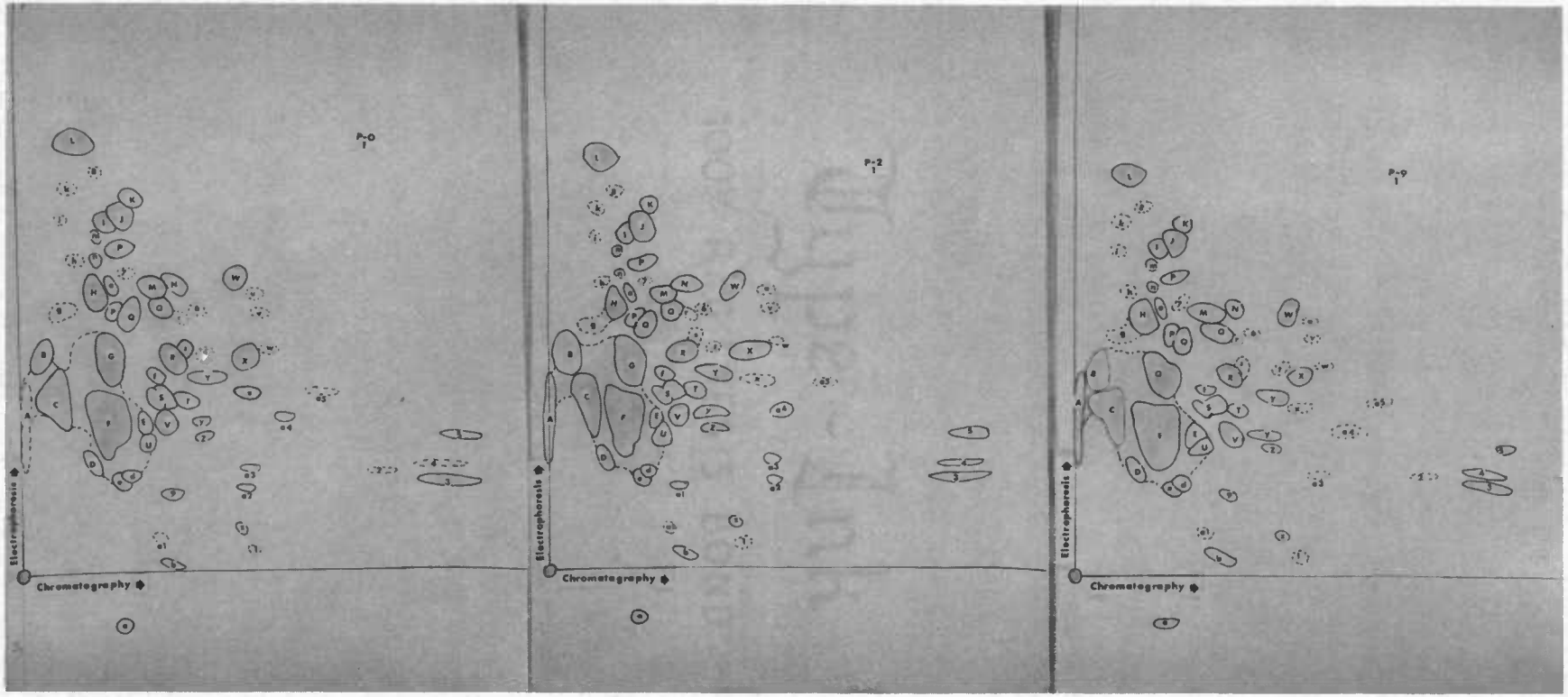


Figure 7. Peptide maps of tryptic digests of P-preparations from cow No. 1.

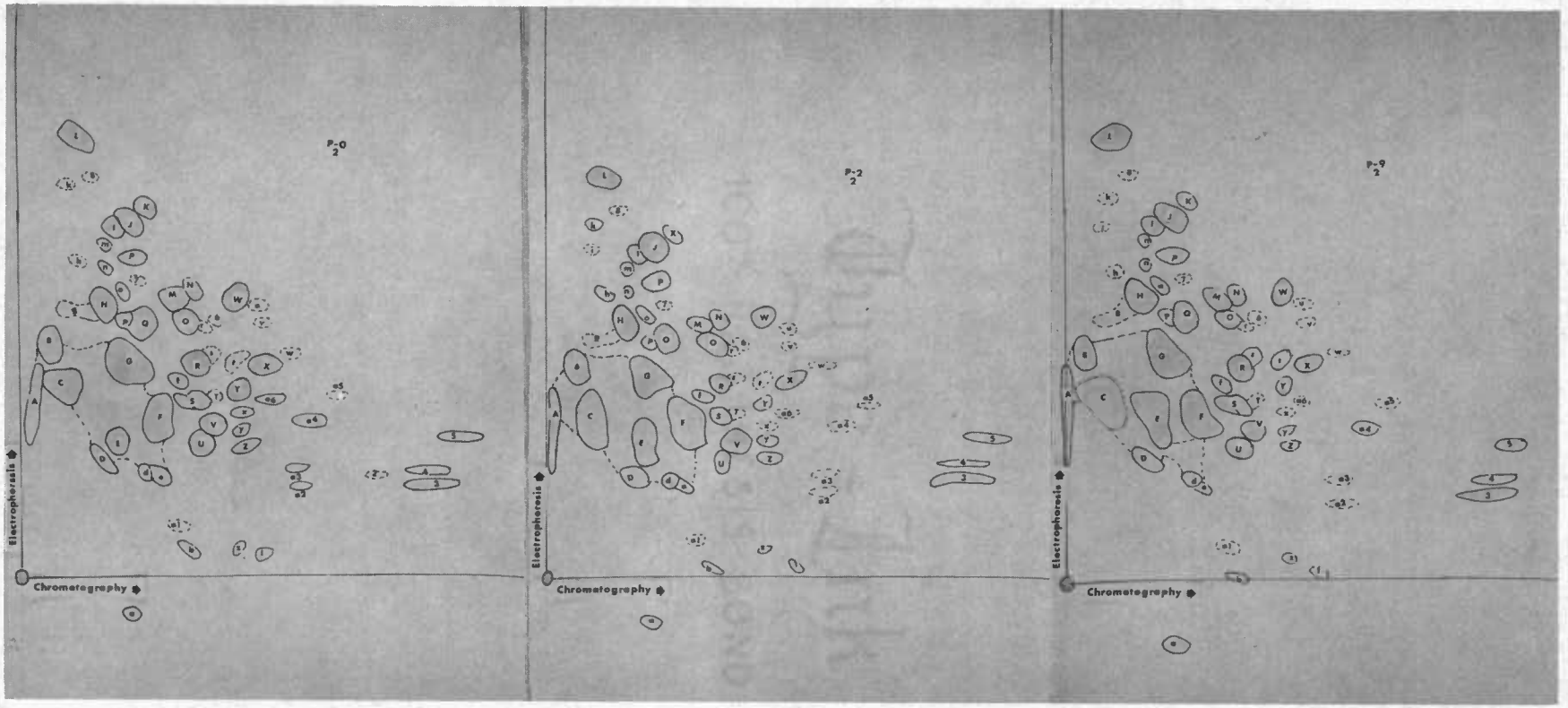


Figure 8. Peptide maps of tryptic digests of P-preparations from cow No. 2.

with a dotted line.

The remarkable similarity in peptide maps qualitatively indicates close similarity in the relative purity of tropomyosin preparations from the same extraction method. Since the purification scheme required to produce tropomyosin of a high degree of purity (Hartshorne and Mueller, 1969) was not carried through in this work, the uniformity of the tropomyosin preparations as seen on the peptide maps is noteworthy. The failure to produce "pure tropomyosin" may also have contributed to the greater number of peptides observed in this work as compared to the previous investigators (Carsten, 1968; Katz and Converse, 1964).

Katz and Converse observed two peptides which were fast moving on chromatography (1964). Carsten failed to observe these and reported that they had probably moved off the paper with the solvent front due to the length of the chromatographic run (1968). In the present work four peptides -- '2', '3', '4', and '5' -- were observed in this region of the peptide map which would correspond to the area of the two fast moving peptides of Katz and Converse.

Also of interest is the one peptide, spot 'a', found on all peptide maps in the present work, which migrated toward the anode during high-voltage electrophoresis at pH 3.7. No peptide of this nature was reported by the previous workers.

The ninhydrin spray solution used to develop the peptide maps in

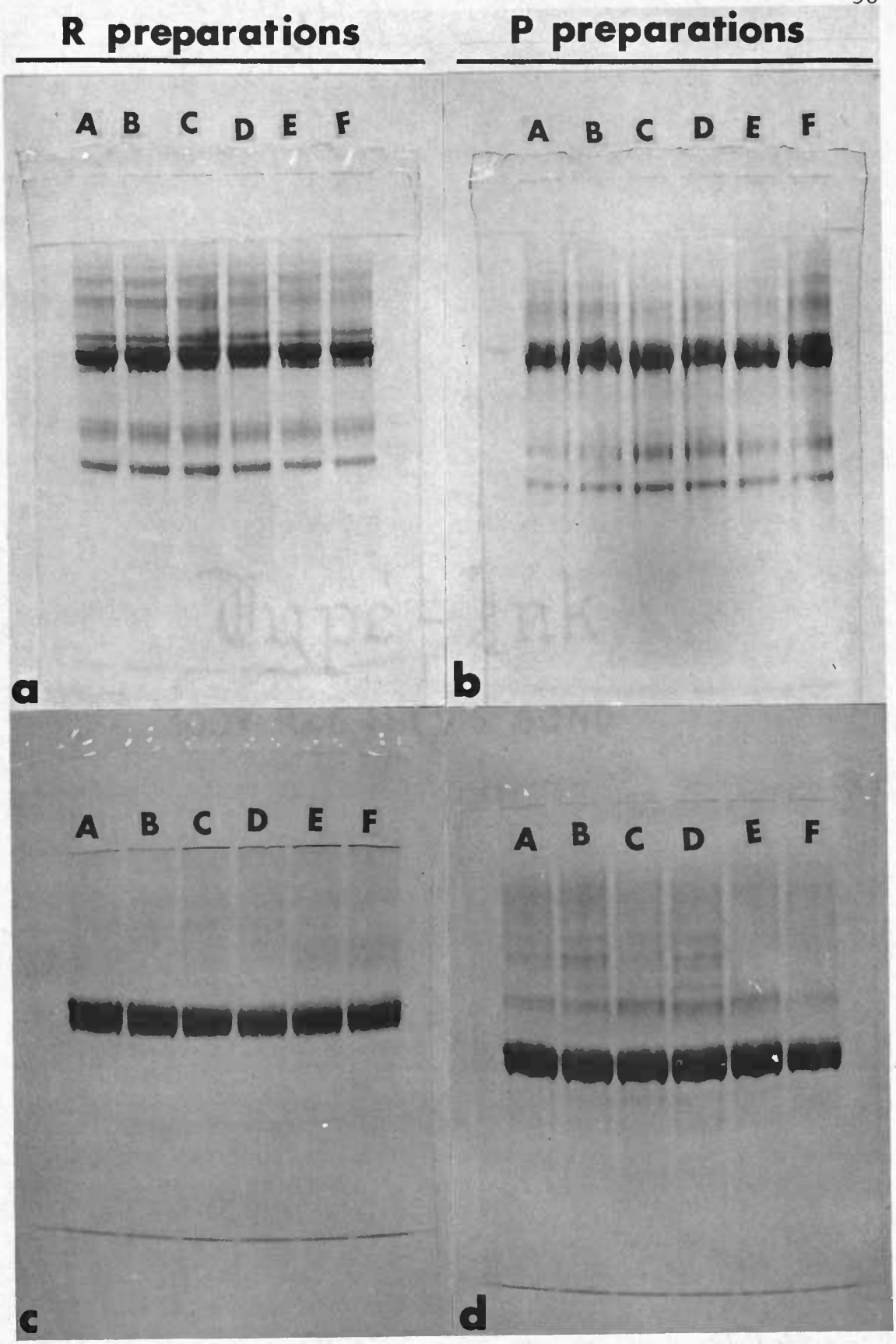
this work produced spots which varied in color from red to yellow. This enabled the detection of distinct spots which were very close together and might otherwise have been considered to be one larger spot. This may account for the greater number of peptides observed in this work as compared to that of Katz and Converse (1964) and Carsten (1968).

The variation in color of spots also enabled detection of a difference between preparations from the two animals. Spot 'd' appeared yellow in all preparations while spot 'e' was orange. Spot 'e' was below and to the left of spot 'd' in all preparations from cow No. 1 and was below and to the right of spot 'd' in all preparations from cow No. 2. This may imply a genetic difference in the amino acid composition of one of these peptides. What that difference is cannot be determined from these data.

Polyacrylamide-Gel Electrophoresis

Patterns obtained from polyacrylamide-gel electrophoresis of the tropomyosin preparations are shown in Figure 9. The patterns shown in Figure 9 a and b resulted when β-mercaptoethanol was deleted from the system. The patterns shown in Figure 9 c and d resulted when 2 m*M* β-mercaptoethanol was added to all solutions used in the electrophoresis. Tris-glycine electrode buffer, pH 8.65, with 5 *M* urea was used for all the patterns shown in Figure 9.

Figure 9. (a and b) Polyacrylamide-gel electrophoresis patterns of tropomyosin preparations obtained with a running buffer consisting of 5 M urea - 0.0165 M Tris - 0.039 M glycine, pH 8.65. (c and d) Polyacrylamide-gel electrophoresis of the same preparations in the same buffer with 2 mM β-mercaptoethanol added to the system. Letters designate meat aging time and animal from which the sample was obtained: A, 0-days aging, cow No. 1; B, 0-days aging, cow No. 2; C, 2-days aging, cow No. 1; D, 2-days aging, cow No. 2; E, 9-days aging, cow No. 1; F, 9-days aging, cow No. 2.


R preparations
P preparations
A B C D E F
a
A B C D E F
b
A B C D E F
c
A B C D E F
d

No differences between tropomyosin preparations from either extraction method (R or P) are detectable by electrophoresis in the absence of β-mercaptoethanol (Figure 9 a, b). Some differences in protein concentration in the lighter bands can be seen in patterns obtained with 2 mM β-mercaptoethanol added to the system (Figure 9 c, d). Although these differences in protein concentration in the lighter bands are easily observable, it is important to note that few, if any, of the bands seen in one preparation from either extraction method can be considered to be completely missing in any other preparation from the same extraction method. Some bands, particularly in preparations P-9/1 and P-9/2, do not show well in the photograph but were detectable in the gel when it was illuminated from below by an uniform, diffuse light.

The appearance of a new protein band in the electrophoresis pattern of tropomyosin preparations from aged meat or the loss of a band which was present in the electrophoresis pattern of 0-day tropomyosin preparations would have been evidence for the hydrolysis of tropomyosin with aging of meat. Hydrolytic cleavage of the tropomyosin molecule could be expected to produce tropomyosin fragments which would migrate at a rate different from that of tropomyosin during polyacrylamide-gel electrophoresis since the net charge and size of these fragments would most probably differ from the net charge and size of the tropomyosin molecule. The band or bands formed in the gel by

tropomyosin would be expected to be eliminated or reduced in intensity due to the elimination or reduction in concentration of the intact molecule. Although some evidence of the latter nature is seen for preparations P-9/1 and P-9/2 in Figure 9 d, it was the opinion of Dr. J. H. Rampton (1970), who has had some experience with the use of sulfhydryl protecting agents in the electrophoresis of tropomyosin, that the lighter bands seen in Figure 9 c and d are due to incomplete reduction by β-mercaptoethanol of oxidized, disulfide-linked forms of tropomyosin. Loss of the lighter bands in preparations P-9/1 and P-9/2 (Figure 9 d) is due to a more complete reduction of the oxidized forms of tropomyosin in these preparations. In light of his experience it was concluded that these experiments show no evidence of the hydrolysis of tropomyosin during aging of meat.

It is noteworthy that the heavy bands observed in Figure 9 c and d had a faster migration rate than the heavy bands observed in Figure 9 a and b. This is further evidence for reduction of disulfide-linked forms of tropomyosin by β-mercaptoethanol to yield a lower molecular weight form. Therefore, the dark bands observed in Figure 9 c and d may be due to dissociation of tropomyosin into subunits in 5 M urea-2 mM β-mercaptoethanol (Woods, 1967; McCubbin et al., 1967).

Oxidation artifacts have been reported in polyacrylamide-gel electrophoresis in gels polymerized by ammonium persulfate as was done in this work (Fantes and Furminger, 1967; Brewer, 1967). In

the disc electrophoresis of enolase in polyacrylamide gels polymerized by ammonium persulfate, Brewer (1967) was able to reduce two characteristic bands to one by adding thioglycolate to the system.

A beneficial effect of thioglycolate has been reported by Woods in the characterization of tropomyosin by disc electrophoresis in 7.5% polyacrylamide gel with a Tris-glycine buffer, pH 9.5, 8 M urea added (1967). He observed two bands for each of six tropomyosin preparations pre-treated with N-ethyl maleimide, though the slower moving band was very faint in five of these. When these tropomyosin preparations were treated with 0.01 M thioglycolic acid and 1 mM thioglycolic acid was added to the electrophoresis buffer system one band resulted. Woods concluded that the slower moving bands were due to disulfide-bonded species (1967).

Many workers have observed multiple bands on electrophoresis patterns of tropomyosin (Rampton, 1969; Parsons et al., 1969; Carsten, 1968; Woods, 1967; Arai and Watanabe, 1968b; Yasui et al., 1968; Arai and Watanabe, 1968a; Rampton, 1970). Reduction of bands on electrophoresis patterns of tropomyosin by the addition of a sulfhydryl protecting agent to the system has also been reported by several investigators (Parsons et al., 1969; Woods, 1967; Yasui et al., 1968; Rampton, 1970). Parsons et al. (1969) reported ten peaks in densitometer tracings (by laser beam) of starch-gel electrophoresis patterns in 8 M urea of tropomyosins from the longissimus dorsi and

psoas major muscles of the pig. Nine, nine, and seven peaks were observed in similar tracings for tropomyosins from the longissimus dorsi of sheep, ox, and rabbit, respectively, suggesting species differences. The extraction of tropomyosin from the longissimus dorsi with 8 M urea containing 2% β-mercaptoethanol reduced the number of peaks in the tracings to two for pig, sheep, and ox preparations and one for the rabbit preparation (Parsons et al., 1969).

In the work of Arai and Watanabe (1968a) disc electrophoresis in 7% polyacrylamide gel using 5 M urea in a Tris-glycine buffer, pH 8.4, produced five bands from tropomyosin. Addition of 1 mM dithiothreitol to their system reduced the number of bands to two (1968b). Also reported were two bands from tropomyosin when both urea and dithiothreitol were left out of their system (1968a). In the present electrophoresis experiments tropomyosin would not penetrate the 4% polyacrylamide gel when urea was deleted from the system. The observed high viscosity of the tropomyosin preparations prior to urea addition suggests that in the absence of urea the protein forms an aggregate too large to penetrate the porous gel. Aggregation of tropomyosin in low ionic strength solutions is well known (McCubbin et al., 1967; McCubbin and Kay, 1969).

Yasui et al. (1968) employed disc electrophoresis in 6% polyacrylamide gel with 4 M urea in diethylbarbituric acid buffer, pH 7.5, to characterize tropomyosin. From tropomyosin prepared by

the method of Mueller (1966) but deleting the sulfhydryl-protecting agent (dithiothreitol) two bands were observed in the electrophoresis pattern. Tropomyosin prepared by the same method with 0.5 mM dithiothreitol in all extraction media gave three bands in the electrophoresis pattern. The third band had a migration rate which corresponded to that of troponin (Yasui et al., 1968). No difference in the two characteristic bands from tropomyosin was observed with the addition of dithiothreitol to the extraction media. Tropomyosin prepared by the method of Yasui et al. (1968) with 0.5 mM dithiothreitol in all extraction media gave four bands in the electrophoresis pattern which could be reduced to two by allowing the protein to stand in 1 M KCl containing 0.1 M dithiothreitol for 40 hours in the cold (Yasui et al., 1968).

Carsten (1968) used disc electrophoresis in polyacrylamide gel with 4 M urea in Tris-glycine buffer, pH 8.1, to characterize tropomyosin preparations. Rabbit skeletal tropomyosin and human uterus tropomyosin gave two similar bands in the electrophoresis pattern. Sheep uterus tropomyosin gave three bands, none of which had a migration rate corresponding to that of troponin (Carsten, 1968).

Rampton (1969) employed disc electrophoresis in 6.5% polyacrylamide gel with 7 M urea in a Tris-glycine buffer to characterize tropomyosin prepared by four different methods. Two or three characteristic bands were reported for the different preparations.

In more recent work, however, he indicated that only one band would result if care was taken to protect the tropomyosin sulfhydryl groups from oxidation during electrophoresis by adding dithiothreitol to the system (1970).

Viscometric Analyses

Results of viscometric analyses of tropomyosin preparations are shown in Figures 10-17. Figures 10-13 show the analyses of tropomyosin preparations in 0.039 M borate buffer, pH 7.1, $\mu = 0.003$. The non-linearity of the graphs of reduced viscosity versus concentration is most likely due to aggregation of tropomyosin with increasing protein concentration in the low ionic strength buffer (Jirgensons and Straumanis, 1954; Van Holde, 1970; Dyson, 1970). The aggregation is probably an end-to-end polymerization of the rod shaped tropomyosin molecules, increasing the axial ratio in the aggregate and thereby causing a pronounced viscosity increase (Tsao et al., 1951; Van Holde, 1970; Jirgensons and Straumanis, 1954; Tanford, 1965). The anomalous viscosity of tropomyosin is well known and can be enhanced by troponin contamination in tropomyosin preparations (Ebashi and Kodama, 1965; Ebashi and Kodama, 1966).

Polymerization of tropomyosin in neutral solvents has been reported by several investigators (Iida and Ooi, 1967; Mueller, 1966; McCubbin and Kay, 1969). Increase in the asymmetry of tropomyosin

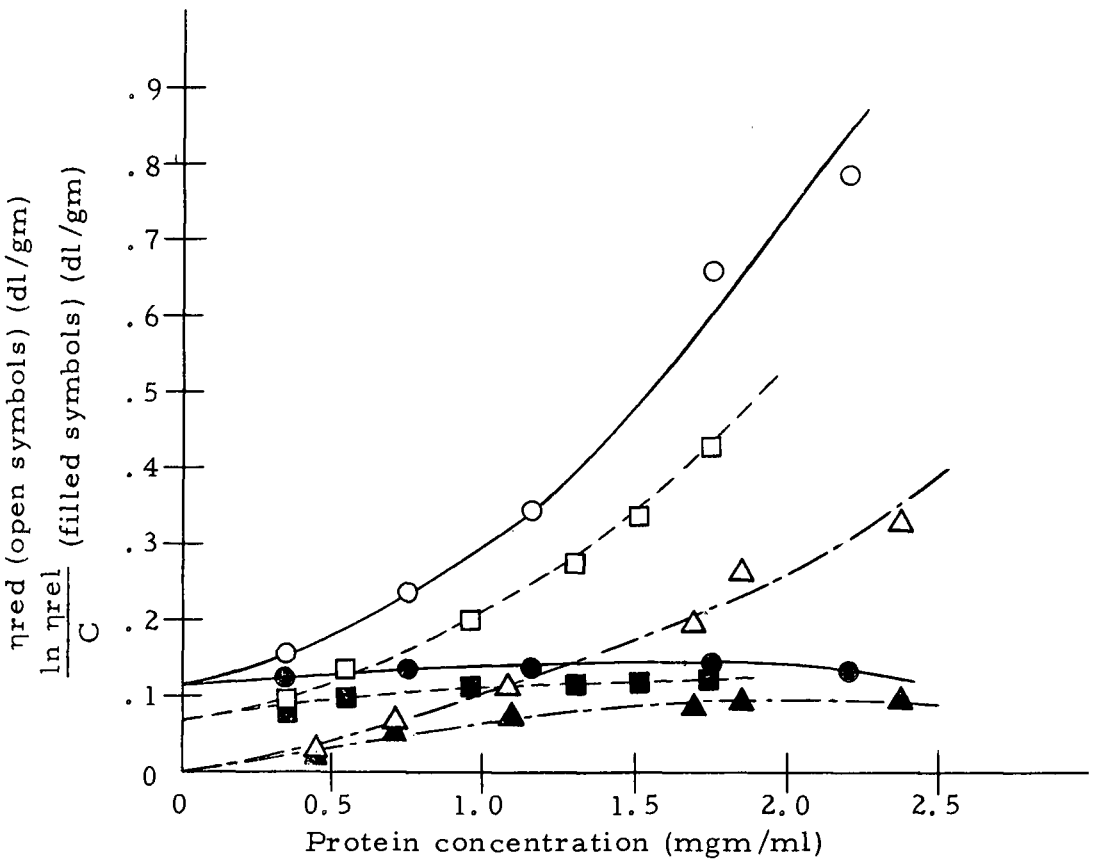


Figure 10. Plot of the reduced viscosity versus protein concentration (open symbols) and of the natural logarithm of the relative viscosity divided by protein concentration versus protein concentration (filled symbols) from viscometric analyses of tropomyosin preparations R-0/1 (circles), R-2/1 (squares), and R-9/1 (triangles) in 0.039 M borate, pH 7.1.

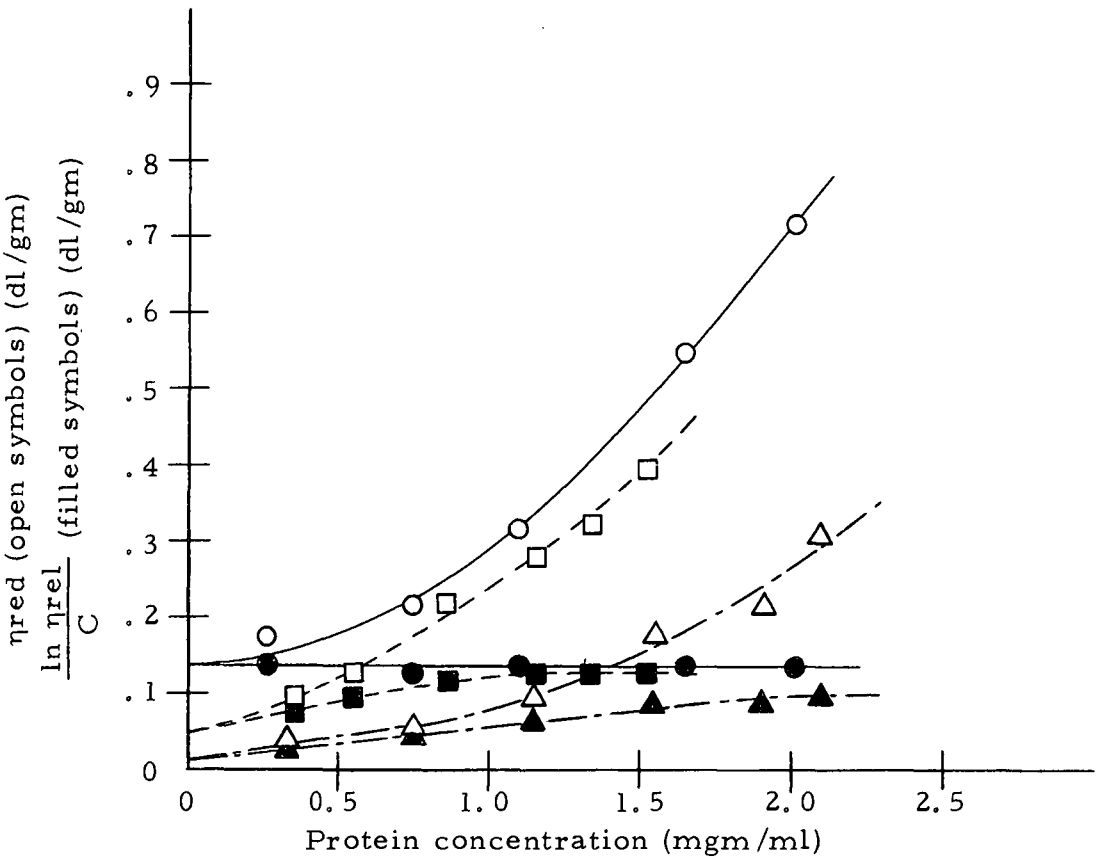


Figure 11. Plot of the reduced viscosity versus protein concentration (open symbols) and of the natural logarithm of the relative viscosity divided by protein concentration versus protein concentration (filled symbols) from viscometric analyses of tropomyosin preparations R-0/2 (circles), R-2/2 (squares), and R-9/2 (triangles) in 0.039 M borate, pH 7.1.

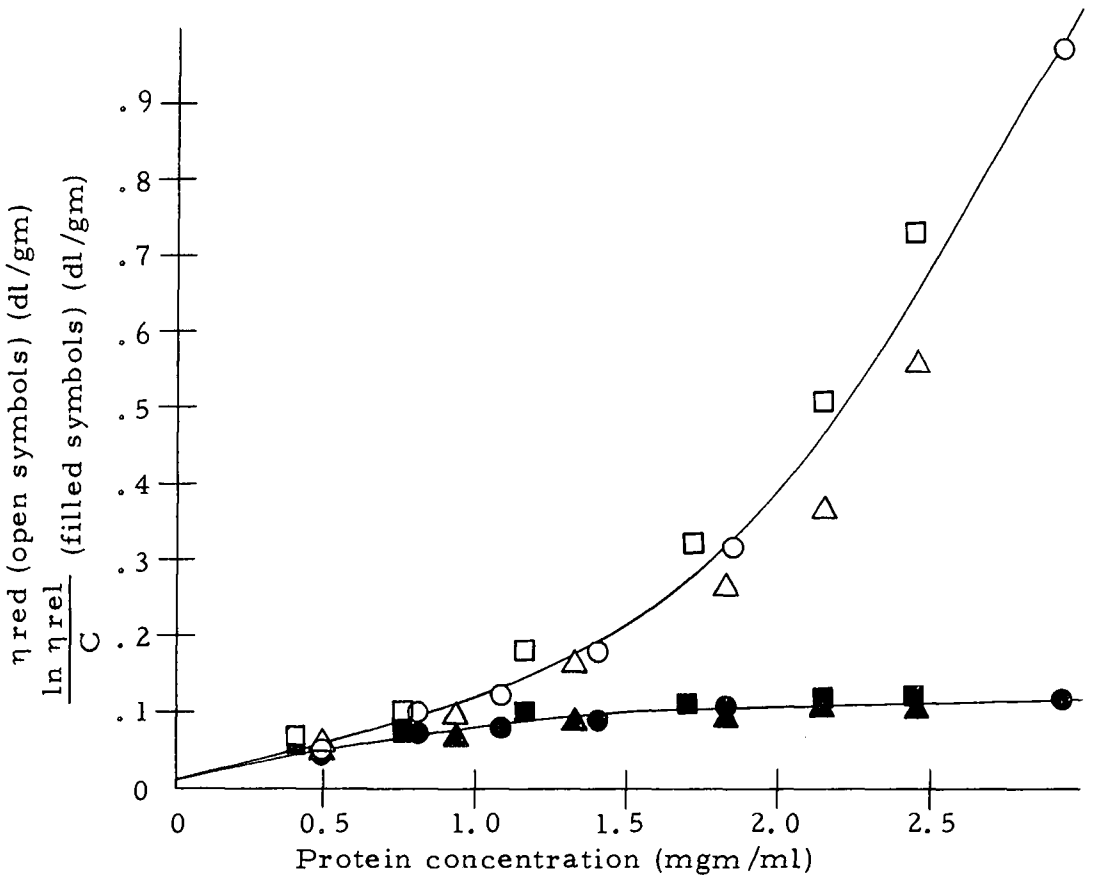


Figure 12. Plot of the reduced viscosity versus protein concentration (open symbols) and of the natural logarithm of the relative viscosity divided by protein concentration versus protein concentration (filled symbols) from viscometric analyses of tropomyosin preparations P-0/1 (circles), P-2/1 (squares), and P-9/1 (triangles) in 0.039 M borate, pH 7.1.

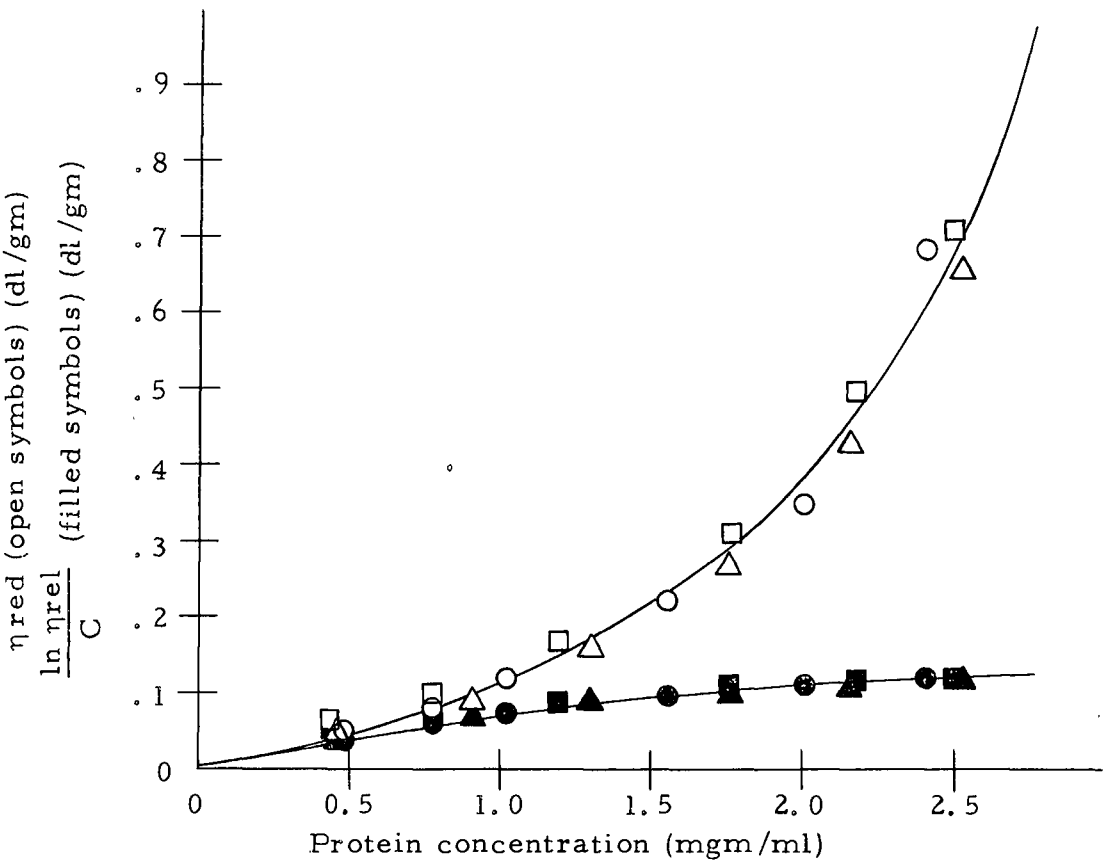


Figure 13. Plot of the reduced viscosity versus protein concentration (open symbols) and of the natural logarithm of the relative viscosity divided by protein concentration versus protein concentration (filled symbols) from viscometric analyses of tropomyosin preparations P-0/1 (circles), P-2/1 (squares), and P-9/1 (triangles) in 0.039 M borate, pH 7.1.

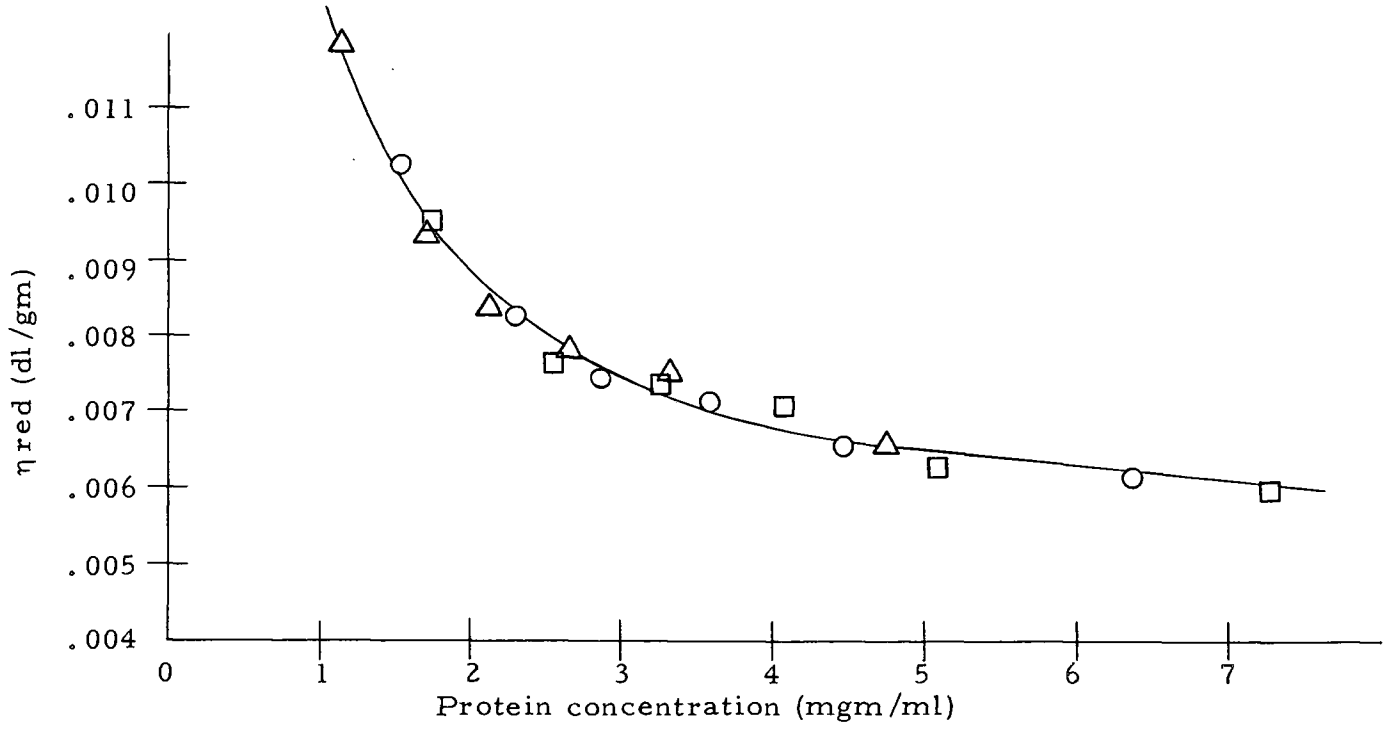


Figure 14. Plot of the reduced viscosity versus protein concentration from viscometric analyses of tropomyosin preparations R-0/1 (circles), R-2/1 (squares), and R-9/1 (triangles) in 5 M urea-0.0165 M Tris-0.039 M glycine-2 mM β-mercaptoethanol, pH 8.65.

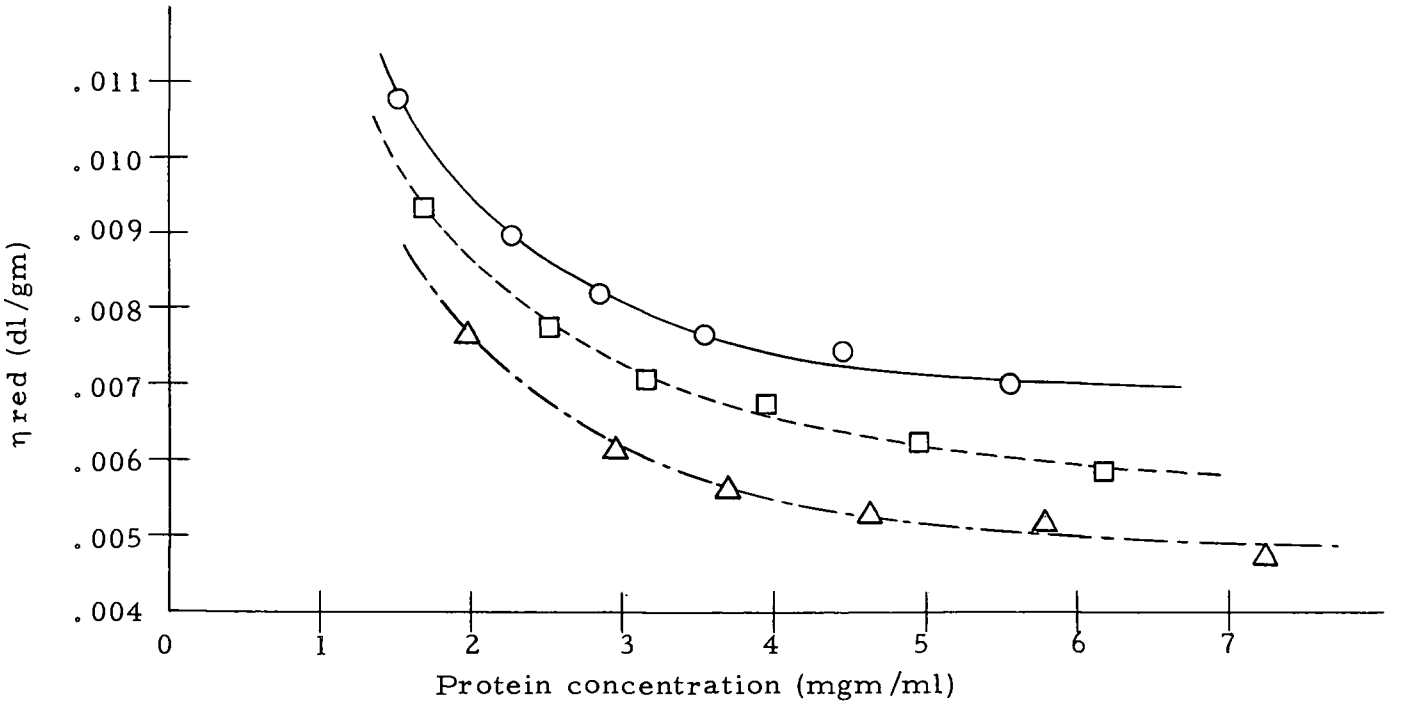


Figure 15. Plot of the reduced viscosity versus protein concentration from viscometric analyses of tropomyosin preparations R-0/2 (circles), R-2/2 (squares), and R-9/2 (triangles) in 5 M urea-0.0165 M Tris-0.039 M glycine-2 mM β-mercaptoethanol, pH 8.65.

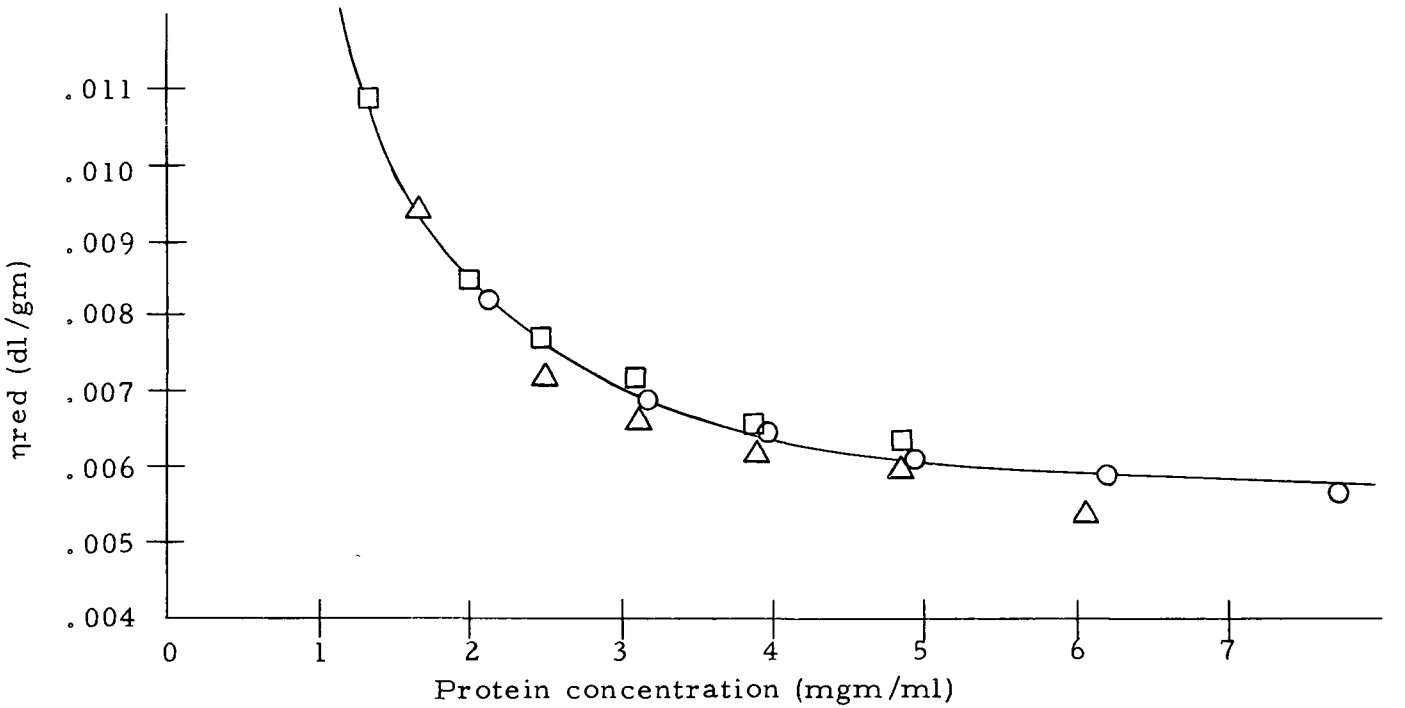


Figure 16. Plot of the reduced viscosity versus protein concentration from viscometric analyses of tropomyosin preparations P-0/1 (circles), P-2/1 (squares), and P-9/1 (triangles) in 5 M urea-0.0165 M Tris-0.039 M glycine-2 mM β-mercaptoethanol, pH 8.65.

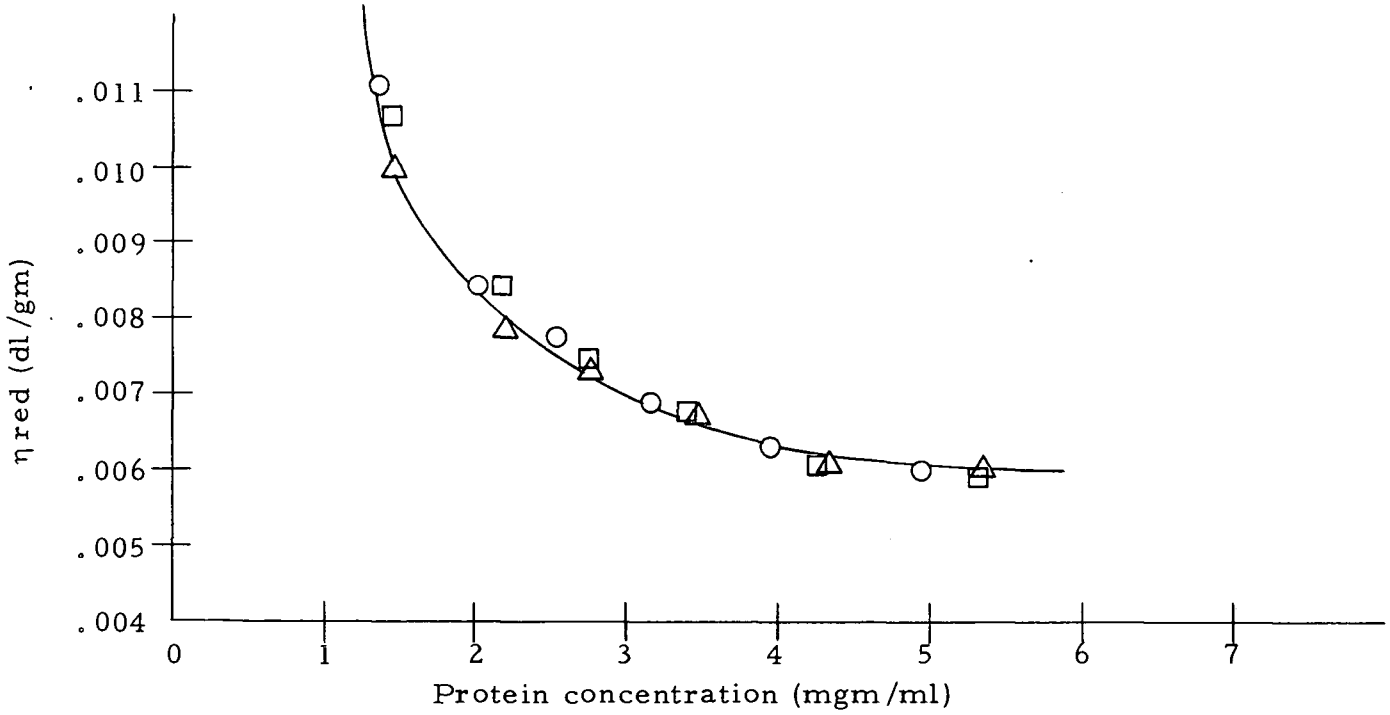


Figure 17. Plot of the reduced viscosity versus protein concentration from viscometric analyses of tropomyosin preparations P-0/2 (circles), P-2/2 (squares), and P-9/2 (triangles) in 5 M urea-0.0165 M Tris-0.039 M glycine-2 mM β-mercaptoethanol, pH 8.65.

aggregates has been interpreted as indicative of end-to-end polymerization (Tsao et al., 1951). McCubbin and Kay observed a linear aggregation effect for tropomyosin on graphs of reduced viscosity versus concentration in neutral solvents (1969). In their experiments the ionic strength was varied from 0.1 to 1.1. Constancy of their optical rotatory dispersion data suggested that the aggregation must be an end-to-end polymerization and that high ionic strength neutral solvents do not disrupt the helical content of the molecule. Helical content is in excess of 90% (McCubbin and Key, 1969). The axial ratio of tropomyosin at 25% hydration has been reported to decrease from 45 to 26 as the ionic strength increased from 0.1 to 1.1 at pH 6.5 (Tsao et al., 1951).

The curvature seen in the graphs of reduced viscosity versus concentration (Figures 10-13) was particularly undesirable here since the immediate objective of these analyses was to obtain the intrinsic viscosity or limiting viscosity number. The limiting viscosity number is obtained by extrapolation of the reduced viscosity versus concentration curve to zero protein concentration (Yang, 1961; Kragh, 1961; Tanford, 1965). The limiting viscosity number is directly related to the average molecular weight of the solute particles (Yang, 1961; Jirgensons and Straumanis, 1954; Kragh, 1961). Therefore, a reduction in the limiting viscosity number for tropomyosin preparations from aged meat could be evidence of hydrolysis of tropomyosin

during meat aging.

The limiting viscosity number is usually obtained by regression analysis of linear data on a reduced viscosity versus concentration plot to locate the best straight line through the data points. This line is then extrapolated to zero protein concentration to obtain the limiting viscosity number. Since extrapolation of a curved line is difficult, a plot of the natural logarithm of the relative viscosity divided by concentration versus concentration was made on the same graph. This plot can also be extrapolated to zero protein concentration to determine the limiting viscosity number (Yang, 1961; Kragh, 1961). Therefore, the two curved lines obtained for each tropomyosin preparation (Figures 10-13) should meet at zero concentration to give a good estimate of the limiting viscosity number (Van Holde, 1970).

The results obtained for P-preparations (Figures 12, 13) indicate no differences in tropomyosin preparations as meat aging time increased. The aggregation phenomenon is evident. The results obtained for R-preparations (Figures 10, 11) show marked differences in preparations from aged meat. A definite reduction in the tendency to form aggregates is observed for the preparations as meat aging time increased. A reduction in the limiting viscosity number as meat aging time increased is also apparent but may be a further manifestation of the differences in aggregating properties and cannot be interpreted as evidence of hydrolysis of tropomyosin.

The reduction in limiting viscosity number observed for R-preparations as aging progressed correlates well with the observed increase in actomyosin contamination (Table 2) and decrease in nucleotide contamination (Table 3). This suggests a possible steric hindrance to tropomyosin aggregation by the actomyosin. However, failure to observe a similar correlation for the P-preparations (Figures 12, 13 and Tables 2, 3) would tend to argue against this proposal.

Factors which may affect the aggregation of tropomyosin are the extent of oxidation of sulfhydryl groups on the protein and the presence of another protein, e. g., troponin, which could promote the aggregation (Woods, 1967; Ebashi and Kodama, 1965; Ebashi and Kodama, 1966). Differences in the two extraction procedures could easily explain the differences observed between R-preparations and P-preparations. Although sulfydryl protecting agents were present in all solutions used up to the final dialysis in both procedures there are points in both procedures at which oxidation of sulfhydryl groups could have occurred. The removal of ether by evaporation to near dryness is one such point. The tropomyosin preparations were not carried through to a high level of purity and variation between the two extraction procedures in the amount of troponin contamination is possible.

To eliminate the aggregation problem and the possible oxidation

of sulfhydryl groups during the analyses, viscosity determinations were also performed in a solvent containing 5 M urea-2 mM β-mercaptoethanol. The solvent employed was the electrode buffer used for polyacrylamide-gel electrophoresis (5 M urea-0.0165 M Tris-0.039 M glycine-2 mM β-mercaptoethanol, pH 8.65, μ=0.0135). Results of these experiments are shown in Figures 14-17.

It is of primary importance to note that the reduced viscosity coordinate has been greatly expanded in these graphs in order to show the curvature in the plots which would not be detectable on a conventional scale. The reproducibility of the data suggests that the observed curvature is real and noteworthy. Curvature of the type seen in Figures 14-17 is known to occur in associating systems in which the association of macromolecules leads to a more nearly spherical aggregate. The resultant reduction in axial ratio of solute particles is evidenced as a viscosity decrease (Jirgensons and Straumanis, 1954; Van Holde, 1970; Dyson, 1970).

In either 6.67 M urea-0.1 M β-mercaptoethanol or 6 M guinidine hydrochloride-0.1 M β-mercaptoethanol tropomyosin is known to dissociate into subunits of molecular weight approximately 34,000 (McCubbin et al., 1967; Woods, 1967). Optical rotatory dispersion data indicate that the helical content is reduced to about 5% in 6.67 M urea-0.1 M β-mercaptoethanol. This was interpreted to indicate the subunits were in the random coil form (McCubbin et al., 1967).

In 6.67 M urea-0.075 M phosphate-0.2 M KCl, pH 6.5, μ=0.3, the axial ratio of tropomyosin at 25% hydration was reported to be 25 (Tsao et al., 1951). Therefore, an ordered array for the molecule in 6.67 M urea seems apparent.

It is difficult to predict the helical content of tropomyosin under conditions employed in the present viscometric analyses. It is quite likely, however, that the molecule has been dissociated into subunits in 5 M urea. These subunits are thought to associate side-to-side in the native molecule (McCubbin et al., 1967). A concentration dependent association of these subunits to form an aggregate with a reduced axial ratio would explain the observed curvature in plots in Figures 14-17 (Van Holde, 1970; Dyson, 1970).

With the exception of the R-preparations from cow No. 2 (Figure 15), no differences were seen in preparations from aged beef in these data. The small differences observed in Figure 15 may be a manifestation of the differences in aggregation tendency observed in borate buffer (Figures 10, 11). The reason for the discrepancy in results from cow No. 1 and cow No. 2 (Figures 14, 15) is unknown.

It was anticipated that linear plots would be obtained from viscometric analyses of the tropomyosin preparations in 5 M urea. Extrapolation in the usual manner to obtain the limiting viscosity number was the immediate objective. A lower limiting viscosity number for preparations from aged meat would have indicated a lower

average molecular weight and would have been evidence of hydrolysis of tropomyosin during aging of meat. Evidence of this nature was not observed in these analyses.

SUMMARY AND CONCLUSIONS

Studies of the possible hydrolysis of tropomyosin during aging of psoas major muscle of beef were carried out. Tropomyosin was prepared by direct extraction from meat and by extraction of initially prepared myofibrils. Extractions were performed at zero, two, and nine days of aging at 4°C. N-Terminal amino acid analysis, peptide maps of tryptic digests, polyacrylamide-gel electrophoresis, and viscometric analysis were employed to detect hydrolytic changes. Comparisons of actomyosin and nucleotide contamination in the preparations were also made. No evidence of hydrolysis of tropomyosin during aging of beef was observed.

N-Terminal amino acid analysis showed three characteristic spots for all tropomyosin preparations on starched-paper chromatograms. These spots were attributed to an experimental artifact and it was concluded that N-terminal amino acids were not present in the preparations in significant amounts.

Peptide maps of tryptic digests revealed 54-60 spots, some perhaps originating in contamination in the preparations. No significant differences were seen in peptide maps as meat aging time increased. In fact, a remarkable similarity in the peptide maps qualitatively implied a high degree of similarity in the relative purity of the preparations.

Polyacrylamide-gel electrophoresis patterns exhibited no new

bands or loss of bands as meat aging progressed. The influence of a sulfhydryl-protecting agent was dramatically illustrated as the addition of 2 mM β-mercaptoethanol to the system resulted in one major band whereas many were observed in the absence of β-mercaptoethanol. The heavy band observed in the patterns obtained with β-mercaptoethanol had a faster migration rate than the darkest band in patterns developed without β-mercaptoethanol.

Viscometric analysis showed a pronounced concentration-dependent aggregation of tropomyosin in 0.039 M borate, pH 7.1. No differences between preparations from myofibril extraction were observed. Preparations from direct extraction of meat exhibited a reduction in aggregation tendency as meat aging time increased. A reduction in limiting viscosity number was also apparent but may have been a further manifestation of reduced aggregation and cannot be interpreted as indicating a reduction in average molecular weight. A reduction in reduced viscosity with increase in protein concentration was observed for all preparations in 5 M urea-0.0165 M Tris-0.039 M glycine-2 mM β-mercaptoethanol, pH 8.65. This suggested that aggregation resulted in the formation of a particle of lower asymmetry under these conditions.

Actomyosin content increased with aging time in preparations from direct extraction of meat. Nucleotide content decreased with aging in these preparations. The increased actomysin content and decreased

nucleotide content may play an undefined role in the observed reduction in aggregation tendency.

Actomyosin content was maximum and nucleotide content was minimum in the 2-day preparations of tropomyosin obtained from myofibril extraction. The differences observed with aging were less pronounced than for the preparations obtained from direct meat extraction.

BIBLIOGRAPHY

Arai, K. and S. Watanabe. 1968a. Troponin, tropomyosin, and the relaxing protein; myofibrilar proteins of rabbit skeletal muscle. Journal of Biochemistry 64:69-72.

_____ 1968b. A study of troponin, a myofibrillar protein from rabbit skeletal muscle. Journal of Biological Chemistry 243:670-5678.

Bailey, J. L. Techniques in protein chemistry. Amsterdam, Elsevier. 310 p.

Bailey, K. 1948. Tropomyosin: A new asymmetric protein component of the muscle fibril. Biochemical Journal 43:271-278.

_____ 1951. End-group assay in some proteins of the keratin-myosin group. Biochemical Journal 49:23-27.

Bendall, J. R. 1964. Meat proteins. In: Symposium on Foods: Proteins and Their Reactions, ed. by H. W. Schultz and A. F. Anglemier. Westport, Avi. p. 225-254.

Bodwell, C. E. and A. M. Pearson. 1964. The activity of partially purified bovine catheptic enzymes on various natural and synthetic substrates. Journal of Food Science 29:602-607.

Bodwell, C. E., A. M. Pearson and M. E. Spooner. 1965. Post-mortem changes in muscle. I. Chemical changes in beef. Journal of Food Science 30:766-772.

Brewer, J. M. 1967. Artifact produced in disc electrophoresis by ammonium persulfate. Science 156:256-257.

Briskey, E. J. 1967. Myofibrillar proteins of skeletal muscle. In: Proceedings of the Meat Industry Research Conference. Chicago, American Meat Institute Foundation. p. 1-28.

Carsten, M. E. 1968. Tropomyosin from smooth muscle of the uterus. Biochemistry 7:960-967.

Caspar, D. L. D., C. Cohen and W. Longley. 1969. Tropomyosin: Crystal structure, polymorphism and molecular interactions. Journal of Molecular Biology 41:87-107.

Chibnall, A. C. and P. R. Spahr. 1958. Determination of the N-terminal residues in proteins with methoxycarbonyl chloride. Biochemical Journal 68:135-142.

Cohen, C. and K. C. Holmes. 1963. X-ray diffraction evidence for α-helical coiled-coils in native muscle. Journal of Molecular Biology 6:423-432.

Davey, C. L. and K. V. Gilbert. 1966. Studies in meat tenderness. II. Proteolysis and the aging of beef. Journal of Food Science 31:135-140.

_____ 1968a. Studies in meat tenderness. 4. Changes in the extractability of myofibrillar proteins during meat aging. Journal of Food Science 33:2-7.

_____ 1968b. Studies in meat tenderness. 6. The nature of myofibrillar proteins extracted from meat during aging. Journal of Food Science 33:343-348.

_____ 1969. Studies in meat tenderness. 7. Changes in the fine structure of meat during aging. Journal of Food Science 34:69-74.

Davies, R. E. 1967. Recent theories on the mechanism of muscle contraction and rigor mortis. In: Proceedings of the Meat Industry Research Conference. Chicago, American Meat Institute Foundation. p. 39-53.

Disney, J. G., M. J. Follet and P. W. Ratcliff. 1967. Biochemical changes in beef muscle post mortem and the effect of rapid cooling in ice. Journal of the Science of Food and Agriculture 18:314-321.

Drabikowski, W. and J. Gergely. 1962. The effect of temperature of extraction on the tropomyosin content in actin. Journal of Biological Chemistry 237:3412-3417.

Dyson, R. D. 1970. Assistant Professor, Oregon State University, Dept. of Biochemistry and Biophysics. Personal communication. Corvallis, Oregon. January 23.

Ebashi, S. and A. Kodama. 1965. A new protein factor promoting aggregation of tropomyosin. Journal of Biochemistry 58:107-108.

Ebashi, S. and A. Kodama. 1966. Interaction of troponin with F-actin in the presence of tropomyosin. Journal of Biochemistry 59:425-426.

E-C Apparatus Corporation. 1966. Electrophoresis and countercurrent. Philadelphia. n. p. (E-C Bulletin Vol. II, No. 1).

Endo, M., Y. Nonomura, T. Masaki, I. Ohtsuki and S. Ebashi. 1966. Localization of native tropomyosin in relation to striation patterns. Journal of Biochemistry 60:605-608.

Fantes, K. H. and I. G. S. Furminger. 1967. Proteins, persulphate and disc electrophoresis. Nature 215:750-751.

Haga, T., M. Yamamoto, K. Maruyama and H. Noda. 1966. The effect of myosin and calcium on the solubilization of F-actin from muscle mince. Biochimica et Biophysica Acta 127:128-139.

Hamoir, G. 1951. Fish tropomyosin and fish nucleotropomyosin. Biochemical Journal 48:146-151.

Hamoir, G. and L. Laszt. 1962. La Tropomyosine B de carotides de bovidé. Biochimica et Biophysica Acta 59:365-375.

Hanson, J. and J. Lowy. 1963. Structure of F-actin and actin filaments isolated from muscle. Journal of Molecular Biology 6:46-60.

Hartshorne, D. J. and H. Mueller. 1967. Separation and recombination of the ethylene glycol bis (β-aminoethyl ether)-N, N'-tetraacetic acid-sensitizing factor obtained from a low ionic strength extract of natural actomyosin. Journal of Biological Chemistry 242:3089-3092.

_____ 1969. The preparation of tropomyosin and troponin from natural actomyosin. Biochimica et Biophysica Acta 175:301-319.

Huxley, H. E. 1957. The double array of filaments in cross-striated muscle. Journal of Biophysical and Biochemical Cytology 3:631-648.

_____ 1963. Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle. Journal of Molecular Biology 7:281-308.

Huxley, H. E. 1965. The mechanism of muscular contraction. Scientific American 213:18-27.

Huxley, H. E. and J. Hanson. 1957. Quantitative studies on the structure of cross-striated myofibrils. I. Investigations by interference microscopy. Biochimica et Biophysica Acta 23: 229-249.

Iida, S. and T. Ooi. 1967. Polymerization of tropomyosin: Dependence on temperature and pH. Archives of Biochemistry and Biophysics 121:526-528.

Jen, M. H., T. C. Hsu and T. C. Tsao. 1965. The sulfhydryl groups of rabbit tropomyosin and the amino acid sequence near these groups. Scientia Sinica 14:81-90.

Jen, M. H. and T. C. Tsao. 1957. A comparative, chemical study of tropomyosins from different sources. I. Amino acid composition and N-terminal structure. Scientia Sinica 6:317-326.

Jirgensons, B. and M. E. Straumanis. 1954. A short textbook of colloid chemistry. New York, John Wiley and Sons. 420 p.

Katz, A. M. and M. E. Carsten. 1963. Actin from heart muscle: Studies on amino acid composition. Circulation Research XIII: 474-477.

Katz, A. M. and R. P. Converse. 1964. Comparison of tropomyosins from cardiac and skeletal muscle. Circulation Research XV:194-198.

Kay, C. M. and K. Bailey. 1960. Light scattering in solutions of native and guanidinated rabbit tropomyosin. Biochimica et Biophysica Acta 40:149-156.

Khan, A. W. 1968. Biochemical changes occurring during aging of poultry and their significance in post-mortem tenderization. Canadian Institute of Food Technology Journal 1:86-89.

Knappeis, G. G. and F. Carlsen. 1962. The ultrastructure of the Z-disc in skeletal muscle. Journal of Cell Biology 13:323-335.

Kominz, D. R., F. Saad, J. A. Gladner and K. Laki. 1957. Mammalian tropomyosins. Archives of Biochemistry and Biophysics 70:16-28.

Kragh, A. M. 1961. Viscosity. In: Determination of the size and shape of protein molecules, ed. by P. Alexander and R. J. Block. New York, Pergamin. p. 173-209. (A Laboratory Manual of Analytical Methods of Protein Chemistry [including Polypeptides] Vol. 3.)

Laki, K., K. Maruyama and D. R. Kominz. 1962. Evidence for the interaction between tropomyosin and actin. Archives of Biochemistry and Biophysics 98:323-330.

Lawrie, R. A. 1966. Meat science. Oxford, Pergamon. 368 p.

Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. In: Methods in enzymology, ed. by S. P. Colowick and N. O. Kaplan. Vol. III. New York, Academic. p. 447-454.

Locker, R. H. 1954. C-Terminal groups in myosin, tropomyosin and actin. Biochimica et Biophysica Acta 14:533-542.

_____ 1960. Proteolysis in the storage of beef. Journal of the Science of Food and Agriculture 11:520-526.

Marsh, B. B. 1954. Rigor mortis in beef. Journal of the Science of Food and Agriculture 5:70-75.

Marsh, B. B. and A. Snow. 1950. A simple tissue homogenizer. Journal of the Science of Food and Agriculture 1:190.

Martins, C. B. and J. R. Whitaker. 1968. Catheptic enzymes and meat tenderization. I. Purification of cathepsin D and its action on actomyosin. Journal of Food Science 33:59-64.

Maruyama, K. 1966. Effect of β-actinin on the particle length of F-actin. Biochimica et Biophisica Acta 126:389-398.

Masaki, T., M. Endo and S. Ebashi. 1967. Localization of 6S component of α-actinin at Z-band. Journal of Biochemistry 62:630-632.

McCubbin, W. D. and C. M. Kay. 1969. Physicochemical studies on the aggregation of bovine cardiac tropomyosin with ionic strength. Canadian Journal of Biochemistry 47:411-414.

McCubbin, W. D., R. F. Kouba and C. M. Kay. 1967. Physico-chemical studies on bovine cardiac tropomyosin. Biochemistry 6:2417-2425.

Morrison, R. T. and R. N. Boyd. 1959. Organic chemistry. Boston, Allyn and Bacon. 948 p.

Mueller, H. 1966. EGTA-Sensitizing activity and molecular properties of tropomyosin prepared in presence of a sulfhydryl protecting agent. Biochemische Zeitschrift 345:300-321.

Needham, D. M. and J. M. Williams. 1963. Proteins of the uterine contractile mechanism. Biochemical Journal 89:552-561.

Ohtsuki, I., T. Masaki, Y. Nonomura and S. Ebashi. 1967. Periodic distribution of troponin along the thin filament. Journal of Biochemistry 61:817-819.

Okitani, A., T. Nakamura and M. Fujimaki. 1968. The changes in "myosin B" during storage of rabbit muscle. Part VI. Effect of Ca-chelating agent on ATPase activity of intact and trypsin-treated "myosin B." Agricultural and Biological Chemistry 32:683-688.

Ooi, T. 1967. Tryptic hydrolysis of tropomyosin. Biochemistry 6:2433-2439.

Parsons, A. L., J. L. Parsons, J. M. V. Blanshard and R. A. Lawrie. 1969. Electrophoretic differentiation of myofibrillar proteins in the pig. Biochemical Journal 112:673-678.

Penny, I. F. 1968. Effect of ageing on the properties of myofibrils of rabbit muscle. Journal of the Science of Food and Agriculture 19:518-524.

Pepe, F. A. 1966. Some aspects of the structural organization of the myofibril as revealed by antibody-staining methods. Journal of Cell Biology 28:505-525.

Perry, S. V. and A. Corsi. 1958. Extraction of proteins other than myosin from the isolated rabbit myofibril. Biochemical Journal 68:5-12.

Perry, S. V. and T. C. Grey. 1956. A study of the effects of substrate concentration and certain relaxing factors on the magnesium-activated myofibrillar adenosine triphosphatase.

Biochemical Journal 64:184-192.

Petropakis, H. J. 1968. Gel electrophoresis analysis of bovine sarcoplasmic proteins. Master's thesis. Corvallis, Oregon State University. 75 numb. leaves.

Rampton, J. H. 1969. Separation, identification and characterization of some myofibrillar proteins. Ph. D. thesis. East Lansing, Michigan State University. 143 numb. leaves.

_____ 1970. Captain, U.S. Army Natick Laboratories. Natick, Massachusetts. Personal communication during visitation. Corvallis, Oregon. February 18.

Reedy, M. K. 1964. Discussion following: The structure of actin filaments and the origin of the axial periodicity in the I-substance of vertebrate striated muscle, J. Hanson and J. Lowy. Royal Society of London Proceedings B 160:458-460.

Ritchie, R. F., J. G. Harter and T. B. Bayles. 1966. Refinements of acrylamide electrophoresis. Journal of Laboratory and Clinical Medicine 68:842-850.

Saad, F. M. and D. R. Kominz. 1961. The N-terminal group of rabbit tropomyosin. Archives of Biochemistry and Biophysics 92:541.

Sanger, F. 1945. The free amino groups of insulin. Biochemical Journal 39:507-514.

Schroeder, W. A. 1967. Degradation of peptides by the Edman method with direct identification of the PTH-amino acid. In: Enzyme structure, ed. by C. H. W. Hirs. New York, Academice. p. 445-461. (Methods in Enzymology. Vol. XI.)

Slayter, H. S. and S. Lowey. 1967. Substructure of the myosin molecule as visualized by electron microscopy. Proceedings of the National Academy of Sciences 58:1611-1618.

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. Journal of Cell Biology 34:431-445.

Stromer, M. H., D. J. Hartshorne, H. Mueller and R. V. Rice. 1969. The effect of various protein fractions on Z- and M-line reconstitution. Journal of Cell Biology 40:167-178.

Tanford, C. 1965. Physical chemistry of macromolecules. New York, John Wiley and Sons. 710 p.

Tsao, T. C., K. Bailey and G. S. Adair. 1951. The size, shape and aggregation of tropomyosin particles. Biochemical Journal 49: 27-36.

Ullrick, W. C. 1967. A theory of contraction for striated muscle. Journal of Theoretical Biology 15:53-69.

Van Holde, K. E. 1970. Professor, Oregon State University, Dept. of Biochemistry and Biophysics. Personal communication. Corvallis, Oregon. March 4.

Woods, E. F. 1967. Molecular weight and subunit structure of tropomyosin B. Journal of Biological Chemistry 242:2859-2871.

Yang, J. T. 1961. The viscosity of macromolecules in relation to molecular conformation. Advances in Protein Chemistry 16: 323-400.

Yasui, B., F. Fuchs and F. N. Briggs. 1968. The role of the sulfhydryl groups of tropomyosin and troponin in the calcium control of actomyosin contractility. Journal of Biological Chemistry 243:735-742.

Zak, R., M. Rabinowitz and C. Platt. 1967. Ribonucleic acids associated with myofibrils. Biochemistry 6:2493-2500.