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Investigations were conducted to determine whether the low molecular weight nitrogen compounds and the extra protein complex of bovine psoas major muscle were altered during a 12-day aging period.

Electrophoretic studies of soluble proteins either before or after gel filtration on Sephadex G-25 columns indicated that the soluble proteins were not altered during 12 days of aging. Data of absorbance measurements (280 and 260 nm) of the soluble protein extracts separated by gel filtration showed that the low molecular weight nonprotein nitrogen compounds increased during aging. Results of nitrogen determinations of the gel filtered extracts also supported this finding. Data of ultraviolet scanning, vertical gel electrophoresis and two-dimensional paper chromatography-high voltage electrophoresis of gel

filtration extracts suggested that the low molecular weight nonprotein nitrogen compounds were mostly nucleic acids. No evidence was obtained during the course of this study to indicate the presence of polypeptides in either the 0-day (fresh) or 12-day (aged) samples.

Evidence was obtained which indicated that the extra protein was altered during 12 days of aging. Vertical gel electrophoretic patterns of the 0-day extra protein samples showed one more band than patterns of the 12-day samples. Disappearance of this band during aging might be the result of proteolysis or an alteration in the ability of the protein constituting this band to bind with other muscle proteins. The binding ability of this protein may be weakened during aging which would allow for it to be solubilized and removed during the extraction procedure of the extra protein. This may be an important finding when considering that changes in the binding and anchoring of proteins to the Z-line have been suggested by several workers as a partial explanation of the tenderization of meat during aging.

Verification of the presence of tropomyosin and nucleoproteins in the extra protein complex was obtained by ultraviolet absorbance measurements and viscometric analysis of the extra protein fractions separated by vertical gel electrophoresis and collected by elution convection procedure.

Proteolytic and Chemical Changes of Some Minor Nitrogen Compounds and Extra Protein of Bovine Muscle During Aging

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PROTEOLYTIC AND CHEMICAL CHANGES OF SOME MINOR NITROGEN COMPOUNDS AND EXTRA PROTEIN OF BOVINE MUSCLE DURING AGING

INTRODUCTION

Much of the research concerned with the proteolytic changes occurring in muscle proteins during post-mortem aging has been directed toward the development of procedures or techniques capable of detecting these changes systematically. Accumulation of knowledge concerning these proteolytic changes can lead to a better understanding of the biochemistry of post-mortem tenderization of meat as well as providing guidelines for subsequent practical applications.

Cellulose ion exchange chromatography (Thompson et al., 1968) and vertical gel electrophoresis (Petropakis et al., 1969) have been employed in our laboratories to detect proteolytic changes in sarcoplasmic proteins during post-mortem aging. Results of these studies have indicated the detection of only minor and inconsistent proteolytic changes. Conversely, it has been well established that during post-mortem aging the amount of free amino acids in muscle increases (Niewiarowicz, 1958; Colombo and Gervasini, 1958; McCain et al., 1968; Field and Chang, 1969). It also has been inferred (Lawrie, 1966) that apart from the increase in free amino acids arising from possible proteolysis, their concentration is augmented by breakdown of various peptides (e.g., carnosine, anserine) naturally occurring in

muscle.

During the past decade, numerous studies have been concerned with the post-mortem properties and characteristics of the two major muscle structural proteins, actin and myosin, and with actomyosin, the rigid muscle complex formed during rigor mortis. In 1963, Huxley reported that a third structural protein, tropomyosin, appeared to play a key role in muscle structure by anchoring the actin filaments to the Z-line. More recent studies indicate that this structural anchor point for the actin filaments, the Z-line, disintegrates during aging (Davey and Gilbert, 1969). In view of these findings in addition to the fact that proteolytic enzymes are contained within the lysosomes of living muscle (Tappel, 1966), it has been suggested that the proteolytic hydrolysis of tropomyosin might occur during aging to influence the tenderization of meat (Khan, 1968; Penny, 1968; Davey and Gilbert, 1969). Whether tropomyosin was hydrolyzed during aging was rather thoroughly investigated recently in our laboratories by Dewey (1970). He found no evidence indicative of tropomyosin being hydrolyzed during an aging period of nine days.

Since the increase of free amino acids with aging cannot be explained readily because the proteolytic breakdown of sarcoplasmic proteins has not been detected by fairly sensitive techniques of cellulose ion exchange chromatography and vertical gel electrophoresis (Thompson *et al.*, 1968; Petropakis *et al.*, 1969), intermediate steps

of such a possible breakdown should be investigated. Moreover, according to Lawrie (1966), there is very little evidence to indicate the dissociation of the actomyosin complex or the proteolytic degradation of structural proteins during post-mortem aging. It is obvious, therefore, that additional research should be directed toward other less known and ill-defined structural proteins such as the extra protein fraction to gain further knowledge about the biochemical changes occurring in muscle during aging.

This study was two-fold in purpose. The first part involved the detection, during aging, of the presence and possible increase of polypeptides or low molecular weight nitrogen compounds that might serve as precursors for free amino acids. The second part of the study was to obtain information on the extra protein and to investigate the possibility that it is hydrolyzed during aging.

REVIEW OF LITERATURE

Proteins are biopolymers composed of a large number of repeating units, the amino acid residues, held together by the amide group as the covalent link (Witkop, 1968). In addition to covalent linkage, the intricate structure of proteins is stabilized by several types of bonds or forces as summarized by Jones (1964). An early classification of proteins as either globular or fibrous was based more on physical properties than on knowledge of their chemical structure. The globular proteins are quasi-spherical in gross structure, with a considerable amount of folding of the long polypeptide chain. They are in general soluble and often can be crystallized. The fibrous proteins are composed of elongated, filamentous chains which are joined laterally by cross-linkages to form a fairly stable structure. They are insoluble or, if soluble, show anomalous viscosity and often the tendency to form gels (Bernal, 1962).

Although the fibrous or globular state of proteins may reflect structural conformation, this classification is not adequate to define a complicated macromolecule such as a protein in descriptive terms. Therefore, the classification of proteins is now directed toward levels of structural organization in terms of primary, secondary, tertiary and quaternary structures (Jones, 1964).

Muscle Proteins

The muscle proteins form a specialized class of proteins. They include proteins capable of rapid contraction and relaxation which are surrounded by a connective protein tissue network to bind them into place and a complex system of enzymes which supply the muscle elements with energy (Bendall, 1964). Muscle proteins can be broadly divided into those which are soluble in water or dilute salt solutions, those which are soluble in concentrated salt solutions and those which are insoluble (Lawrie, 1966). Differences in the extractability of muscle proteins forms a basis for their classification into the following four major fractions; sarcoplasmic proteins, proteins of the granules, myofibrillar proteins, and stroma proteins (Szent-Gyorgyi, 1960). The distribution of these proteins and their abundance (% concentration) in muscle is shown in Table 1, together with other data on the chemical composition of a typical mammalian muscle after rigor mortis but before the onset of marked degradative changes (Lawrie, 1966).

Sarcoplasmic Proteins

These proteins occupy all the spaces of the muscle cell not taken up by formed elements and the contractile system (Briskey, 1967). They can be brought into solution readily with either water or

Table 1. Chemical composition (proteins and nonprotein substances) of typical muscle (percent wet weight). *

			(%)	
PROTEIN				18.0%
Myofibrillar	myosin, tropomyosin, X protein		7.5	
	actin		2.5	
Sarcoplasmic	albumins, globulins (glycolytic enzymes)		5.6	
	myoglobin		0.36	
	haemoglobin		0,04	
Mitochondrial	cytochrome C	ca.	0.002	
Sarcoplasmic reticulum	collagen			
Sarcolemma	elastin		2.0	
Connective tissue	"reticulin"			
	insoluble enzymes			
SOLUBLE NON-PROTEIN SUBSTANCES				3.5%
Nitrogenous	creatine		0.55	
	inosine monophosphate		0.30	
	di- and tri- phosphopyridine nucleotides		0.07	
	amino acids		0.35	
	carnosine, anserine		0.30	
Carbohydrate	lactic acid		0.90	
	glucose-6-phosphate		0.17	
	glycogen		0.10	
	glucose		0.01	
Inorganic	total soluble phosphorous		0.20	
	potassium		0.35	
	sodium		0.05	
	magnesium		0.02	
	calcium		0.007	
	zinc		0.005	
Traces of glycolytic intermediates, trace metals, vitamins, etc.		ca.	0.10	

*Abridged from Table 4.1, Lawrie (1966).

neutral salt solutions of low ionic strength ($\Gamma/2 < 0.2$). A solution thus obtained has a low viscosity (Szent-Gyorgyi, 1960). Because of the ease with which these proteins are extracted, they are frequently mentioned as the "soluble proteins" of muscle. A large share of the soluble muscle proteins consists of the glycolytic enzymes, including all the components of the glycolytic system plus creatine kinase and myoglobin. Other soluble nonprotein nitrogenous substances and inorganic material are also present in the sarcoplasmic extract (Table 1). The glycolytic and associated enzymes are not evenly distributed in quantity in the sarcoplasm. It has been reported that one enzyme alone, glyceraldehyde phosphate dehydrogenase, accounts for over 20% of the sarcoplasmic protein (Czok and Bucher, 1960). This, together with aldolase, enolase, kinases and lactate dehydrogenase make up about half of the sarcoplasmic protein. Several of these enzymes consist of more than one molecular species (Scopes, 1970). Nevertheless, some proteins have been isolated from the sarcoplasm of various species that have not been identified. According to Scopes (1966, 1968), there must be many uncharacterized proteins in muscle sarcoplasm, mostly in minute quantity, to carry out the normal functions of cellular metabolism in addition to glycolysis. Czok and Bucher (1960) estimated that up to 40% as final yield of sarcoplasmic extract could be obtained in crystalline forms.

In water extracts of muscle, which are dialyzed to very low

ionic strength, globulins are precipitated while the so-called albumins remain in solution. During this procedure considerable denaturation results by the lowering of pH due to dissolved CO_2 in the dialysate. The amount of precipitating globulin is critically dependent on pH as well as ionic strength because dialysis to very low ionic strength while maintaining the pH at 7.0 or above, did not cause any significant precipitation (Scopes, 1970).

The sarcoplasmic proteins do not contribute significantly to the filamentous organization of muscle. Their function is mainly concerned with the metabolic activities of the cell. Nevertheless, because of their change in quantity during continued contraction or training there is the possibility of participation or involvement of an unknown component of this fraction in the contractile system (Perry, 1965; Briskey, 1967).

A very interesting property of sarcoplasmic proteins is their variation, both quantitatively and qualitatively, between closely related species whereas the structural proteins show a universal similarity This variation has been used in comparative studies of different species for which normal electrophoretic patterns of sarcoplasmic proteins have been established (Scopes, 1968). Apart from charge differences implicit in their electrophoretograms, the sarcoplasmic proteins differ in various other parameters including their relative susceptibility to denaturation (Lawrie, 1966).

Granule Proteins

Most of the granules are extracted along with the sarcoplasmic proteins by solvents of low ionic strength. The granular fraction is comprised of nuclei, mitochondria, microsomes and lysosomes. The mitochondria carry enzymes of the oxidative cycle where ATP is produced while the "relaxing factor" and the magnesium activated ATPases appear to originate from the microsomal fraction (Szent-Gyorgyi, 1960). The synthesis of protein takes place in the microsomes and the lysosomes contain various hydrolytic enzymes important in degradative activities (Whitaker, 1966). Several studies indicate that the intracellular proteolytic enzymes, the cathepsins, may be partially responsible for the increase in tenderness that occurs during aging or storage of muscle (Randall and McRae, 1967).

The granules are localized between the myofibrils and occasionally are found within the Z-membranes (Perry, 1956).

Myofibrillar Proteins

These proteins have been grouped as those which are soluble in concentrated salt solutions However, recent discoveries of the highly water soluble proteins, α-actinin, β-actinin and troponin (Maruyama, 1965; Ebashi and Kodama, 1966), even though they have been shown to exist for the most part in the myofibril (Goll *et al.*,

1967), makes it particularly essential to be cautious with this protein classification (Briskey, 1967). Therefore, it is better to refer to these proteins as those which are responsible for the filamentous organization of muscle and function directly in contraction and relaxation. For their extraction, neutral salt solutions of high ionic strength ($\Gamma/2 > 0.5$) are required (Szent-Gyorgyi, 1960) even though after extraction, some of them as mentioned previously are highly soluble at lower ionic strength. Their resistance to extraction is partly the result of intimate associations and interactions between the proteins within the myofilaments. The high viscosity of the extract indicates the fibrous nature of the proteins brought into solution (Szent-Gyorgyi, 1960).

The proteins of the myofibril that have been reported include myosin, actin, tropomyosin, α-actinin, β-actinin, troponin and extra protein. The protein composition compiled from various sources is approximately 55% myosin, 20% actin, 7% tropomyosin, 2% troponin, 10% α-actinin, 2% β-actinin and 4% other unknown proteins (Ebashi, 1966; Rampton, 1969).

The micro-structure of the myofibril has been reviewed by Bendall (1964) and Briskey (1967). Skeletal muscle is composed of long muscle fibers running its length which are further subdivided into long, unbranching threads, the myofibrils. Myofibrils are striated and exhibit alternate light and dark bands when viewed through

the light microscope. Adjacent myofibrils lie with their light and dark bands in register, conferring a striated structure upon the entire fiber (Briskey, 1967). The light or I bands consist of thin filaments and have a thin dark line running across the center, the Z-line. The dark or A bands consist of thick filaments and have a clear zone in the middle, the H-zone. As reported by Bendall (1964), the ultrafine structure of the myofibril as viewed through the electron microscope is built up of two sets of filaments; the thick myosin filaments of the A band and the thin actin filaments of I band which are running from each Z-line, where they are attached, toward the middle of A band in the H-zone. The thick and thin filaments thus overlap and when viewed in cross section six thin filaments lie in hexagonal array around each thick filament (Huxley, 1965). Electron microscopy studies of the Z-line showed that it is a complex lattice in which the thin filaments (actin) do not extend directly from one sarcomere to the next. Instead, four filamentous projections proceed from the end of each actin filament into the tetragonal, zig-zag configuration of the Z-line (Knappeis and Carlsen, 1962). Reedy (1964) confirmed the evidence for this model.

Myosin is the most abundant of the myofibrillar proteins. It is extractable from a muscle mince with strong salt solutions and then can be precipitated free of the various water soluble proteins upon reduction of ionic strength (Briskey, 1967). A modified Hasselbach-

Schneider (1951) solution, which is composed of 0.47 M KCl, 0.1 M potassium phosphate buffer, 0.01 M sodium pyrophosphate and 10^{-3} M $MgCl_2$, pH 6.5, extracts myosin with almost no contamination from actin (Perry and Zydowo, 1959a). Richards et al. (1967) reported that myosin preparations have been plagued by the presence of myokinase, AMP deaminase, nucleic acids, myosin-nucleic acid complexes and unidentified proteins. The molecule of myosin is highly asymmetric and the ratio of length to diameter being about 100:1. Because of its high content of glutamic and aspartic acids, and of dibasic amino acids, it is highly charged and has a strong affinity for calcium and magnesium ions.

The myosin molecule is built from two types of sub-units, light (L-) and heavy (H-) meromyosin. The H-meromyosin has a molecular weight of about 380,000 and is less than 45% helical. It is sited on the periphery of the myosin filaments. Most of the -SH groups and proline-residues of myosin are found on the H-meromyosin which contains all of the ATPase and actin-combining properties of myosin. The L-meromyosin is nearly 100% helical and appears to be of purely structural importance. It has been suggested that one dimer molecule of L-meromyosin combines with one molecule of H-meromyosin to form a molecule of myosin (Bendall, 1964). This simplification of the myosin molecule has been opposed by Perry (1967a) who reported that L- and H-meromyosins are fragments rather than subunits.

Actin is the other major protein of the myofibril. It can exist in two forms, the globular of G-form and the fibrous or F-form. The G-actin consists of relatively small separate globular beads (MW 70,000) which are aggregated end to end to form a double chain, the F-actin, in the presence of salts and small amounts of ATP. There are four reactive -SH groups in G-actin and only two in F-actin and these -SH groups play a part in the polymerization process (Bendall, 1964). It is the F-actin which combines with myosin to form the contractile actomyosin of active or pre-rigor muscle and the inextensible actomyosin of muscle in rigor mortis (Lawrie, 1966). According to Huxley (1965), the myosin and actin filaments are at sites connected by tiny cross-bridges. During relaxation, ATP can act as lubricant, and the thin filaments slip passively over the thick ones while the cross-bridges detach and reattach themselves at new sites.

α-Actinin was first isolated and studied by Ebashi and Ebashi (1964, 1965). The amino acid composition of α-actinin resembles that of actin, thus it has been suspected of being denatured actin. Ebashi (1966) indicated that α-actinin is located in the Z-band and perhaps in the H-band while Goll _et al_. (1967) and Masaki _et al_. (1967) reported that it was present in the Z-band.

β-Actinin was discovered by Maruyama (1965) who reported that this protein regulates the length of F-actin filaments (inhibits network

formation in F-actin) at the length at which they occur *in vivo*. Ebashi (1966) suggested that β-actinin may function in muscle development rather than in muscle contraction. The amino acid composition of this protein is similar to that of actin.

Tropomyosin was initially isolated by Bailey (1946, 1948) and when prepared according to his procedure, it is referred to as tropomyosin B. Tropomyosin A (paramyosin) is formed in certain muscles capable of prolonged tetanic contraction (Poglazov, 1966). Tropomyosin B is characterized by the formation of highly polymerized (end to end polymerization) viscous solutions at neutral pH in the absence of salts. It contains low levels of tryptophan and proline but the presence of large amounts of dicarboxylic and basic amino acids in its amino acid composition (Poglazov, 1966) provides resistance to denaturing effects of organic solvents and acid pH (Briskey, 1967). It has molecular asymmetry and high α-helical content. The question of its location is still undecided. Huxley (1963) suggested the presence of tropomyosin at the Z-line. Such a location would explain the difficulty of extracting actin free of tropomyosin although recent reports indicate that tropomyosin is not a Z-line component (Stromer *et al.*, 1969; Caspar *et al.*, 1969). However, it has not been disputed that this protein may serve to anchor the thin filament to the Z-line. The presence of tropomyosin B in the I band has been reported by Pepe (1966).

Troponin was isolated and studied by Ebashi and Kodama in 1965. Previously Szent-Gyorgyi and Kaminer (1963) had isolated a muscle protein, which they called metin on the basis of its metachromatic property. This protein was subsequently shown to be a complex of tropomyosin B and troponin (Azuma and Watanabe, 1965a, b). Ebashi et al. (1967) called the complex of tropomyosin B and troponin, native tropomyosin, and reported that this complex is essential for the regulating action of calcium on the contractile system of muscle. They added that at least four proteins, myosin, actin, tropomyosin and troponin are involved in the process of physiological contraction.

Arai and Watanabe (1968a) indicated that tropomyosin B and troponin form a complex which behaves exactly like the relaxing protein (Ebashi and Ebashi, 1964) and that the relaxing protein preparation is actually dissociated by 5 M urea into troponin and tropomyosin B. The inhibition of the magnesium stimulated ATPase activity of myofibrils by calcium chelating agents is mediated by the troponin-tropomyosin B complex. Troponin can be dissociated into two components, troponin B and troponin A (Hartshorne et al., 1969). Troponin B, in the absence of EGTA (ethanedioxy-bis(ethylamine)tetraacetate), inhibits the magnesium activated ATPase of desensitized actomyosin (natural actomyosin from which the EGTA-sensitizing factor has been removed) and troponin A which when added to the inhibitor renders it effective only in the presence of EGTA. The

inhibitor component of troponin has similar properties with the inhibitory factor reported earlier by Hartshorne et al (1966). Recently Schaub and Perry (1969) reported that troponin in the presence of dissociating reagents is dissociated into inhibitory and calcium sensitizing factors and that the relaxing protein system of muscle may contain another component (protein) yet not characterized.

Troponin is a globular protein found in the I band (Pepe, 1966) but not in the Z-line (Endo et al., 1966). Preparations of troponin when added to tropomyosin B increase the tendency of the latter to aggregate. The aggregated complex has a higher viscosity and larger sedimentation constant than tropomyosin B (Ebashi and Kodama, 1965; Ebashi, 1966). According to Perry (1967), troponin preparations usually contain tropomyosin. Recently, Schaub and Perry (1969) described a method for the preparation of the troponin free of tropomyosin from low-ionic strength extracts of myofibrils.

The existence of the extra protein complex was first indicated by Szent-Gyorgyi et al. (1955). They found that when myosin was extracted from myofibrils with high ionic strength salt solutions, considered to be selective for myosin, other proteins were also extracted. They claimed that, in addition to the myosin, protein material amounting to approximately 20% of the total myofibrillar nitrogen passed into solution. Subsequent studies indicated this additional protein material was considerably less when well-washed myofibrils were used (Perry

and Corsi, 1958). On lowering the ionic strength to about 0.05 M, myosin precipitated, leaving the extra protein in solution (Perry and Zydowo, 1959a; Poglazov, 1966). Corsi (1957) presented electrophoretic evidence showing that the extra protein contains several components, of which he considered tropomyosin to be the most abundant. Perry and Zydowo (1959a) confirmed that the extra protein is heterogeneous. Using DEAE-cellulose chromatography they separated the extra protein into four fractions. They reported that fraction I contained sarcoplasmic components while fraction II was composed of water-soluble and water-insoluble components not yet identified. Fraction III contained tropomyosin B plus some other protein while a considerable amount of bound ribonucleic acid was found in fraction IV. It was suggested later (Perry and Zydowo, 1959b) that the ribonucleoprotein might be associated with protein synthesis. Maruyama (1965) reported that the extra protein possibly consists in part of α- and β-actinins.

The extra protein accounts for approximately 7% of the total myofibrillar protein (Rampton, 1969).

Stroma Proteins

These proteins make up the connective tissues. They are often called the insoluble proteins because they are retained in the residue after extensive extraction of well-homogenized muscle with strong

salt solutions (Szent-Gyorgyi, 1960). Collagen, elastin and reticulin are the major stroma proteins.

Changes in Muscle Proteins During Aging

During the rigor process anaerobic metabolism continues to produce ATP until the energy reserves of the muscle are depleted or lactic acid accumulation inhibits glycolysis. Oxidative phosphorylation is terminated, and calcium ions are released by the shock to the nervous system. Because of the presence of calcium, ATPases are able to function and the muscle goes into continuous contraction and fails to relax since calcium ions are not pumped away. Finally, the ATPases deplete the ATP supply and the muscle remains in the contracted state (Lawrie, 1966). 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.

The biochemical changes occurring during post-mortem aging have not been elucidate. The widely-held view that aging is a consequence of proteolytic action had been supported by the findings that nonprotein nitrogen accumulates within the tissue during aging and that muscle tissues contain proteolytic enzymes, the cathepsins (Balls, 1938). Landmann (1963) studied the muscle cathepsins and reported that within the pH range of muscle in rigor mortis (pH 5.4-6.8), the

activity of these enzymes should be expected. Many studies favoring proteolytic changes during post-mortem aging have been reported. Niewiarowicz (1956) demonstrated that the nonprotein nitrogen increases during aging. Zender at al. (1958) reported that autolysis and degradation of the fine structure of muscle fibers occurred during prolonged aging. Solovjev et al. (1962) showed that N-terminal groups of myosin increased after 6 days of aging. Sharp (1963) reported autolytic effects of muscle cathepsins during aseptic storage and concluded from histological evidence that autolysis mainly affects the sarcoplasmic proteins.

That proteolytic changes occur in muscle proteins during aging has been disputed by many investigators. Wierbicki et al. (1954) did not detect an increase in nonprotein nitrogen indicative of proteolysis in muscle aged for 15 days. Locker (1960), using terminal group analysis, found no significant increase in N-terminal groups during aging which would indicate protein breakdown in either the sarcoplasmic or myofibrillar proteins. Bodwell and Pearson (1964) pointed out that an increase of N-terminal groups would not necessarily be an indication of the amount of protein breakdown if reactions of transpeptidation and peptide elongation catalyzed by proteolytic enzymes occurred during aging.

Increases in the nonprotein nitrogen and free amino acids during aging of muscle have been reported by many investigators

(Niewiarowicz, 1956; Colombo and Gervasini, 1958; Davey and Gilbert, 1966; McCain et al., 1968; Field and Chang, 1969). Lawrie (1966) reported that apart from the increase in free amino acids arising from proteolysis, their concentration is also augmented by the breakdown of various peptides such as carnosine and anserine which are hydrolyzed to β-alanine and histidine.

Whether or not proteolytic changes occur in the sarcoplasmic proteins during aging is not clear. Bodwell and Pearson (1964) and Sharp (1963) concluded that the water soluble proteins were the major substrates for the natural proteolytic enzymes. Zender et al. (1958) reported that electrophoretic patterns of proteins were modified after prolonged storage. On the other hand, Kronman and Winterbottom (1960) studied the alteration of the water soluble proteins of the bovine muscle aged for seven days and reported that the observed changes were not consistent. Aberle and Merkel (1966) reported that electrophoretograms obtained from starch gel electrophoresis of sarcoplasmic proteins were very similar at 1, 7 and 14 days of aging. Petropakis et al. (1969) employed vertical gel electrophoresis to detect proteolytic changes of sarcoplasmic proteins with post-mortem aging. They found no evidence of proteolysis of these proteins.

Little is known about the post-mortem proteolytic reactions in contractile proteins. Wierbicki et al. (1954) suggested that post-tenderization may be caused by a dissociation of actomyosin to actin

and myosin or by a redistribution of ions within the muscle causing increased hydration and tenderness. The same investigators later reported that no evidence exists for the dissociation of actomyosin with aging (Wierbicki et al., 1956). Lawrie (1966) reviewing the changes occurring in the myofibrillar proteins concluded that aging does not involve dissociation of actin from myosin. However, significant and consistent biochemical changes have been observed in the myofibrillar components only, thus is now thought that during aging morphological changes occur in these structures (Davey and Gilbert, 1968a).

Guenther and Turba (1969) confirmed previous reports (Fujimaki et al., 1965) that myosin decreases during post-mortem aging. They added that the disappearance of myosin was accompanied by the appearance of degradation products after 4 days, one of which was still present after 7 days. The peptide maps that they obtained from myosin and from the degradation products showed a lack of 18 peptide spots of the original 75 myosin peptides and the appearance of 8 new peptides in the degradation products. Penny (1968) reported that, when myofibrils from aged muscle were treated with 1 M potassium chloride or 0.1 M pyrophosphate, more protein was extracted than from those prepared from unaged muscle due to increasing extraction of actin, which may have been released by disruption of the Z-bands. The weakening and final disruption of the Z-line during aging has been

also suggested by Davey and Gilbert (1968b). They showed that more actin could be extracted from the aged muscle because of the disrupted Z-line structure, and that this was accompanied by increasing amounts of a complex mixture of extra protein, soluble at low ionic strength. The location of this extra protein within the structure of the myofibrils was not ascertained.

Recently Davey and Gilbert (1969) reaffirmed the disruption and possible dissolution of Z-line material during aging. They reported that during aging, in addition to the weakening of lateral attachments normally maintaining the myofibrils in register within the muscle, changes occur within the myofibrils themselves. The most notable was the fading of Z-lines during a short period (1 hour) of microscope examination. Both the weakening of lateral attachments and disappearance of Z-lines during aging were inhibited by EDTA (ethylenediaminetetraacetic acid). Haga et al. (1966) reported that the presence of small amounts of calcium (1-10 mM) during the extraction of protein from myofibrils helped to detach actin from the Z-lines. This can to some extent explain the above protective property of EDTA which complexes with calcium and by so doing removes these ions from the myofibrillar environment. When the muscle is aged without the presence of EDTA, as normally happens, the calcium released during rigor can possibly induce in some way the fragility of the Z-line and a weakening of the lateral attachments between myofibrils in

the Z-line regions. Penny (1970a, b) reported that when myofibrils from muscles aged 8 and 15 days were extracted by Hasselbach-Schneider solution (0.5 M KCl, 1 mM $MgCl_2$, 10 mM tetrasodium pyrophosphate, 10 mM KH_2PO_4, pH 6.5) increasing amounts of myosin, actin, tropomyosin and troponin were obtained. Similarly, Tris-HCl solution (pH 8.2) extracted more of a complex mixture containing actin and α-actinin. Using DEAE-cellulose chromatography and starch-gel electrophoresis Penny concluded that there was no evidence of breakdown or disappearance of any particular protein and he suggested that one of the effects of aging may have been an alteration in the binding of some proteins to each other in the myofibril.

The connective tissue proteins undergo very little change during aging (Sharp, 1963; Lawrie, 1966) and thus, are not assumed to exert much influence upon the post-mortem tenderization of muscle.

EXPERIMENTAL PROCEDURE

The experimental work in this study was divided in two parts. The first part deals with research on polypeptides and low molecular weight nitrogen compounds while the second part concerns studies on extra protein preparations. To determine the influence of post-morten aging, samples were obtained at 0 day of aging (1-2 hrs. post-mortem) and were aged up to 12 days at 3-4°C. The zero-day and twelve-day aged samples are denoted by the prefix 0- and 12-, respectively. Sample preparation and most of the experimental work was performed in a cold room (3-4°C). Deionized water was used for preparation of buffers. Reagent grade chemicals were used unless otherwise stated.

Analytical and instrumental methods used in the first experimental part were gel filtration, ultrafiltration, spectrophotometric scanning, paper chromatography, vertical gel and high voltage electrophoresis. In the second part, ultracentifugation, vertical gel electrophoresis, electrophoretic elution-convection and viscometric measurements were employed. Other complimentary analytical methods used are stated during the description of the experimental procedure. An outline of sample preparation and experimental procedure is presented in Figure 1.

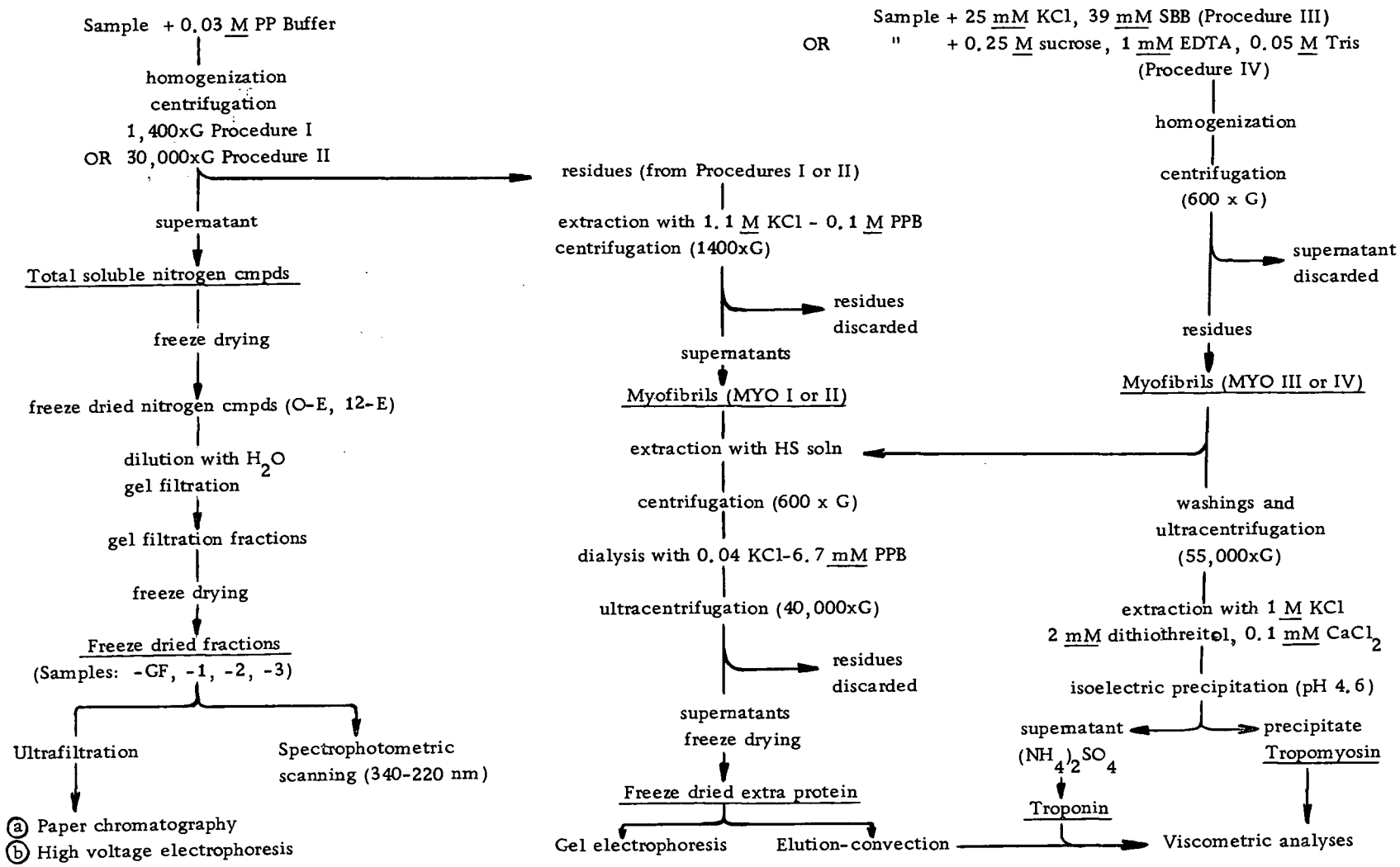


Figure 1. Outline of experimental procedure.

Preparation of Samples

Sections of psoas major muscle were obtained from local meat processing plants and held at 4°C until used.

Fifty grams of muscle, excluding as much intramuscular fat and connective tissue as possible, were diced and homogenized with 150 ml of cold 0.03 M potassium phosphate buffer (PPB), pH 7.4, for 90 seconds in a water-jacketed Waring Blendor cooled with flowing tap water (10-12°C). Homogenization was continued for an additional 30 seconds to thoroughly mix the homogenate. Then 350 ml of the same buffer (0.03 M PPB) were added, and the mixture was gently stirred for 30 minutes in the cold room. This mixture was centrifuged at 1400 x G (Procedure I) for 20 minutes at 4°C. Centrifugation at 30,000 x G was also applied to duplicate samples of muscle (Procedure II). The supernatant was retained after filtering through glass wool or Whatman No. 12 filter paper to remove floating fat particles. The centrifugate was suspended in 500 ml of cold 0.03 M PPB, the mix was stirred for 30 minutes and centrifuged for 20 minutes at 1400 x G. The supernatant was filtered and retained. The centrifugate was once again suspended in additional 500 ml of 0.03 M buffer solution (PPB). The mixture was stirred, centrifuged and the new supernatant was again retained. The combined supernatants containing the soluble nitrogen compounds were freeze dried and stored at 0°C. These

freeze dried samples (0-E and 12-E) were used to determine total soluble nitrogen compounds (Lawrie, 1961) and to study the soluble proteins and low molecular weight nitrogen compounds.

Myofibrils Preparation

Residues from extractions with 0.03 M buffer (Procedure I or II) were suspended in 500 ml of a cold mixture of 1.1 M KCl and 0.1 M potassium phosphate buffer (KCl-PPB), pH 7.4. They were stirred gently for 1 hour and centrifuged at 1400 x G for 20 minutes at 4°C. The suspension of the residue in 500 ml KCl-PPB mixture, stirring and centrifuging were repeated twice. Supernatants of each of the centrifugations were retained. The combined supernatants containing the myofibril proteins were dialyzed against deionized water for 24 hours at 4°C. After dialysis, the myofibrillar suspensions were freeze dried. These freeze dried myofibril samples (MYO I or MYO II) were used for determination of total myofibrillar proteins (Lawrie, 1961) and for the extraction of extra protein.

Myofibrils were also prepared according to the method of Perry and Zydowo (1959a). Chilled and minced psoas major muscle (400 g) was homogenized for 90 seconds with 5 volumes (2000 ml) of 25 mM KCl containing 39 mM sodium borate buffer (SBB), pH 7.1 (Perry, 1953; Perry and Grey, 1956), and the homogenate was centrifuged at 600 x G for 15 minutes (4°C). The supernatant was discarded and the

residue resuspended with an additional 2000 ml of KCl-SBB and homogenized for 2 minutes (Procedure III). The homogenate was centrifuged for 15 minutes at 600 x G and the supernatant was again discarded. The loose upper layer of the sediment was removed with a minimum of buffer and retained while the residue was discarded. Alternatively, the initial homogenization of muscle was carried out with 12 volumes of a solution containing 0.25 M sucrose, 1 mM EDTA and 0.05 M Tris-(Hydroxymethyl)-aminomethane (Tris buffer) at pH 7.6 (Czok and Bucher, 1960; Goll and Robson, 1967). After standing 10 minutes, the homogenate was centrifuged at 20,000 x G for 15 minutes at 4°C. The supernatant was discarded and the residue was washed a second time. The washed muscle residue was suspended in 9 volumes of 0.1 M KCl-39 mM sodium borate buffer, pH 7.1, then centrifuged at 600 x G for 15 minutes. The supernatant was discarded and the upper layer of the sediment was removed as in Procedure III with a minimum of buffer and retained (Procedure IV). The retained upper layer (Procedure III or IV) was diluted with 7 volumes (2800 ml) of 0.1 M KCl-39 mM sodium borate buffer, pH 7.1 (Perry and Zydowo, 1959a). The suspension, consisting mainly of myofibrils, was centrifuged for 15 minutes at 500 x G to remove coarse material. The sediment was discarded and the suspension centrifuged for 15 minutes at 600 x G to sediment the myofibrils. These myofibrils were resuspended and centrifuged three more times

to remove as much sarcoplasmic proteins as possible. Finally, the suspension of washed myofibrils was freeze dried. A part (1/5 of total) of these freeze dried, so called crude myofibril preparations (MYO III and MYO IV), was used for extraction of extra protein as in the case of MYO I and MYO II. The remaining myofibrils were used for the preparation of troponin.

Extra Protein Preparation

Freeze dried myofibrils obtained by Procedures I, II, III or IV (MYO I, MYO II, MYO III and MYO IV) were extracted with 4-5 volumes of Hasselbach and Schneider (1951) solution containing 0.1 M potassium phosphate buffer, pH 6.5, 0.47 M KCl, 1 mM $MgCl_2$ and 0.01 M sodium pyrophosphate (HS solution). The mixture was gently stirred for 20-30 minutes in the cold room and centrifuged at 600 x G for 30 minutes (4°C). The insoluble material was discarded and the turbid supernatant was dialyzed against 6.7 mM potassium phosphate buffer, pH 7.2, containing 0.04 M KCl (Perry and Zydowo, 1959a) for up to 48 hours with several changes of dialysis medium. This dialysis was adequate to remove most of the salts from the buffer used for the extraction of extra protein. A gelatinous precipitate was formed during dialysis which was removed by ultracentrifugation for 20 minutes, at 40,000 x G in a Beckman preparative ultracentrifuge, Model L-2HV, equipped with a type 30 rotor. The centrifugate was

discarded and the clear supernatant containing the extra protein was concentrated by freeze drying.

Troponin Preparation

The preparation of troponin from myofibrils was adapted from the procedure of Schaub and Perry (1969). Freeze dried myofibrils (MYO III crude preparation) were washed twice with 3-4 volumes of 2 mM Tris-HCl buffer, pH 7.4, containing 15 mM 2-mercaptoethanol, followed by dialysis against 20 volumes (two changes) of the same solution (4°C). After 24 hours the viscous suspension was centrifuged at 55,000 x G for 30 minutes at 4°C. The residue was diluted and centrifuged again and the combined supernatants were freeze dried. The freeze dried samples were suspended in 3-4 volumes of a solution containing 1 M KCl, 2 mM dithiothreitol and 0.1 mM $CaCl_2$. The mixture was gently stirred for 1-2 hours in the cold room to accomplish the extraction of tropomyosin-troponin complex. The material that did not dissolve was removed by centrifugation and the pH of the supernatant was lowered slowly from approximately 7.5 to 4.6 by adding drops of 0.1 M HCl. At this step of isoelectric precipitation of tropomyosin (Ebashi and Kodama, 1965), most of the protein was precipitated, although a small part remained in the supernatant. After 1 hour the precipitate was collected by centrifugation and retained. The supernatant was adjusted to pH 7 by adding drops of

2 M Tris and then solid $(NH_4)_2SO_4$ was added to 70% saturation. After 1 hour the precipitate was collected by centrifugation and then dialyzed against 100 volumes of 15 mM Tris-HCl buffer, pH 7.4, containing 0.5 mM dithiothreitol and 0.1 mM $CaCl_2$. The dialysis fluid was changed twice and the slight precipitate formed was centrifuged off. Troponin suspensions thus obtained were freeze dried and stored for future use. The precipitate of the isoelectric precipitation step was washed with 1 M KCl-2 mM dithiothreitol-0.1 mM $CaCl_2$, at pH 7.5, and precipitated again at pH 4.6. The precipitate was dispersed in 5 volumes of deionized water, the pH adjusted to 7.0 and solid $(NH_4)_2SO_4$ was added slowly to give 70% saturation. The fraction that precipitated between 47-70% saturation (Bailey, 1948) was stored at 0°C until used as the tropomyosin preparation.

All of the freeze dried samples were stored at 4°C.

Part I. Techniques Utilized in Studying Soluble Proteins and Low Molecular Weight Nitrogen Compounds

Gel Filtration

Gel filtration was used to fractionate the low molecular weight nitrogen compounds. A Sephadex laboratory column (2.5 x 100 cm) was packed to a height of 80 cm with Sephadex G-25 medium gel which had been suspended in 0.03 M potassium phosphate buffer, pH 7.4. After several hours, the column bed was stabilized by passing 2-3 bed volumes of buffer through the packed column. Ten ml

of an aqueous solution of Blue Dextran 2000 were eluted through the column to check the homogeneity of the column bed (Pharmacia, 1969).

One g of freeze dried 0-E or 12-E material was added to 15 ml of cold deionized water (4°C). The mix was gently stirred and filtered through glass wool to remove any floating particles. The sample (16 ml) was placed in the sample applicator on top of the column bed and eluted with 0.03 M potassium phosphate buffer, pH 7.4, at a flow rate of 40-45 ml/hour. Effluent emerging from the column was passed into flow-through cells of Gilson UV absorption meters where absorbance of the fluid was monitored at 265 and 280 nm and recorded with either Beckman Potentiometric or Texas Instruments "recti-riter" recorders. The effluent, after leaving the UV monitors, was collected in 10 ml fractions with LKB fraction collectors. Each fraction was marked on the appropriate absorption recording chart by automatic fraction markers to facilitate identification of fractions. Effluents of these fraction areas were either freeze dried or desalted immediately after gel filtration. All of the work involved in gel filtration was carried out at 4°C.

Spectrophotometric Scanning

Freeze dried eluants of gel filtration fractions were diluted with deionized water and scanned automatically in a Beckman DB recording

spectrophotometer. Samples were scanned from 340 to 220 nm against a 0.03 M potassium phosphate buffer, pH 7.4, blank.

Desalting and Ultrafiltration

Several methods were employed to remove potassium phosphate from the effluent. In preliminary experiments, a TORBAL-BLT Chromatography Desalting apparatus (Model CD-1, The Torsion Balance Co., Clifton, N.J.) was used (Blainey and Yardley, 1956) without satisfactory results although repeated sample dilutions, and desaltings were tried. The extent of desalting was checked by assaying for the presence of potassium phosphate in the samples with a 0.1 M solution of $AgNO_3$ (Hogness and Johnson, 1954; Pierce et al., 1959). The formation of white-yellow precipitate of silver phosphate was sufficient to establish the presence of phosphate.

The ion retardation resin, AG11A8, which has been recommended for the chromatographic desalting of biochemicals such as amino acids, polypeptides, nucleic acids, proteins, etc. (Bio-Rad Laboratories, 1963; Rollins et al., 1962) was also tried. Columns (1.2 cm diameter) were packed, equilibrated and operated by procedures similar to those described previously for gel filtration. Aqueous dilutions of samples to be desalted were eluted through stabilized resin columns of AG11A8 with deionized water. Since the resolution of salts and organic components is increased with increased

column length and with decreased flow rate, columns as long as 100 cm (bed 1.1 cm^2 x 100 cm) and flow rates as low as 0.3 $ml/cm^2/min$ were tried. Also, to increase resolution, sample loading in many runs was only 5 ml of fluid containing approximately 0.3 M potassium phosphate. Effluents were passed into the flow-through cell of a Beckman DB recording spectrophotometer and monitored at 260 nm. One ml fractions were collected using an ISCO Model 270 fraction collector and its companion Model V volumeter. After the peak was reached in the desalting pattern, drops of effluent were checked by the qualitative $AgNO_3$ method for the presence of phosphate ions. When white-yellow precipitate was formed the collection of desalted sample was stopped and the remainder of some effluents was passed through a column a second time.

Ultrafiltration was also employed to remove potassium phosphate from the samples. An Amicon ultrafiltration cell, Model 401 (Amicon Corp., Lexington, Mass.), and Amicon UM Diaflo membranes were used for ultrafiltration. Originally, UM-05 membranes having a molecular weight exclusion limit of 500 were used without success. Diaflo UM-2 membranes (exclusion limits of 1000 MW) were finally used with very satisfactory results. Samples diluted (1:5 v/v) with deionized water were applied in order to avoid destruction of membranes by the phosphate buffer. As the retentate volume decreased during filtration, it was diluted with deionized water to the

original volume and re-ultrafiltered (diafiltration). This was repeated several times to obtain samples practically free of salts. Table 2 shows the sequence of diafiltration dilutions along with the other operating characteristics used. The progress of desalting was followed by ultraviolet scanning of retentate volume and by the $AgNO_3$ method. During ultrafiltration samples were continuously mixed by means of a built-in magnetic stirrer. Ultrafiltrations were performed in a cold room at 4° C. Desalted samples were freeze dried and used for paper chromatography-high voltage electrophoresis runs.

Table 2. Diafiltration dilutions and other operating characteristics.

Dilution		v/v	Salt Concentration	
			Initial	Final
a	First	1:5[a]	0.03 M	0.005 M
b	Second	1:1[b]	0.005 M	0.0025 M
c	Third	1:4[b]	0.0025 M	0.0005 M
d	Fourth	1:4[b]	0.0005 M	0.0001 M
e	Fifth	1:4[b]	0.0001 M	0.02 mM
f	Sixth	1:4[b]	0.02 mM	0.004 mM
g	Final	1:5[b]	0.004 mM	<0.001 mM

Membranes: Diaflo UM-2
Pressure: 80 psi (N_2)
Flow rate of diluted samples: 1.8-2.2 ml/min
Retentate volume: 40-60 ml
Total volume: 300-360 ml

[a]One volume of gel filtration eluant plus five volumes of deionized water.

[b]One retentate volume plus one, four or five volumes of deionized water.

Paper Chromatography-High Voltage Electrophoresis

Freeze dried desalted samples of gel filtration fractions were studied by two-dimensional chromatography and electrophoresis (Katz et al., 1959). To prepare samples, 0.010 g of dry material was diluted in 0.20 ml of deionized water. One hundred μl of this preparation was applied to Whatman No. 3 MM filter paper (46 x 57 cm sheets) to form a small origin spot of less than 7 mm diameter. Descending paper chromatography in n-butanol-acetic acid-water (4:1:5 v/v/v), upper phase from the mixture equilibrated overnight, was run 21 hours in a Chromatocab at room temperature. At the end of chromatography the paper was dried in a hood for 3-4 hours and prepared for electrophoresis by moistening the entire paper with pyridine-acetic acid-water (1:10:289 v/v/v) buffer solution, pH 3.7. Using the same buffer, high voltage electrophoresis was run at 3000 V for 45 minutes in a High Voltage Electrophorator (Model D, Gilson Medical Electronics, Middleton, Wisconsin) cooled by tap water flowing through stainless steel coils. After electrophoresis the papers were dried and sprayed with a solution made up of cadmium acetate (100 mg), water (10 ml), acetic acid (5 ml), acetone (100 ml), and ninhydrin (1 g) (Heilmann et al., 1957; Bailey, 1962), dried in the hood, and developed 3-5 minutes in a 100°C oven. Alternatively, the papers were sprayed with 0.5% solution of ninhydrin in butanol

(Ninspray, NBC, Cleveland, Ohio).

Polyacrylamide-Gel Electrophoresis

A vertical gel electrophoresis apparatus (Model EC-470, E-C Apparatus Corporation, Philadelphia, Pa.) and companion power supply (Model EC-454) were used for electrophoretic runs by the discontinuous technique (Petropakis *et al.*, 1969; E-C Apparatus Corporation, 1966). Samples were mixed with appropriate volume of a "sample mixture" to obtain protein concentrations of 5 mg/ml. A 4% spacer gel, 10% running gel and 20% plug gel were employed. Tris-HCl spacer buffer (0.062 M Tris-0.048 M HCl, pH 6.7), Tris-HCl running buffer (0.38 M Tris-0.048 M HCl, pH 8.9), and Tris-glycine electrode buffer (0.005 M Tris-0.039 M glycine, pH 8.3) were the buffers used. The experimental procedure and methods of preparation of "sample mixture," buffers, gel staining and destaining were described in detail by Petropakis (1968).

Nitrogen Determination

Total nitrogen contents of the tissue and myofibrils were determined by the micro- and macro-Kjeldahl procedures (A.O.A.C., 1960). Nitrogen determinations of the remaining samples were carried out by the micro-Kjeldahl method. Protein concentrations were calculated by multiplying percent nitrogen by the factor 6.25.

Samples 0-E and 12-E were used for the determination of total soluble nitrogen compounds. After the addition of 20% trichloroacetic acid to precipitate the protein, the above samples were centrifuged and the amount of soluble nonprotein compounds in the supernatants (samples 0-E (np), 12-E (np)) were determined. The difference between total soluble nitrogen and soluble nonprotein nitrogen represented the nitrogen due to sarcoplasmic proteins.

The protein content of samples was also estimated by ultraviolet absorption at 280 and 260 nm. The protein concentration was calculated by the following equation: protein concentration (mg/ml) $= F \times \frac{1}{d} \times$ optical density at 280 nm where d is the cuvette width in centimeters. The factor F was determined from the ratio of optical density at 280 nm over 260 nm and is given in tables (Layne, 1957) which also list the corresponding percentage of nucleic acids.

Part II. Techniques Utilized in Studying Extra Protein Preparations

Protein Determination

The protein content of the extra protein preparations was determined by the micro-Kjeldahl method (A. O. A. C., 1960) and the biuret method of Layne (1957) using bovine serum albumin to produce a reference calibration curve. Ultraviolet absorption measurements at 280 nm and 260 nm were also used to estimate protein and nucleic

acid contents as described previously.

Vertical Polyacrylamide Gel Electrophoresis

The discontinuous technique described in Part I was used with appropriate modifications for the fractionation of extra protein preparations. It soon became obvious that the discontinuous technique used for the separation of sarcoplasmic proteins (Petropakis *et al.*, 1969) could not be utilized for the fractionation of extra protein samples since a majority of the protein remained at the site of sample application. In a series of preliminary trials, such experimental variables as gel concentration, buffer composition, pH, field strength and other factors which might affect the resolution of extra protein were examined. From these experiments it was concluded that an 8% running gel was optimal for the fractionation of extra protein and that the use of urea and dithiothreitol (Arai and Watanabe, 1968b) in preparing buffers and gels greatly improved the resolution. It was also found that after the addition of urea the electrode buffer lacked suitable buffering capacity. This deficiency was corrected by increasing the ionic strength of the electrode buffer.

In a typical run the spacer gel solution (4%) was prepared by dissolving 4 g of Cyanogum-41, 30 g (5 M) of urea and 0.0154 g (1 mM) dithiothreitol in 0.62 M Tris-HCl, pH 6.7, spacer buffer to make 100 ml. Plug and running gel solutions (8%) were prepared by

dissolving 16 g Cyanogum-41, 60 g (5 M) urea and 0.0308 g (1 mM) dithiothreitol in 0.76 M Tris-HCl running buffer, pH 9.0 to make 200 ml. The addition of TMED (N, N, N', N'-tetramethylethylene diamine), Tween 80, AP (ammonium persulfate) and the polymerization of gels was performed as usual. Electrode buffer, pH 8.6, was prepared by dissolving 2.4 g (0.01 M) Tris and 5.8 g (0.039 M) glycine in deionized water to make 2 liters of buffer. To a portion of this buffer, 600 g (5 M) of urea and 0.3086 g (1 mM) of dithiothreitol were added to make 2 liters of solution which was used for the electrophoretic run. Although buffer solutions were prepared in stock quantities for later use, 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). A "sample mixture" consisting of 15% sucrose in deionized water plus 5 M urea was used to dilute samples to the desired protein concentration of 5 mg/ml. Occasionally, a 20% sucrose solution or 8 M urea in 10% sucrose solution were used as "sample mixtures." A small quantity of bromophenol blue dye was added to each "sample mixture" to serve as migration guide during electrophoresis. The application of sample was done as usual. During the prerun, 200 V was applied until the sample was completely stacked and the sharp sample layer had moved to the surface of the running gel. The main electrophoretic run was carried out at 400 V. Buffer was not circulated during electrophoresis. Staining

and destaining of gels were done as described by Petropakis (1968).

Several electrophoretic runs of specified duration were carried out without samples to study the formation and migration of the leading ion-trailing ion boundary (Williams, 1964). In these runs the discontinuous buffer and gel systems described by Petropakis (1968) were used. Immediately after electrophoresis, gels were cut in horizontal strips 1 cm wide. These strips were dipped and eluted in deionized water and the resulting dilutions were used to form spots on chromatographic paper. The presence of the trailing ion, glycine, in the spots was detected by spraying the papers with 0.5% solution of ninhydrin in butanol. The chromatograms were dried in the hood and developed for 3-5 minutes in a 100°C oven. Pink color developed for positive tests.

All vertical gel electrophoresis runs were performed in a cold room (4-5°C). Gel patterns were photographed with a Polaroid MP-3 camera, type 55 P/N Polaroid film, red filter and a fluorescent light box for illumination.

Elution Convection

The elution convetion technique (Raymond, 1964; Raymond and Jordan, 1966) was employed to recover the proteins separated by vertical gel electrophoresis. An elution convection cell (Model EC-760, E-C Apparatus Corporation, Philadelphia, Pa.) and companion

power supply (Model EC-735) were used for this work. Instructions provided by the manufacturer were followed to assemble and operate the cell. A cold water-ethyl alcohol mixture (3°-4°C) was circulated continuously from an adjacent constant temperature refrigeration system through the cooling channels of the cell during the elution convection run. Electrophoresis preceding elution convection was performed on a 6 mm thick gel slab which was twice the thickness of the standard analytical gel. With the 6 mm gel a 4-slot form was used to produce slots accommodating up to 0.25 ml of sample solution in each slot. The gel slab to be eluted was trimmed so that it fitted securely in the elution grid. Compared with its original orientation in the electrophoresis cell, the gel slab was rotated 90° before being placed in the grid so that the protein bands were aligned in a vertical position above the tubules in which the eluants were to be recovered. Since the electrophoretic pattern must be eluted while proteins are in the undenatured state, the gels were not stained and the bands, with the exception of the dye zone which migrates ahead of proteins, were not visible. For this reason during the trimming of the gel slab a vertical strip was excised and stained for use as a guide for identification of the eluted fractions. Elution convection was performed as soon as possible after termination of the electrophoresis run to avoid extensive diffusion of protein bands and therefore, deterioration of the pattern.

The buffer employed in elution convection had the same composition as the 0.05 M electrode buffer (pH 8.6) used in the electrophoresis runs with the exception that it contained 4 M rather than 5 M urea. The use of buffer containing 4 M urea was necessary because a protein buffer solution (vertical gel electrophoresis was performed in 5 M urea medium) with a higher density than the protein-free buffer facilitates the downward movement of eluted bands to the collection tubules. Four liters of buffer were used for each elution convection run. Buffer was recirculated continuously during elution.

A constant potential of 20 volts (100 mA) was applied for a period of 8-10 hours. After completion of the elution convection run the gel slab was stained to detect whether any protein remained on the gel slab. Fractions of 1.0 to 1.5 ml were collected into the 31 tubules of the elution grid. The fractions were removed with a syringe fitted with polyethylene tubing, placed in small test tubes and stored at 2°C for future use.

The absorbance of fractions collected by elution convection was measured at 260 and 280 nm. Because of the small quantity of these fractions (1-1.5 ml) special micro-cuvettes were used.

Viscometric Measurements

Measurements of viscosities of a series of samples was carried out at 25°C in 0.01 M Tris buffer, pH 7.6 (Sugita *et al.*, 1969).

Ostwald viscometers which had water flow-through times of 80.9, 73.0, and 67.1 seconds were employed for the viscosity determinations. The viscosity of fractions obtained by elution convection was measured after these fractions were dialyzed against 0.01 M Tris buffer, pH 7.6 (T buffer) for 24 hours. Dialysis was necessary to rid the samples of urea as well as diluting them in the desired medium (T buffer). In dialyzed fractions, denoted by their tube number, increasing quantities of troponin preparation (TN) were added to check for a possible increase in viscosity which would indicate the presence of tropomyosin in these fractions. Dialyzed fractions were also freeze dried and the dried samples were added to tropomyosin preparations diluted to 0.025 mg protein/ml with T buffer, to check again for a possible increase of viscosity which would indicate the presence of troponin in these fractions. To study the effect of KCl concentration on the viscosities of tropomyosin preparations, increasing quantities of KCl were added and the viscosity of the resulting samples was measured. Tropomyosin preparations used for these measurements were dialyzed overnight against T buffer and then diluted with the same buffer to obtain a concentration of 3.0 mg protein/ml.

The relative viscosity (n_r) of the tropomyosin samples was calculated from the formula $n_r = \frac{n}{n_o} = \frac{d_o}{d} \cdot \frac{t}{t_o}$ where t and t_o are the flow times of the sample and water, respectively, and d, d_o equal densities of the sample and water (Kragh, 1961; Moore,

1964). For these measurements it was assumed that $\frac{d_o}{d} = \delta$ remained constant. The specific viscosity (n_{sp}) of elution convection samples was calculated from the equation $n_{sp} = \frac{d_o}{d} \cdot \frac{t}{t_o} - 1$ (Kragh, 1961; Moore, 1964) assuming that the density of samples does not differ significantly from the density of water ($\frac{d_o}{d} \simeq 1$) over the range of protein concentrations employed in these experiments.

RESULTS AND DISCUSSION

Preparation of Samples

In designing the experimental procedure, sampling times of 0 and 12 days were selected for studying changes occurring during aging. Such a sampling procedure would allow enough time for proteolytic changes to occur at a level pronounced enough to compare differences during aging. Proteolysis, if it actually proceeds at a significant level, is a progressive change occurring during aging. The sample at 0 day can be considered to represent the state at which little or no chemical or physical changes have yet occurred in the muscle system.

The increase in concentration of free amino acids during postmorten aging has been reported by many investigators (Niewiarowicz, 1956; Colombo and Gervasini, 1958; Davey and Gilbert, 1966; McCain et al., 1968; Field and Chang, 1969). According to Niewiarowicz (1956) the most common amino acids, with the exception of tryptophan, were detected on the first day of aging. Tryptophan, however, was found after 12 days (Niewiarowicz, 1956). After 2 weeks of aging the increase of most amino acids, excluding those from microbial action, tends to level off (McCain, et al., 1968). An increase in the percentage of myofibrillar proteins extracted during aging has also been

reported (Aberle and Merkel, 1966; Davey and Gilbert, 1968a). As it is shown in Figure 2, adapted from Davey and Gilbert (1968a), the extractability of myofibrils is at maximum after approximately 12 days of aging. For these reasons a 12 day of aging sampling was selected.

In all of the above references the changes occurring during aging were considered to be independent of bacterial action. In order to limit bacterial spoilage in this study, samples aged in the cold room (3-4°C) were occasionally sprayed with a solution containing 100 ppm aureomycin and 100 ppm chloroamphenicol (Davey and Gilbert, 1968b).

In extracting the soluble nitrogen compounds with 0.03 M potassium phosphate buffer, centrifugation at 30,000 x G (Petropakis et al., 1969) was alternatively employed for duplicate samples. This was to assure a better separation of the soluble nitrogen compounds from the residues which were used later for the preparation of myofibrils.

For the extraction of myofibrillar proteins, the potassium iodide solution recommended by Lawrie (1961) was not used but one modified to contain KCl instead of KI was employed. This change was necessary because iodide is oxidized by air especially in the presence of light (Day and Underwood, 1964) and on occasion, iodine was found to be liberated during the several hours of sample preparation. A solution containing 0.25 M sucrose, 1 mM EDTA and 0.05 M Tris,

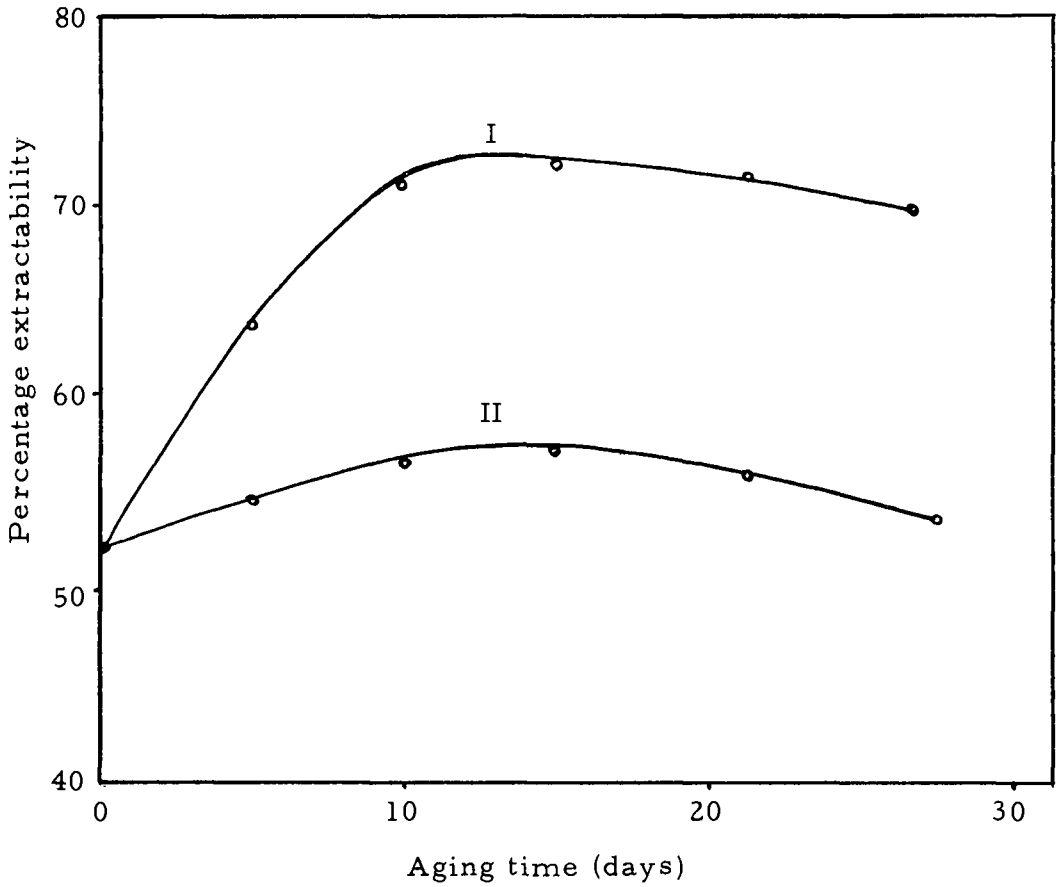


Figure 2. Effect of aging on the percentage extractability of myofibrils. Curve I, ultimate pH 6.03. Curve II, ultimate pH 5.50. Adapted from Davey and Gilbert (1968a).

pH 7.6, was also used (procedure IV) to extract the myofibrils. According to Goll and Robson (1967), sucrose should be included in the extraction solution because of its universal use in the preparation of subcellular organelles such as mitochondria and lysosomes. In this study, three different solutions were used to extract myofibrillar proteins in order to elucidate the possible effects of the extraction medium on myofibrillar preparations.

In a series of perliminary experiments, no significant differences were found in the nitrogen content of the samples obtained by procedures I and II. In addition, the electrophoretic and gel filtration patterns of these samples were very similar. The same trends were evident for the samples obtained by procedures III and IV. Thus, only procedures I and III were followed for the preparation of samples in the main experiments.

In preparing extra protein, dialysis against 6.7 mM potassium phosphate buffer, pH 7.2, containing 0.04 M KCl, proved to be a critical step. When subjected to vertical gel electrophoresis, samples containing large amounts of salts produced poorly resolved patterns. The reduction of the ionic strength of samples to 0.03-0.05 yields superior resolution (Ritchie, 1967). For this reason, after the determination of protein content, the freeze dried extra protein preparations were diluted with deionized water to a concentration of approximately 15 mg protein/ml. These diluted extra protein

preparations were dialyzed against the above dialysis solution for 24 hours (4°C) and then were freeze dried again. Freeze dried samples thus obtained were diluted with 3 volumes of "sample mixture," and used for the electrophoretic separation of extra protein. The extra protein samples so prepared contained approximately 5 mg protein/ml and had an ionic strength within the desired limits. The use of these samples greatly improved the electrophoretic patterns.

In the preparation of troponin and tropomyosin, 2-mercapto-ethanol and dithiothreitol were used to prevent oxidation of sulfhydryl groups (Mueller, 1966; Woods, 1967). Dithiothreitol was also used as a sulfhydryl protective agent in the electrophoretic separation of extra protein and tropomyosin samples. Tropomyosin obtained during the preparation of troponin by isoelectric precipitation at pH 4.6 was the Bailey-type tropomyosin (tropomyosin B). In preparing troponin the amount of solid $(NH_4)_2SO_4$ needed to obtain the required percentage of saturation was calculated by the nomogram method of Dixon and Webb (1964).

Generally, after preparation, samples were freeze dried. By this means, concentrated samples were obtained which were stable during storage at 3-4°C and were convenient to handle (weigh, dilute, etc.).

Part I. Soluble Proteins and Low Molecular Weight Nitrogen Compounds

Gel Filtration

Sephadex G-25 having exclusion limits in a molecular weight range of 1,000-5,000 was used to separate the peptides and low molecular weight nitrogen compounds from the larger sized protein components. In a previous study (Petropakis *et al.*, 1969), water soluble (sarcoplasmic) proteins were separated by vertical gel electrophoresis using the discontinuous technique. Since the mobility of sarcoplasmic proteins in a 20% gel is very low, a 20% plug gel should retard or "catch" low molecular weight proteins that might migrate unobstructed and very rapidly in a 10% running gel. Electrophoretic patterns obtained from these runs of several hours duration did not show the presence of such proteins. However, the possibility still existed that some relatively low molecular weight polypeptides might possibly avoid the sieving effect of a 20% gel. For this reason the use of Sephadex G-25 was justified. Moreover, the main objective of this phase was to determine whether low molecular weight polypeptides were present which could be degraded during aging and thus serve as a source of free amino acids. If such were the case then the increase in the concentration of amino acids during aging could be explained.

In gel filtration, samples having a high viscosity can cause irregular flow and therefore, patterns of very poor resolution (Pharmacia, 1969). Thus a series of preliminary experiments were completed wherein several sample dilutions were tried. A dilution of 1 g of freeze dried sample with 15 ml of deionized water produced satisfactory results. Samples were diluted to this concentration for the main experiments. Gel filtration patterns were highly reproducible provided that well-packed columns were used.

Typical gel filtration patterns of samples 0-E and 12-E are presented in Figure 3. These patterns were obtained by monitoring the effluents of each gel filtration run at both 280 nm and 265 nm using two UV-monitors connected in series. The simultaneous monitoring at 280 and 265 nm gave similar patterns. With the exception of the main peak, however, absorbance at 280 nm was much less than at 265 nm. The pattern for the 0-day sample (Figure 3a) shows that the main peak (0-GF) was followed by a definite peak (0-1) plus two additional peaks (0-2 and 0-3) which are somewhat difficult to detect. The 12 day-sample pattern (Figure 3b) shows, in addition to the main peak (12-GF), three definite peaks designated as 12-1, 12-2 and 12-3. A somewhat pronounced shoulder appearing on the left slope of peak 12-1 might be considered as a fourth peak (?) in this pattern. The components of the main peaks, 0-GF and 12-GF (Figure 3a and b), are assumed to be sarcoplasmic proteins. This conclusion

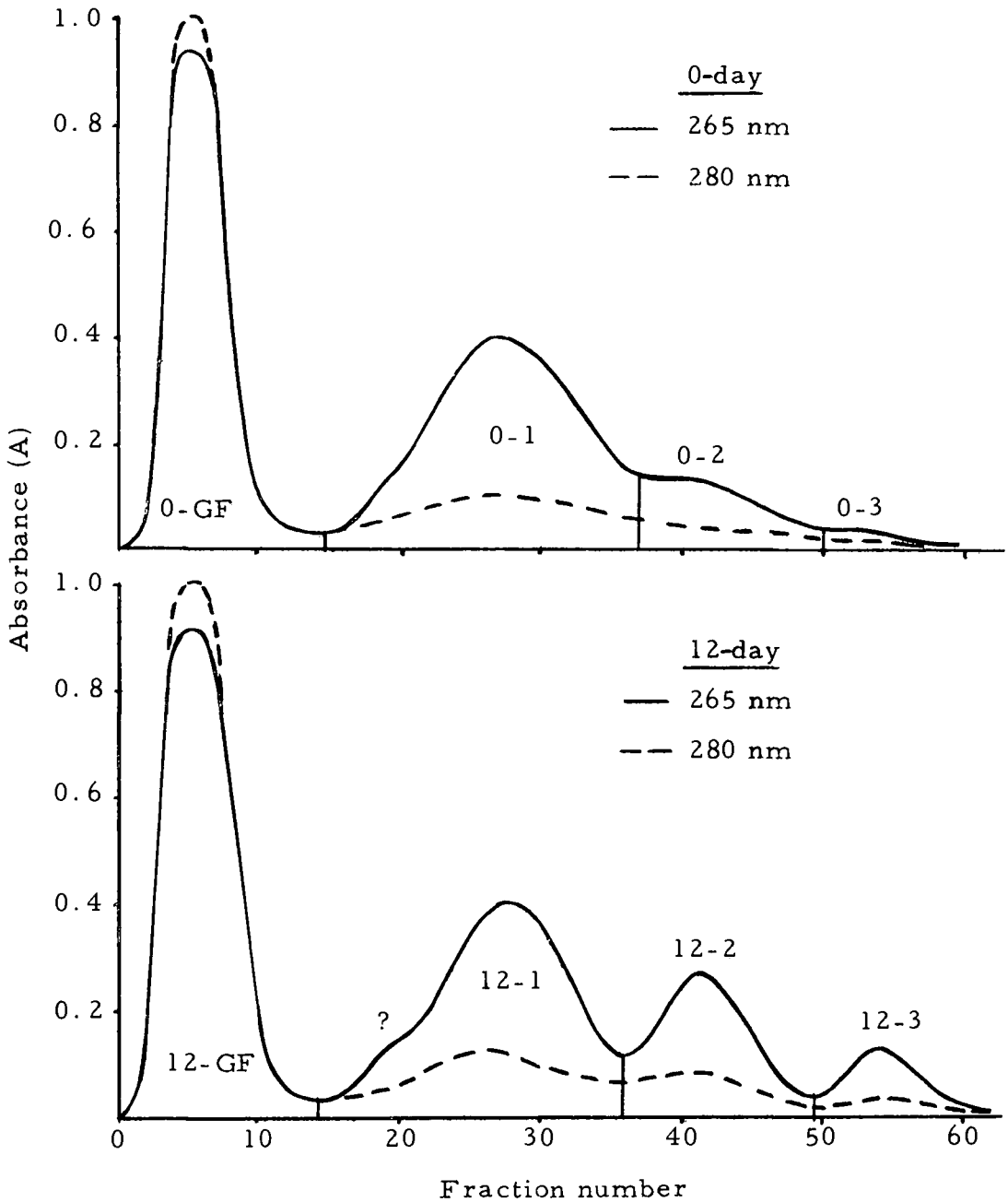


Figure 3. Elution patterns of extracts of samples 0-E and 12-E separated on Sephadex G-25 columns. Vertical lines indicate division of fraction areas. Flow rate of 45 ml/hr.

was based on the high absorbance of these peaks at 280 nm plus the fact that this material was eluted first during gel filtration. After elution of the main peaks (0-GF and 12-GF), absorbance of eluants at 280 nm was quite low. This low absorbance can be interpreted as evidence that protein-like nitrogen compounds are present in very small amounts, if at all, in the eluants of fraction areas 0-1 and 12-1 and practically absent in fraction areas 0-2, 0-3, 12-2 and 12-3. The much higher absorbance of these fractions at 265 nm suggests the presence of nucleic acid material which absorbs much more strongly at 260 nm than at 280 nm (Layne, 1957; Wetlaufer, 1962).

As indicated above, peaks 12-2 and 12-3 appear more pronounced than peaks 0-2 and 0-3 (Figure 3a and b). This finding would indicate that the amount or at least the presence of low molecular weight soluble compounds increase during aging. An increase of non-protein nitrogen during aging has been noted by Davey and Gilbert (1966).

Spectrophotometric scanning from 340 and 220 nm of the gel filtration fraction areas resulted in absorbance spectra that support the above remarks. Typical spectrophotometric scanning patterns of fraction areas 0-1, 0-2, 0-3, 12-1, 12-2 and 12-3 are presented in Figure 4. From these data, it can be seen that the peaks of absorbance were closer to the optimum ultraviolet absorption region of nucleic acids. The ratio of absorbance (A_{280nm}/A_{260nm}) indicates a

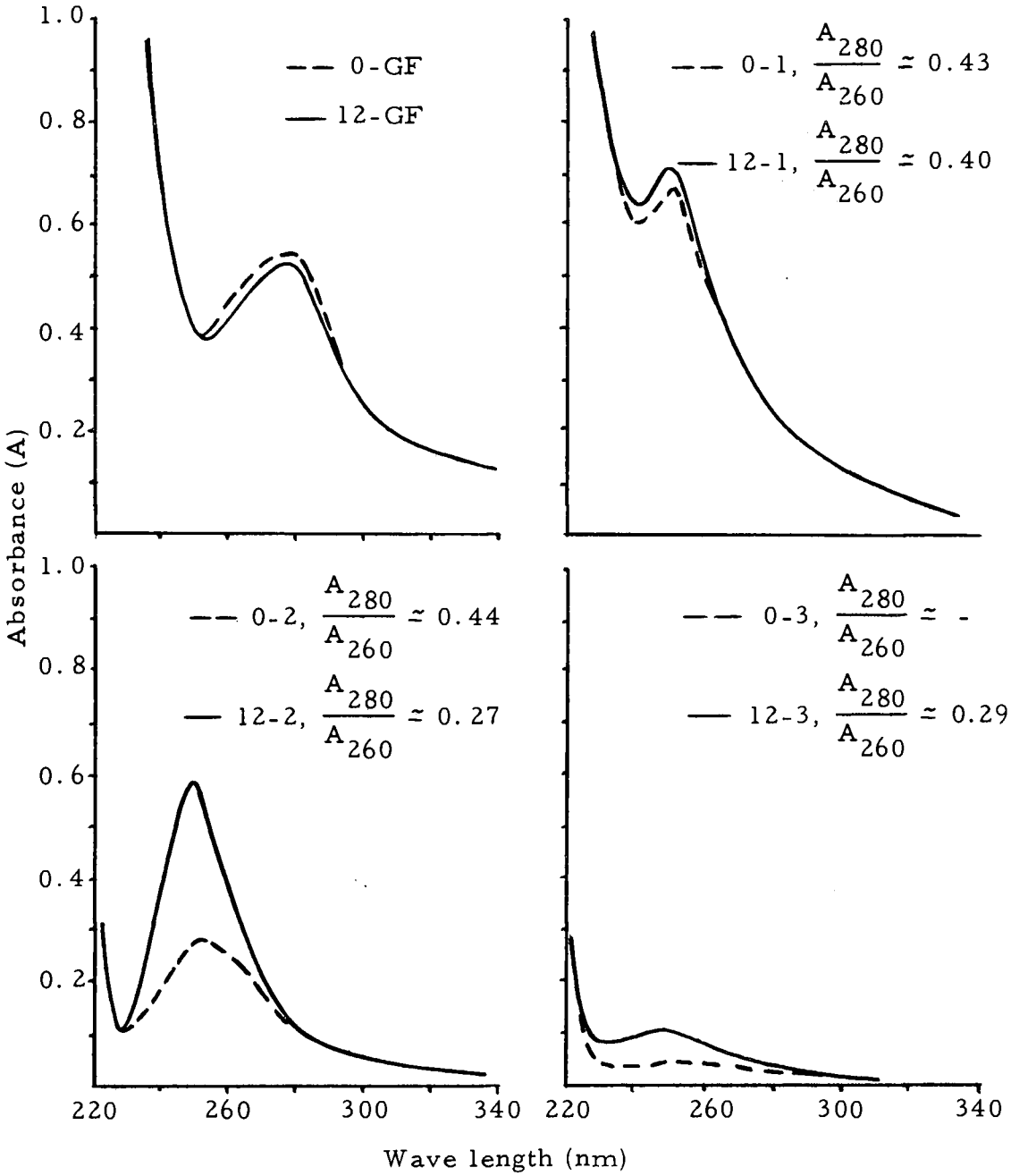


Figure 4. Ultraviolet absorption spectra of gel filtration fractions. Dilutions: Samples 0-GF, 12-GF; 0.1 g freeze dried sample in 60 ml water. Samples 0-1, 12-1, 0-2, 12-2, 0-3, 12-3; 0.04 g freeze dried sample in 6 ml water.

higher nucleic acid content in fractions 12-2 and 12-3. Figure 4 also shows the ultraviolet absorption spectra of fractions 0-GF and 12-GF. The high absorbance at 280 nm indicates once again the presence of protein in these fractions.

Protein and Nitrogen Content of Samples

Protein contents of samples 0-E, 12-E and 0-GF, 12-GF are presented in Table 3. These data were used to determine the appropriate dilution of samples with "sample mixture" before gel electrophoresis. As shown in the same table the nitrogen content of samples 0-GF (np) and 12-GF (np) was found to be zero. This indicates that the main peak of the gel filtration patterns corresponds to the protein fraction of samples 0-E and 12-E while the other peaks contained the separated nonprotein compounds.

The total nitrogen content of the muscle tissue is presented in Table 4. Nitrogen contents of soluble nonprotein nitrogen compounds (samples 0-E (np), 12-E (np)), sarcoplasmic proteins, myofibrillar proteins (samples 0-MYO, 12-MYO) and stroma proteins are given also in Table 4. The nitrogen due to sarcoplasmic proteins was obtained by subtracting the nonprotein nitrogen (sample 0-E (np) or 12-E (np)) from total soluble nitrogen (sample 0-E or 12-E). Stroma protein nitrogen was determined by subtracting the sum of total soluble nitrogen and total myofibrillar nitrogen from the total nitrogen of the muscle tissue.

Table 3. Protein content of soluble protein extracts before and after gel filtration.

Sample	Kjeldahl		Ultraviolet Absorption		
	% N	Protein Content[a]	$\frac{A_{280\,nm}}{A_{260\,nm}}$	Nucleic Acid %	Protein Content[b]
0-E	4.25	265.5	0.80	6.5	275.0
0-E (np)	1.32	82.5	--	-	---
0-GF	4.79	299.0	1.25	1.5	306.0
0-GF (np)	0	0	--	-	---
12-E	4.50	281.0	0.79	6.6	277.5
12-E (np)	1.54	96.0	--	-	---
12-GF	4.55	284.5	1.26	1.4	296.5
12-GF (np)	0	0	--	-	---

[a]Protein content = % N x 6.25, mg/g freeze dried sample.

[b]Protein content in mg/g freeze dried sample.

Table 4. Nitrogen fractions in 0 and 12 days of aging muscle.

Nitrogen Fraction	0-day Sample		12-day Sample	
	% N	As % Total N	% N	As % Total N
Total	3.02	100.0	3.05	100.0
Sarcoplasmic protein	0.61	20.4	0.63	20.7
Soluble nonprotein	0.27	9.0	0.32	10.5
Myofibrillar protein	1.50	49.7	1.52	50.2
Stroma protein	0.64	20.9	0.58	18.6

As early as in 1953, Ginger et al. reported that two weeks of aging increased the nitrogen content of the nonprotein fraction of muscle. Locker (1960) observed small increases in nonprotein nitrogen after either 3 or 16 days post-mortem aging. Sharp (1963) in studying aseptic autolysis of bovine muscle found an increase of nonprotein nitrogen during extended storage. Kronman and Winterbottom (1960) working with the longissimus dorsi muscle reported that sarcoplasmic proteins were most extractable immediately after death but their solubility decreased during post-mortem aging. Similar findings were reported by Goll et al. (1964) for the semitendinosus muscle but they added that statistical analyses revealed no significant differences among the amounts of sarcoplasmic protein extracted at different post-mortem times. Aberle and Merkel (1966) found that nonprotein nitrogen increases significantly during aging. The same investigators noted that the amount of sarcoplasmic nitrogen of the semitendinosus muscle decreased during post-mortem aging while sarcoplasmic nitrogen of the longissimus dorsi muscle did not change significantly. In general, results of this study agree with the above references. As shown in Table 4, soluble nonprotein nitrogen increases with aging. The increase of nonprotein nitrogen during aging can be related to the increase in the height of peaks 12-2 and 12-3 of the gel filtration pattern of the 12-day sample. Statistical analyses did not show significant differences ($P < 0.05$) between the amount of sarcoplasmic

proteins extracted at 0 and 12 days of aging (Table 4).

According to Davey and Gilbert (1968a) the extractability of myofibrillar proteins increases during aging. They also added that a small decline in extractability on prolonged aging (>17 days) is due probably to incipient denaturation of myofibrillar proteins during storage. Previously, it had been reported (Goll et al., 1964) that statistical analyses revealed no significant differences among the amounts of myofibrillar proteins extracted during 13 days of aging. In this study, a slight increase of myofibrillar nitrogen with aging was observed (Table 4) although again, these differences were not statistically significant ($P < 0.05$).

Vertical Gel Electrophoresis

Vertical gel electrophoretic patterns of samples 0-E, 0-GF, 12-E and 12-GF are presented in Figure 5. As shown in this figure it is difficult to state that consistent electrophoretic changes were noted with aging. Electrophoretic patterns of samples 0-E and 12-E corresponding to the total soluble nitrogen compounds at 0 and 12 days of aging are quite similar. The same is true for patterns of samples 0-GF and 12-GF corresponding to the soluble (sarcoplasmic) protein at 0 and 12 days of aging after separation of the low molecular weight compounds by gel filtration. These findings are in agreement with those reported in a previous study of separation of sarcoplasmic

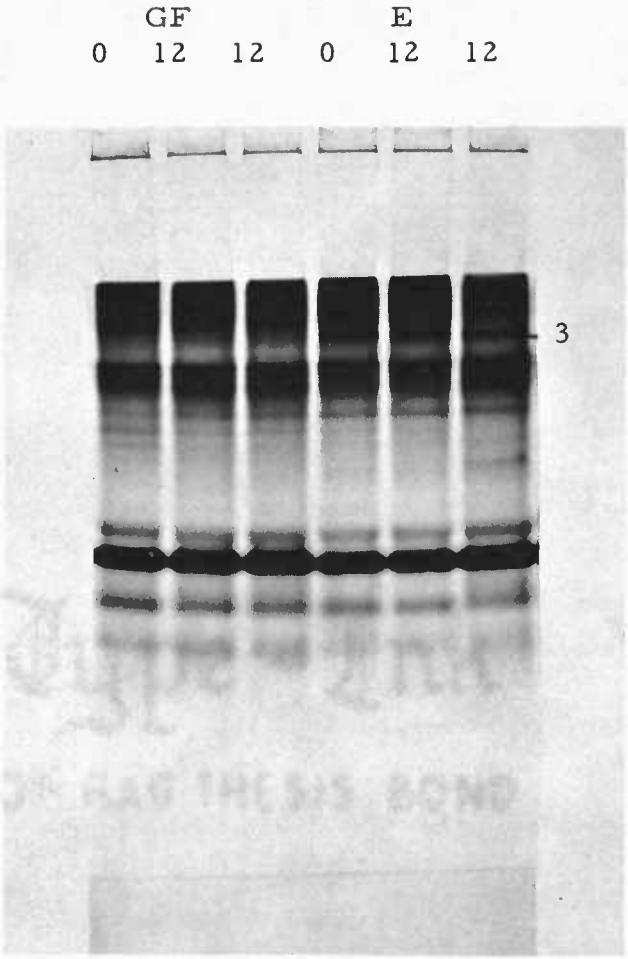


Figure 5. Electrophoretic patterns of samples 0-GF, 12-GF, 0-E, and 12-E. Electrophoretic conditions: buffer, discontinuous system; length of prerun, 1 1/2 hrs at 200 V (spacer gel); length of run, 4 hrs at 400 V (running gel). Band 3 is designated at the right margin of the pattern.

proteins by vertical polyacrylamide gel electrophoresis (Petropakis et al., 1969). Aberle and Merkel (1966) also reported that electrophoretograms obtained from starch gel electrophoresis of sarcoplasmic proteins were very similar at 1, 7 and 14 days post-mortem aging.

Much more evident were pattern differences between samples before (0-E, 12-E) and after gel filtration (0-GF, 12-GF). Petropakis et al. (1969) previously reported that band 3 in the electrophoretic pattern obtained with sarcoplasmic extract dialyzed against distilled H_2O did not appear in the pattern obtained from the undialyzed sample. In the present study the same band is not visible in patterns obtained from the gel filtered samples (Figure 5). Therefore, it can be concluded that this band corresponds to low molecular weight nitrogen compounds lost during dialysis or separated during gel filtration. Since the electrophoretic migration of this band is relatively low in spite of its low molecular weight, its negative charge must be very small. Such a small negative charge at the high pH (pH 8.9-9.4) of the running gel during electrophoresis cannot be easily attributed to a typical polypeptide. No other differences are obvious in the electrophoretograms obtained from the sample before and after gel filtration. Therefore, the presence of polypeptides in detectable amounts in the low molecular weight nitrogen compounds separated by gel electrophoresis is questionable. This same

concludion was drawn from the data of ultraviolet scanning and chromatography-high voltage electrophoresis of the gel filtration fractions. Even if such polypeptides are derived from the possible proteolytic breakdown of proteins during aging their concentration is so small that they cannot be detected by the techniques used although chromatography-high voltage electrophoresis is especially sensitive.

Two-Dimensional Chromatography-High Voltage Electrophoresis

An early attempt to combine electrophoresis with partition chromatography on paper was reported by Haugaard and Kroner (1948). Since then two-dimensional chromatography-electrophoresis (peptide mapping) has been widely used for the separation of peptide preparations (Katz *et al.*, 1959; Katz and Carsten, 1963; Katz and Converse, 1964; Carsten and Katz, 1964; Carsten, 1968). This technique was used in this study because of the hypothesis that peptides and/or low molecular weight charged nitrogen compounds were present in fractions obtained by gel filtration of samples 0-E and 12-E.

In a series of preliminary chromatography-high voltage electrophoresis runs, using partially desalted samples, the results were disappointing. Patterns obtained showed several smeared spots and thus poor resolution. Katz *et al.* (1959) employing the same technique to separate peptides from proteolytic digestion mixtures indicated that satisfactory separation could be obtained only if the final mixture

was free of salts. Therefore, in this study, in order to improve the resolution and obtain clearer patterns, much effort was aimed in the desalting of gel filtration fractions to be separated by two-dimensional chromatography-high voltage electrophoresis. Of the desalting methods described in the experimental procedure, ultrafiltration proved to be the most effective means for removing the potassium phosphate salt from fractions 0-1, 0-2, 0-3, 12-1, 12-2, and 12-3. After repeated dilutions and filtrations the samples obtained were practically free of salt. The final salt concentrations or the number of repetitive dilutions were calculated using the following formula (Amicon Corp., 1969)

$$C_f = \left(\frac{V_o}{V_d}\right)^n \times C_o$$

where

C_f = the final salt concentration

C_o = initial salt concentration

V_o = initial sample volume

V_d = volume after dilution

n = number of repetitive dilutions.

The initial salt concentration was assumed to be approximately 0.03 M since the elution buffer had the same molarity.

In preliminary ultrafiltration experiments, Diaflo UM-05

membranes were used but poor results were obtained. This was attributed to the fact that these membranes have a net anionic charge on their polymeric backbone (Amicon Corp., 1969). Diaflo UM-2 membranes were finally selected since they have a neutral charge and cut-off level at 1000 MW which was within the desired limits. Because of the neutral charge there was no interference with the permeability of salts through the membranes. These membranes were used for all of the main experiments with very satisfactory results.

The progress of filtration was checked qualitatively by $AgNO_3$ and was followed by ultraviolet scanning of dilutions of freeze dried retentate volumes. Typical scanning patterns obtained during desalting of gel filtration fractions are presented in Figure 6. Higher ultraviolet absorbance of the ultrafiltered freeze dried samples indicates an increase of concentration of the macrosolutes (>1000 MW) and a decrease of the concentration of salt (microsolute). The end of ultrafiltration was characterized by samples of high ultraviolet absorbance and negative $AgNO_3$ reaction.

The amount of freeze dried samples obtained after ultrafiltration of fractions 0-2 and 0-3 was minimal. In addition, these fractions were barely distinguishable with considerable overlapping existing between them and fraction 0-1. Thus for the main experiments, fractions 0-1, 0-2 and 0-3 were combined to give sample 0-1, 2, 3. Also, fractions 12-2 and 12-3 were combined into one sample for similar reasons (sample 12-2, 3). Fraction 12-1 and the above combined

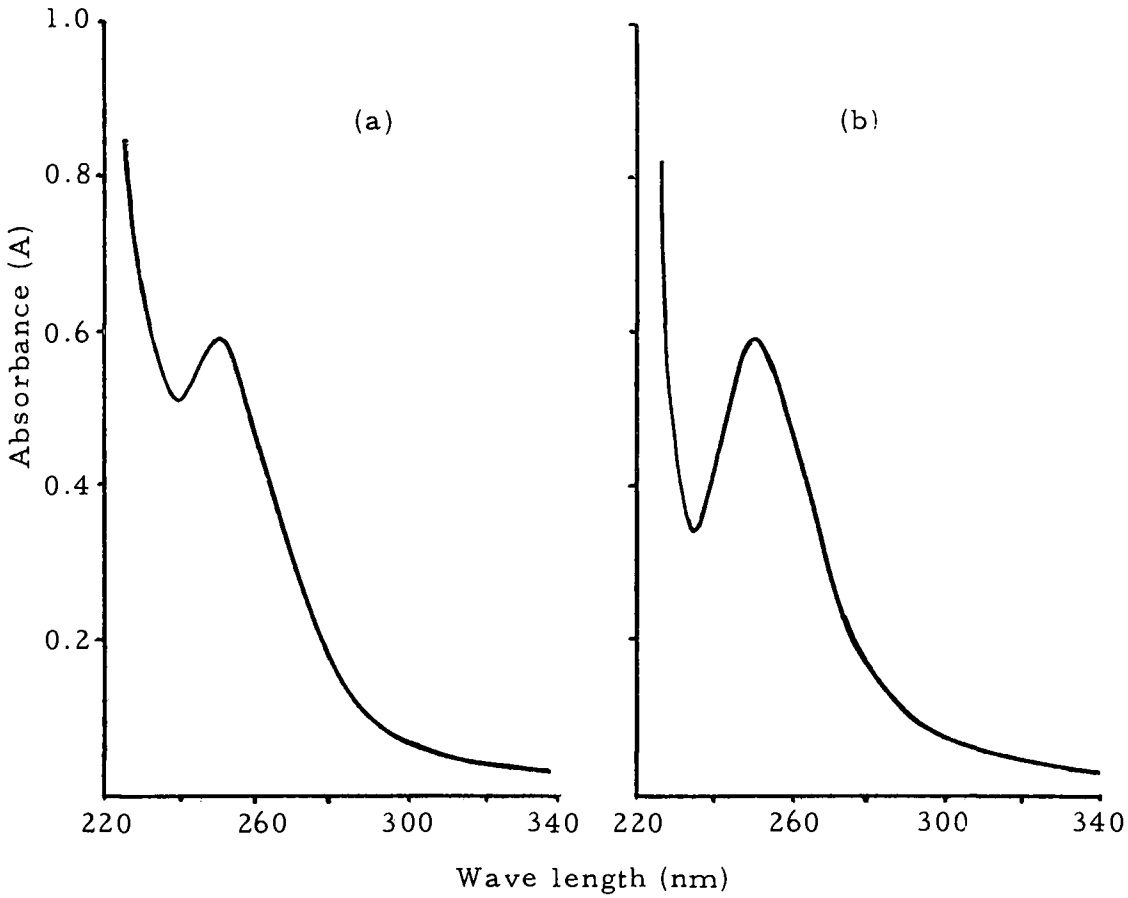


Figure 6. Typical ultraviolet scanning patterns of retentate volumes during ultrafiltration showing the progress of desalting. Sample: 0-1, 2, 3. (a) Eluants from gel filtration (50 ml) were diluted with water (1:5 v/v) and ultrafiltered. The retentate volume (50 ml) was freeze dried, diluted with 2 ml of H_2O and scanned. (b) After repeated ultrafiltrations the last retentate volume (50 ml) was freeze dried, diluted with 40 ml of water and scanned.

samples, after desalting and freeze drying, were diluted with deionized water and used in the main experiments.

Patterns obtained by two-dimensional chromatography-high voltage electrophoresis are presented in Figure 7. Descending paper chromatography was applied from left to the right. The direction of high voltage electrophoresis was from bottom to top with the cathode at the top of the paper. Well-developed spots were outlined with a continuous line and labeled with numbers prior to photography for easier identification. Faint spots were outlined with a dotted line. No variation in color of the developed spots was observed by spraying the paper with cadmium acetate-water-acetic acid-acetone-ninhydrin solution or ninhydrin-butanol solution. In parallel experiments spots of dilutions of commercially available amino acids, RNA, DNA, carnosine, anserine on Whatman No. 3MM chromatographic paper were sprayed with both of the above solutions. After development, a wide variety of colored spots was obtained. Many spots gave different colors for each of the above spray solutions. Comparing these colors with the color of spots obtained by chromatography-electrophoresis it was found that the color of the latter spots was quite similar with that of spots corresponding to nucleic acids. This was evident with both spray solutions. This observation strongly indicates the presence of nucleic acid (nucleotides or fractions of nucleoproteins) in the fractions obtained by gel filtration of samples 0-E and 12-E. As

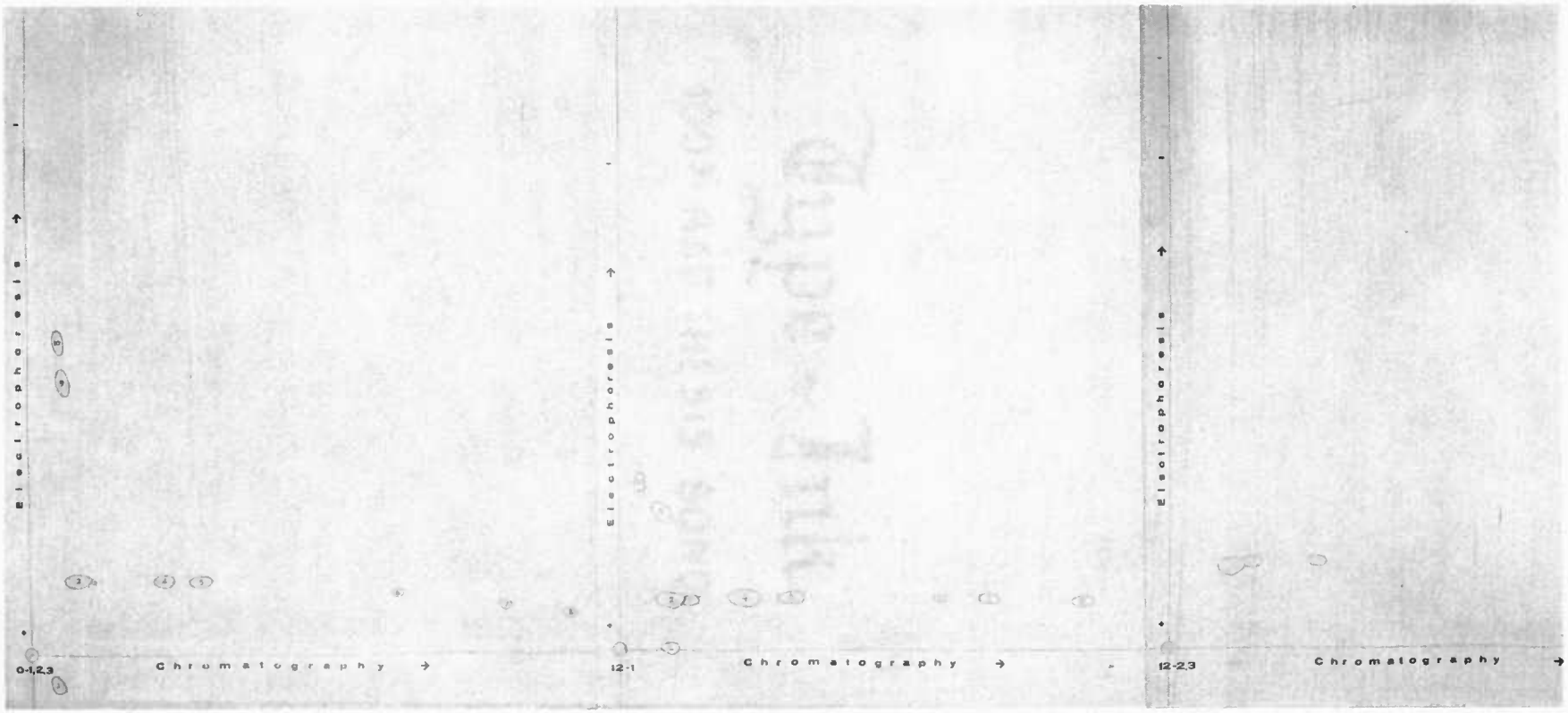


Figure 7. Two-dimensional chromatography-high voltage electrophoresis of samples 0-1, 2, 3 and 12-1, 12-2, 3.

mentioned previously, the same was evident from data obtained by ultraviolet scanning of the same fractions.

Patterns obtained from 0-1, 2, 3 and 12-1 samples appear to be similar (Figure 7). The pattern of sample 12-2, 3 shows three spots which can be interpreted to correspond with spots 2, 3 and 4 of pattern of sample 12-1. The chromatographic system tends to separate the polar from nonpolar peptides while electrophoresis separates the polar peptides according to their charge (Katz et al., 1959). Therefore, spots 2, 3, 4, 5, 6, 7 and 8 which migrated approximately the same distance during electrophoresis can be assumed to have similar charge density but different polarity. Migration towards the cathode indicates that all spots with the exception of spot 1 were positive charged at pH 3.7, the pH of buffer solution used in high voltage electrophoresis.

Differences in patterns observed with aging are not conclusive. In the 12-1 sample pattern the presence of faint spots was more evident than in the pattern of sample 0-1, 2, 3. Differences in the migration rate of the corresponding spots of 0 and 12 day samples should be interpreted with caution. The migration rate of spots during electrophoresis depends heavily on the extent of impregnation of paper with buffer solution, something that cannot be controlled with accuracy. In other words, different migration distances in electrophoresis when all the other experimental conditions are similar does not necessarily

indicate differences in the charge of the spots. Even though the same voltage (3000 V) was applied each time in a number of electrophoretic runs, it was observed that the field strength (current) differed considerably (80-130 mA) from run to run. In summary, differences observed between patterns of 0-day and 12-day samples are not of the magnitude to support specific conclusions about possible changes occurring in the low molecular weight nitrogen compounds during aging.

Part II. Extra Protein

Vertical Polyacrylamide Gel Electrophoresis

As was stated in the experimental procedure in order to improve the electrophoretic resolution of extra protein and tropomyosin preparations the addition of urea and dithiothreitol during the preparation of buffers and gels was necessary. Urea was also used in the preparation of "sample mixtures." Other experimental modifications of the regular discontinuous technique used for the electrophoretic analysis of soluble proteins (Part I) were the use of a new electrode buffer and an 8%, rather than a 10%, running gel. It was also observed that highly resolved and reproducible electrophoretic patterns were obtained only when the ionic strength of the samples was very low.

The use of urea in gel electrophoretic studies of myofibrillar proteins has been suggested by many investigators (Woods, 1965, 1966, 1967; Arai and Watanabe, 1968a, b; Carsten, 1968; Schuab and

Perry, 1969; Champion et al., 1970). Both Dewey (1970) and Rampton (1969) found that tropomyosin would not penetrate polyacrylamide gels when urea was deleted from the system. The high viscosity of tropomyosin preparations in the absence of urea suggests that this protein forms aggregates too large to penetrate the porous gel (Dewey, 1970). In this study, when preliminary electrophoresis runs of several hours duration were performed without the addition of urea to the system it was observed that a majority of extra protein remained at the point of sample application. In other words the sample would not penetrate even the 4% spacer gel. Samples of extra protein, which contained tropomyosin (Perry and Zydowo, 1959a), and the tropomyosin used for the electrophoretic runs had a low ionic strength although aggregation of tropomyosin in such solutions is well known (McCubbin et al., 1967; McCubbin and Kay, 1969). These points explain why the use of urea was necessary. In all of the main experiments urea was used for the preparation of spacer, running and plug gels, electrode buffer and the "sample mixture." Usually the sample preparation was diluted with the "sample mixture" containing urea two hours before electrophoresis. No differences were observed in the electrophoretograms when the sample and "sample mixture" dilutions were left for longer times, up to 8 hours, before electrophoresis.

The use of reducing agents during polyacrylamide gel electrophoresis of proteins sensitive to oxidation has been recommended by

many investigators. In polyacrylamide gel electrophoresis, gels are formed when aqueous solutions of Cyanogum-41 (mixture of 95% acrylamide and 5% methylenebisacrylamide) (E-C Apparatus Corporation, 1966) are polymerized using an oxidation-reduction system, usually an amine such as TMED (tetramethylethylene diamine) with ammonium persulfate (White, 1960). Residues of ammonium persulfate (AP), a strong oxidizing agent, can produce oxidation artifacts during the gel electrophoresis run. Fantes and Furminger (1967) reported that a number of quite diverse proteins were affected even by a low concentration of either acid or alkaline AP present in the polyacrylamide gel or buffer. During disc electrophoresis of yeast enolase in polyacrylamide gels polymerized by AP, Brewer (1967) was able to reduce two characteristic bands to one by adding thioglycolate to the system or using riboflavin as catalyst instead of AP. He concluded that AP can inactivate enolase and produce increased electrophoretic heterogeneity. In using polyacrylamide gel disc electrophoresis to study clostridiopeptidase B, Mitchell (1967) confirmed the oxidizing effects of residual persulfate upon a protein especially susceptible to oxidation. Multiple bands of electrophoretic patterns of tropomyosin have been attributed to artifacts due to residual AP in the gels by many workers (Woods, 1967; Arai and Watanabe, 1968a, b; Yasui _et al._, 1968; Rampton, 1969; Dewey, 1970). It has also been reported that the addition of sulfhydryl protecting agents results in a

reduction of bands in the above electrophoretic patterns (Woods, 1967; Yasui et al., 1968; Parsons et al., 1969; Dewey, 1970). In this study, preliminary electrophoretic runs performed without dithiothreitol to protect the -SH groups, produced patterns with multiple bands and random reproducibility.

Many procedures have been proposed for avoiding artifacts caused by residual persulfate from gel polymerization. Fantes and Furminger (1967) and Brewer (1967) recommended the use of riboflavin instead of AP for gel polymerization. Although riboflavin was used several times in this study its use was finally rejected because riboflavin-TMED is a photocatalyst system and polyacrylamide gels so catalyzed must be exposed to light for relatively long times. Moreover, the persulfate-TMED catalyst system yields gels of more reproducible pore size (Davis, 1964), a property that is very important. With the use of AP, it was also observed that polymerization occurs in shorter time, usually within 15-20 minutes.

It also has been reported that ammonium persulfate can be removed in disc electrophoresis prior to application of sample by an electrophoretic prerun for 2 hours at approximately 4-5 mA per tube and then replacing the contaminated buffer with fresh solution for the main run (Mitchell, 1967). A similar prerunning technique for removing excess AP was reported by Schaub and Perry (1969). Jordan and Raymond (1967) describing the discontinuous technique of vertical

gel electrophoresis reported that catalyst (AP) residues can be removed from gels by pre-running with no samples in place. They also added that the sample solution should be diluted with polymerized acrylamide solution dialyzed against the appropriate buffer until free of catalyst residues. In the case of the prerunning proposed by Mitchell (1967), the upper spacer gel was eliminated and the sharp stacking of sample was compensated by simply giving the sample a lower conductivity than the running buffer (Hjerten et al., 1965). In other words a continuous system was used and therefore, the prerunning while removing the residual AP, does not have any effect on the main electrophoresis run which follows. However, in the case of the discontinuous technique, pre-running without sample to remove catalyst residues cannot be applied for the following reasons. In the discontinuous technique of vertical gel electrophoresis, the polyacrylamide gel consist of 3 sections: a) a large-pore spacer gel (4%) in which the sample is introduced in slots and is electrophoretically concentrated in a sharp layer; b) a small pore running gel in which the sample is separated electrophoretically and by molecular sieving; and c) a small pore plug gel to support in place the gel system and probably catch fast migrating proteins (Petropakis, 1968). This gel system is combined with a discontinuous buffer system which consists of spacer, running and electrode buffers. In Part I of this study, the discontinuous anionic buffer system was used consisting of 0.11 M

Tris-HCl spacer buffer, pH 6.7, 0.42 M Tris-HCl running buffer, pH 8.9, and 0.04 M Tris-glycine electrode buffer, pH 8.3 (Petropakis et al., 1969). In Part II, a 0.05 M Tris-glycine electrode buffer, pH 8.6, was used and urea was added to all three buffers of the system. In these buffer systems, the chloride ion is the leading ion with high electrophoretic mobility (higher than the proteins) independent of pH while glycine (pK = 9.8) is the trailing ion. According to Williams and Reisfeld (1964), at the beginning of the run a leading ion-trailing ion boundary is formed which moves down from the top of the spacer gel as the run progresses. In the spacer gel the trailing ion has a lower mobility than any of the proteins and thus the boundary sweeps up the protein sample which enters the running gel as a sharp layer. In the running gel because of higher pH (the actual running pH is about 9.2-9.4), the trailing ion moves faster than any protein and the boundary leaves the proteins behind. The proteins in a thin starting zone and without the presence of the boundary are then fractionated electrophoretically (Williams and Reisfeld, 1964). From the above discussion it is obvious that prerunning to remove residual AP before sample application would affect the main electrophoretic run which follows.

The prerunning without samples in place was studied in several electrophoretic runs of specified duration. In these runs the migration of leading ion-trailing ion boundary was checked for the presence

or absence of glycine in horizontal strips in which the gels were cut after the prerun. The results of these studies are presented in Figures 8 and 9, and Table 5. As is shown, during the preruns the boundary has moved far from the origin (where the sample is applied) and after 1.5 hours even beyond the spacer gel-running gel interface. After prerunning, when the sample is applied the sweeping boundary does not exist and the protein is not stacked in the spacer gel in a thin layer. Therefore, the advantage of sharp electrophoretic zoning inherent in the discontinuous technique cannot be attained and the use of the discontinuous system becomes ineffective.

In several runs the migration of boundary in the spacer gel was also observed by means of the blue colored band of dye (bromphenol blue) used in place of sample. The plot of migration distance of the boundary versus time is presented in Figure 8 which shows that the boundary moves faster as it enters the running gel because of the pH discontinuity between spacer and running gels and the higher voltage. As shown in Table 5, presenting the pH measurements of the gel strips, the initial pH discontinuity is disturbed after the prerun.

In studying tropomyosin B preparations by disc electrophoresis with polyacrylamide gel, Woods (1967) reported that the protein had to be run in the presence of a reducing agent to avoid the oxidation of -SH groups by residual AP. For this reason, he incorporated 0.001 M thioglycolic acid in the electrode buffer and the protein was diluted

Table 5. Migration of the leading ion-trailing ion boundary during discontinuous vertical gel electrophoresis.

Spacer buffer: 0.11 M Tris-HCl, pH 6.7
Running buffer: 0.42 M Tris-HCl, pH 8.9
Electrode buffer: 0.04 M Tris-glycine, pH 8.3

Strip No.	45 min Run		1-1/2 hrs Run		2 hrs Run		3 hrs Run		Gel
	pH	Ninhydrin Test	pH	Ninhydrin Test	pH	Ninhydrin Test	pH	Ninhydrin Test	
0	8.3	++	8.3	++	8.3	++			spacer 4%
1	8.3	++	8.3	++	8.3	++			
2	7.2	-	8.6	++	8.3	++			
3	6.9	-	9.2	++	8.5	++			200 V
4	8.9	-	9.0	-	8.8	++			
5	8.9	-	8.9	-	9.2	+			
6	8.9	-	8.9	-	8.9	-			running
7	8.9	-	8.9	-	8.9	-	9.0	++	10%
8	8.9	-	8.9	-	8.9	-	9.3	+	400 V
9	8.9	-	8.9	-	8.9	-	8.9	-	
10	8.9	-	8.9	-	8.9	-	8.9	-	

+ or ++ = weak or strong ninhydrin reaction.

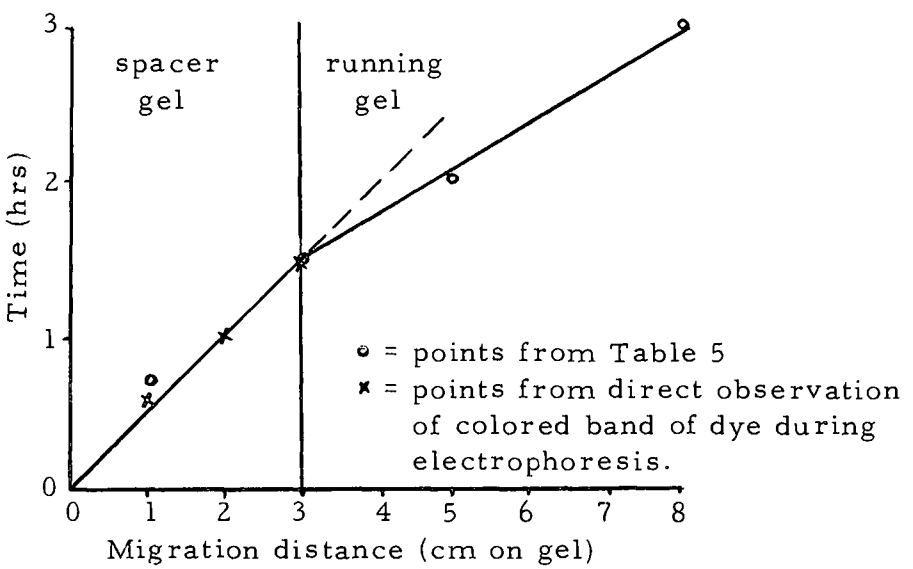


Figure 8. Rate of migration of leading ion-trailing ion boundary in the spacer and running gel.

with 8 M urea-0.01 M thioglycolic acid prior to loading. Schaub and Perry (1969) used 2-mercaptoethanol as a sulfhydryl protecting agent during disc electrophoresis with polyacrylamide gel of the troponin complex. Dewey (1970) working with tropomyosin preparations added 2 mM 2-mercaptoethanol to the polyacrylamide gel electrophoresis system. Arai and Watanabe (1968b) suggested the use of dithiothreitol as sulfhydryl protecting agent in the disc polyacrylamide gel electrophoresis of troponin preparations. In this study 1 mM dithiothreitol was added to spacer, running and plug gels as well as to the Tris-glycine electrode buffer of the main experiments. After the addition of dithiothreitol to the system, reproducible patterns were obtained in which several bands due to oxidation artifacts had disappeared.

In conclusion, a prerun to remove residual AP prior to the application of sample can be applied only when the continuous technique of vertical gel electrophoresis is used. However, in this study the continuous technique was not used. Instead, for the electrophoretic analysis of extra protein and tropomyosin preparations, the discontinuous technique was employed since it gives highly resolved patterns with more discrete bands (Petropakis _et al._, 1969). To apply this technique the oxidizing effects of AP were compensated by using dithiothreitol as a sulfhydryl protecting agent.

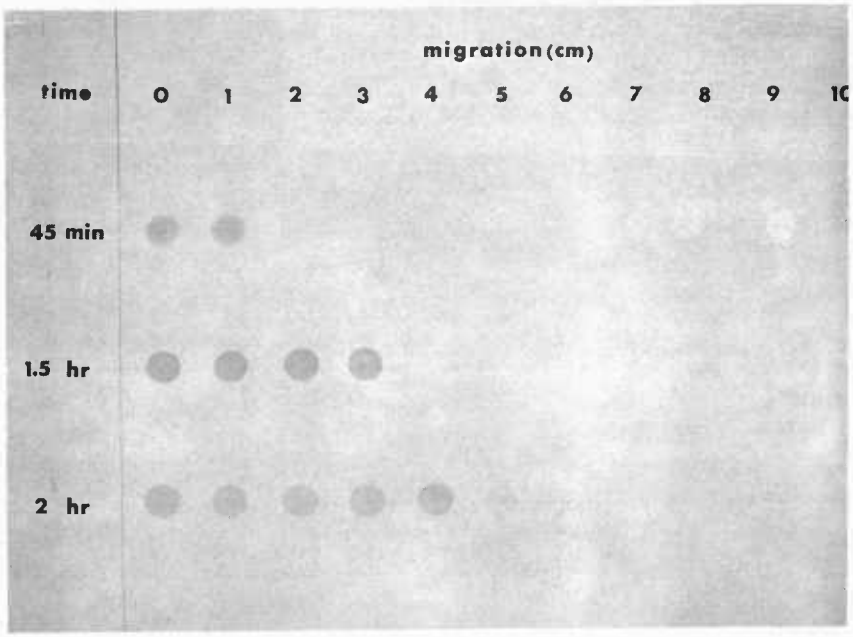
The use of 0.01 M Tris-0.039 M glycine electrode buffer, pH

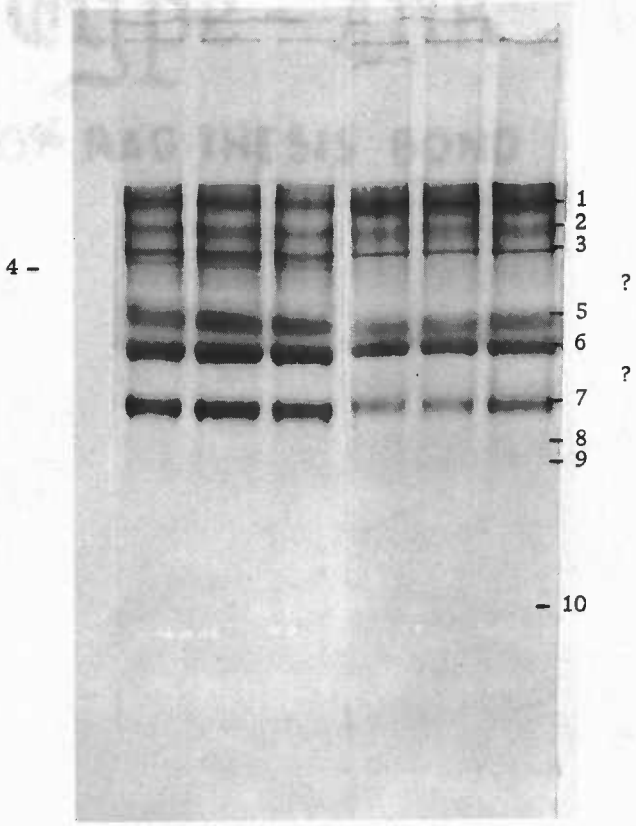
8.6, was necessary to achieve better buffering. In preliminary experiments, 0.005 M Tris-0.039 M glycine electrode buffer (pH 8.3) was used. After the addition of urea in the system during the prerun (sample stacking in spacer gel) at 200 V, the current obtained was only 30-40 mA and formation of leading ion-trailing ion boundary was slow (about 2 hours after the current was turned on). Using the new electrode buffer with higher ionic strength at 200 V and 400 V, currents of 40-45 mA and 70-75 mA were obtained, respectively, and the leading ion-trailing ion boundary was formed and moved to the spacer-running gel interface in approximately 90 minutes after current was applied. It should be noted that for a better stacking the boundary should be formed early enough to sweep the protein in a thin layer before it reaches the spacer gel-running gel interface. Experimentally, the optimum concentration of the running gel was determined to be 8%. Electrophoresis was terminated when the colored dye band reached the lower end of the running gel. Finally, it should be added that poor resolution was experienced when the initial freeze dried extra protein preparations were used for electrophoretic analysis. Reduction of the ionic strength of these preparations by the procedure already described resulted in superior resolution and higher reproducibility.

The patterns obtained by vertical gel electrophoresis of extra protein samples are presented in Figure 10. Stained bands,

Figure 9. Migration of leading ion-trailing ion boundary during discontinuous gel electrophoresis. Spots on the chromatographic paper represent ninhydrin positive reactions with glycine.

Figure 10. Vertical gel electrophoretic patterns of extra protein preparations. Electrophoretic conditions: discontinuous buffer, gel systems containing 5 M urea and 1 mM dithiothreitol; prerun 1 1/2 hrs at 200 V; run 3 hrs at 400 V. (a) 0 day sample. (b) 12 day sample.


migration (cm)
time
0
1
2
3
4
5
6
7
8
9
10
45 min
1.5 hr
2 hr


4 –
1
2
3
?
5
6
?
7
8
9
10

(a) (b)

corresponding to electrophoretically different protein fractions, are designated by numbers at the right margin of the patterns. In these patterns, 9 and 10 different protein bands can be distinguished (Figure 10a and b). Two faintly visible areas (?) might also be interpreted as possible protein bands. Probably the most important item is the difference between patterns (a) and (b) of Figure 10 corresponding to 0- and 12-day aged samples, respectively. Band 4 which clearly appears in patterns of the 0-day extra protein sample does not appear in corresponding patterns of the 12-day sample. This can be considered as evidence that some proteins of the extra protein complex breakdown during aging possibly by proteolytic changes and thus do not appear in the 12-day pattern.

A typical pattern obtained from the DEAE-cellulose (diethylaminoethylcellulose) chromatography of extra protein by Perry and Zydowo (1959a) shows 4 peaks which are identified as fractions I, II, III, and IV (Figure 11). Rampton (1969) reported that disc gel electrophoresis of these fractions revealed that fraction I contained a broad band (Rm. 0.14-0.21) and two sharply defined ones (Rm 0.26, 0.90). Fractions II and III contain three bands (Rm 0.03, 0.31 and 0.25) while fraction IV has two bands (Rm 0.32, 0.51). A total of 9 bands were defined this way by Rampton (1969) while 9-10 well defined bands were also observed in this study. Perry and Zydowo (1959a) reported that sarcoplasmic proteins behaved identically with proteins of

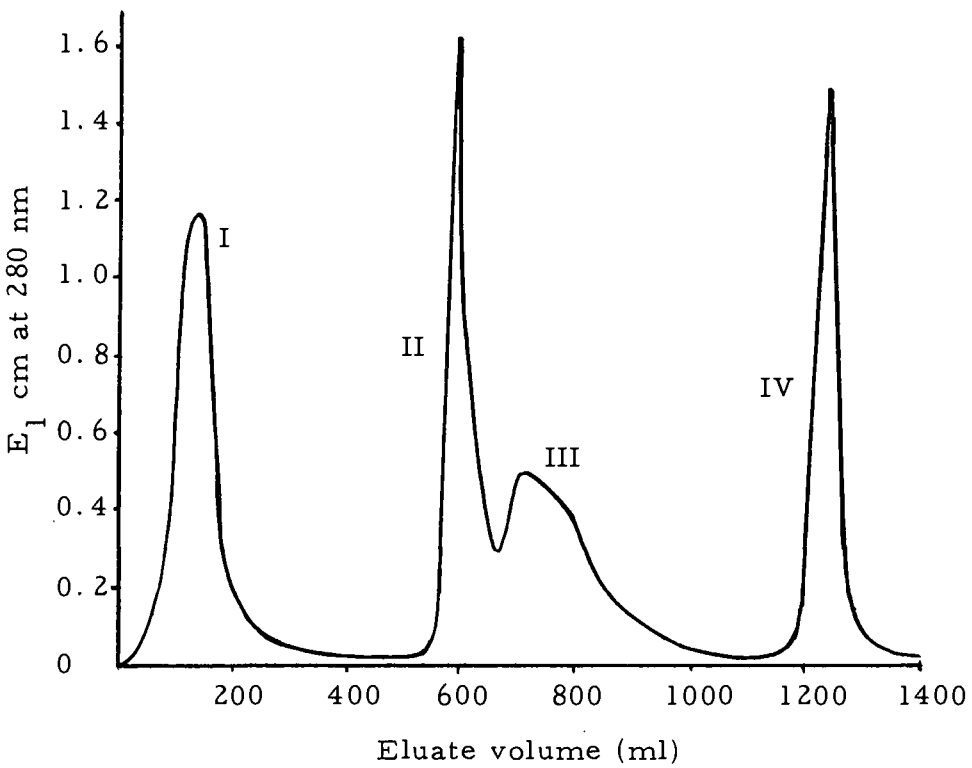


Figure 11. DEAE-cellulose chromatography of extra protein extracted from rabbit myofibrils (Perry and Zydowo, 1959a).

fractions I when they were chromatographed on DEAE-cellulose. They suggested that fraction I contained some, if not most, of the proteins found in sarcoplasm, which had not been removed from the myofibrils prior to extraction of extra protein. In the electrophoresis of sarcoplasmic proteins, Rampton (1969) obtained patterns containing bands with Rm values ranging from 0.15 to 0.29. His findings support the opinion of Perry and Zydowo (1959a) that fraction I consists mainly of sarcoplasmic material. In this study the Rm value of the extra band was 0.15 which is close to the lower Rm value of fraction I given by Rampton (1969). Therefore, it can be argued that the extra band found in the pattern of the 0-day sample was due to sarcoplasmic proteins or proteins with similar Rm that were not removed during washings of myofibrils before extraction of extra protein. However, procedures used for preparation of samples at 0 and 12 days of aging were identical. Therefore, the presence of sarcoplasmic protein in the extra protein preparations in different concentrations cannot be supported except that for some unknown reason the removal of certain sarcoplasmic or other particular material from myofibrils of the 0-day sample is not possible. In other words some proteins which remain with the myofibrils of the 0-day sample during several washings, can be removed easily from myofibrils of the 12-day sample. These findings are supported by the recent work of Penny (1970b) who reported that there was no evidence of a breakdown of any particular

protein in the myofibrillar extracts of longissimus dorsi muscles aged at 4°C for 8 and 15 days. Instead, he suggested that one of the effects of aging may have been an alteration in the binding of some proteins to each other in the myofibril.

The addition of dithiothreitol to sample preparations prior to electrophoresis did not give different electrophoretic patterns. This means tnat the only role of dithiothreitol was the prevention of oxidation of sulfhydryl groups by residual AP.

Protein Determination

The protein contents of freeze dried extra protein samples are presented in Table 6. As shown in this table the estimation of protein content by the biuret method is in good agreement with the results obtained by the micro-Kjeldahl method. However, there are marked differences between the protein content of the above samples determined by the biuret or micro-Kjeldahl methods and that estimated by ultraviolet absorption (Table 6). It has been reported that the extra protein complex contains tropomyosin (Corsi, 1957; Perry and Zydowo, 1959a) and that tropomyosin does not contain tryptophan (Kominz *et al.*, 1954). The strong absorption of proteins at 280 nm is due to the presence of three amino acids, tryptophan, tyrosine and phenylalanine with tryptophan accounting for most of the ultraviolet absorption (Wetlaufer, 1962). Therefore, differences observed

between the protein content determined by the biuret or micro-Kjeldahl methods and ultraviolet absorption support the reported data about the presence of tropomyosin in the extra protein. Also, these differences can provide a rough estimation of the tropomyosin contained in the extra protein preparations of this study.

Table 6. Protein content of freeze dried extra protein samples.

Sample	Biuret Method	Micro-Kjeldahl		UV Absorption
	Protein mg/ g dried sample	% N	Protein mg/ g dried sample	Protein mg/ g dried sample
0-day	120.0	1.76	111.4	65.1
12-day	105.9	1.69	105.0	62.0

The protein content of the extra protein preparations decreased with aging (Table 6). This might be related with the disappearance of at least one protein band in the electrophoretograms obtained with the 12-day samples.

Results of the protein determinations of the extra protein, tropomyosin and troponin samples by the biuret or micro-Kjeldahl method, were used for the dilution of these samples with the appropriate amount of "sample mixture" prior to electrophoresis.

Elution Convection Patterns

The ultraviolet absorption of fractions obtained by elution convection of electrophoretic patterns of extra protein samples was

measured at 260 nm and 280 nm. These values were plotted against the number of the collection tubes which contained these fractions and the resulting patterns are presented in Figure 12. It can be observed in this figure that the peaks of elution convection patterns, in general, correspond to bands of the electrophoretograms (Figure 10). However, it must be emphasized that carry-over of some proteins from one fraction to another may have occurred during the elution convection procedure.

Perry and Zydowo (1959a) reported that four fractions (I, II, III, and IV) were obtained by the DEAE-cellulose chromatography of extra protein preparations. Fraction I has been already discussed in the vertical gel electrophoresis section. Fraction II contained unidentified globulins. Fraction III contained significant amounts of tropomyosin (Perry and Zydowo, 1959a). In this case, some of the bands of the electrophoretic patterns obtained in this study would correspond to tropomyosin. In an attempt to identify these bands, parallel electrophoretic runs of extra protein and tropomyosin preparations were carried out. In several runs troponin preparations were also used. Results of these runs were inconclusive although they did indicate the possible presence of tropomyosin in bands 5, 6 and 7, of the extra protein electrophoretic patterns shown in Figure 10. However, the presence of troponin in the extra protein patterns was not evident. The criterion for this identification was the electrophoretic migration

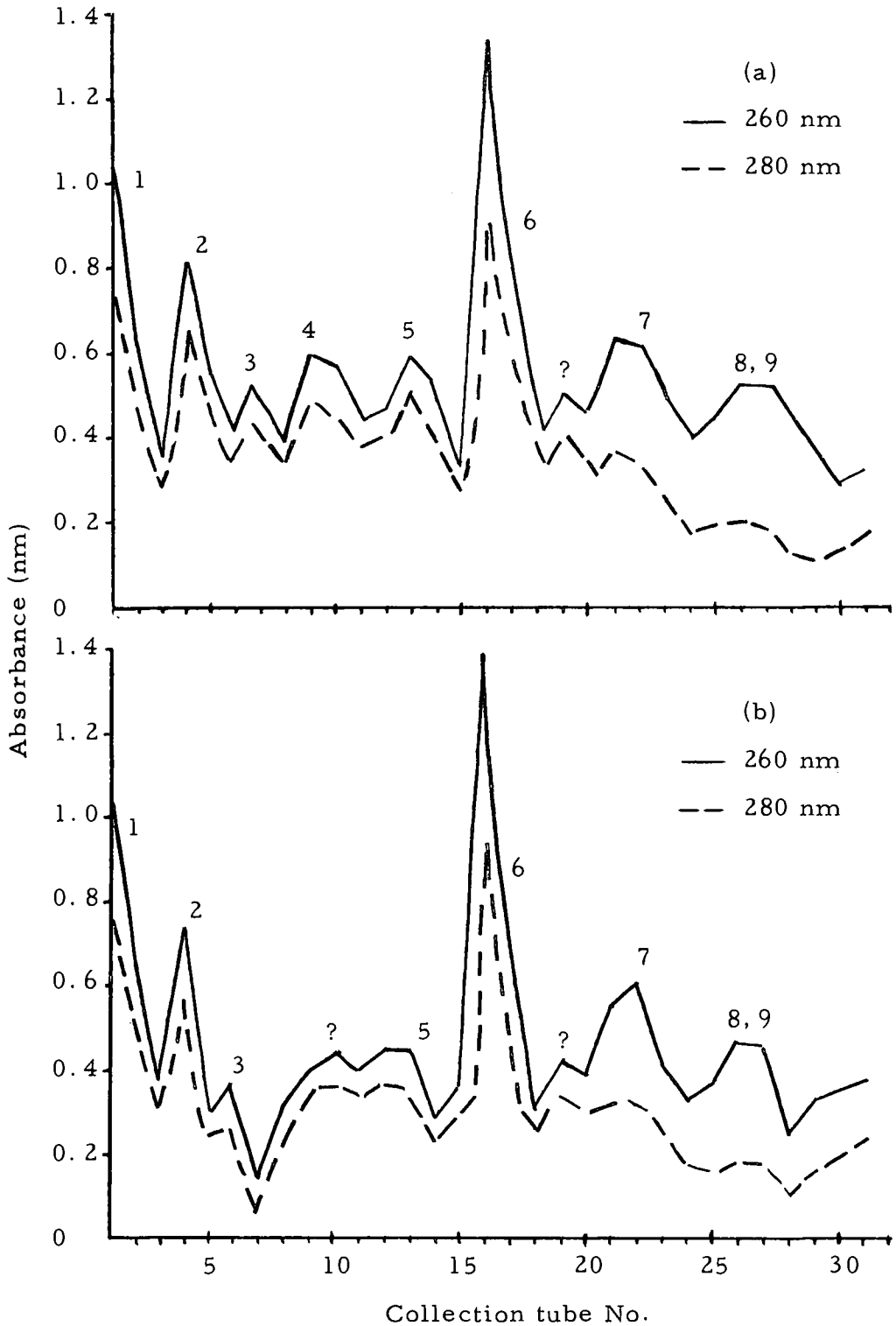


Figure 12. Elution convection patterns of (a) 0-day sample and (b) 12-day sample. The numbers of the peaks designate the corresponding bands of the electrophoretic patterns of Figure 10.

distance assuming that proteins with the same molecular weight and charge have the same migration rate. Because of the combined electrophoretic and sieving effects occurring in gel electrophoresis, it does not exclude the possibility of different proteins having the same or similar migration rates. For this reason fractions obtained from the elution of these bands were subjected to viscometric analysis to obtain additional evidence.

According to Perry and Zydowo (1959a), fraction IV of the DEAE-cellulose chromatography of extra protein differed from the others in that the ratio of absorbance at 280 nm over 260 nm was always the smaller. Because of this fact, they suggested the presence of nucleoproteins in this fraction. The elution convection patterns (Figure 12) show that the same is true for the eluted fractions of tubes 26 and 27. Their peaks have $\frac{A_{280}}{A_{260}}$ ratio of 0.36 and 0.38 for the 0-day and 12-day samples, respectively, which are smaller than that of any other peak. This can be considered as evidence for the presence of nucleoprotein components in these fractions.

Viscometric Analyses

Results of viscometric analyses of elution fractions contained in tubes referred by their number are presented in Table 7. Ebashi and Kodama (1965, 1966, 1967) have reported that the addition of troponin to tropomyosin B preparations elevates the viscosity of the solution.

Table 7. Measurements of the specific viscosity (n_{sp}) of protein fractions obtained by elution convection of extra protein electrophoretic patterns.

	0-Day Sample			12-Day Sample		
	Average time	t/t_0**	$n_{sp}(=t/t_0-1)$	Average time	t/t'_0**	$n_{sp}(=t/t'_0-1)$
Contents of tubes 21 and 22	67.1 sec.	1.000	0.000	81.0 sec.	1.001	0.001
" plus 0.12 ml TN*	67.2 sec.	1.001	0.001	81.1 sec.	1.002	0.002
" plus 0.25 ml TN	68.2 sec.	1.014	0.014	82.0 sec.	1.014	0.014
" plus 0.40 ml TN	69.1 sec.	1.030	0.030	83.4 sec.	1.031	0.031
" plus 0.60 ml TN	---	---	---	84.1 sec.	1.039	0.039
" plus 0.80 ml TN	70.1 sec.	1.044	0.044	84.4 sec.	1.043	0.043
" plus 1.00 ml TN	70.2 sec.	1.045	0.045	84.4 sec.	1.043	0.043

* TN = troponin preparation.

** t_0 = 67.1 seconds (flow through time of Ostwald viscometer A).

t'_0 = 80.9 seconds (flow through time of Ostwald viscometer B).

Sugita et al. (1969) reported a micromethod for the determination of tropomyosin utilizing the viscosity increasing action of troponin on tropomyosin. In this study the above method was used to detect the presence of tropomyosin in several fractions obtained by elution convection. To these fractions, increasing quantities of the toponin preparation were added. An increase of viscosity was observed only in fractions corresponding to tubes 21 and 22 for both the 0-day and 12-day samples (Table 7). This indicates the presence of tropomyosin in the fast moving band 7 of the electrophoretograms of extra protein. It should be noted that Corsi (1957) reported that the fastest moving main component during electrophoresis of extra protein appeared to be tropomyosin.

In Figure 13 the amount of troponin added was plotted against specific viscosity. Plots of curves for 0- and 12-day samples representing the increase of specific viscosity with increasing amounts of troponin are very similar. Since no viscosity changes were observed in the eluted tropomyosin fractions of 0- and 12-day samples, this is evidence that the viscosity of tropomyosin does not change with aging. This, in general, is in agreement with the findings of Dewey (1970). It should be added that accurate measurements of the protein concentration of the eluted samples was not possible. Since dilution of samples cannot be assumed to be absolutely identical, very small differences in the viscosity of tropomyosin of 0- and 12-day samples

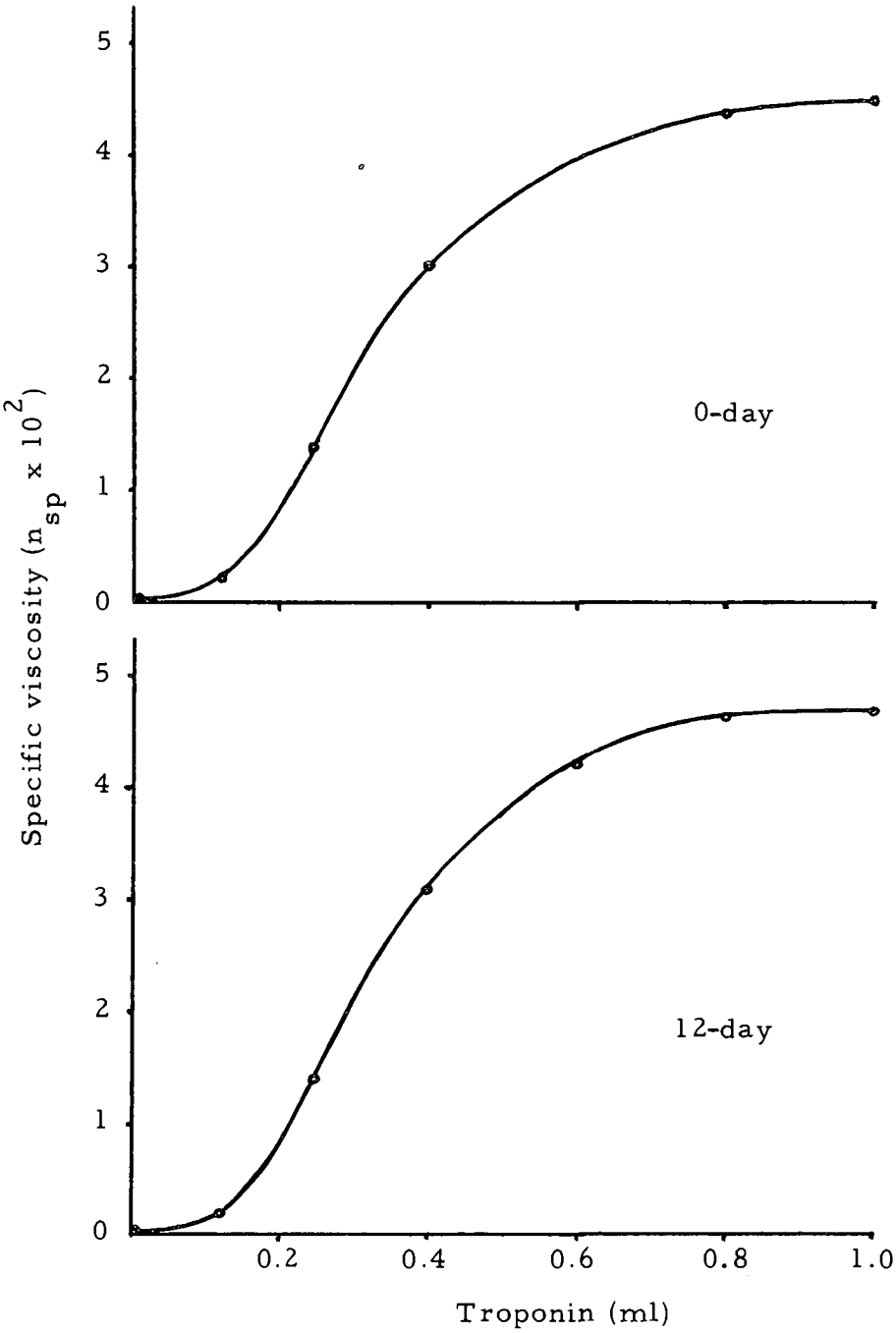


Figure 13. Effect of varied concentrations of troponin preparation (0.5 mg/ml) on specific viscosity of eluted band 7 of electrophoretic pattern of extra protein.

cannot be detected accurately. Thus it can be concluded that only pronounced differences in viscosity of tropomyosin with aging could be detected.

Following a similar procedure freeze dried eluted fractions were added in increasing quantities to tropomyosin preparations. In this case an increase of viscosity would indicate the presence of troponin in the elution fractions. Such increase of viscosity was not observed probably because of the minute amount of protein contained in these fractions or because of the absence of troponin in the extra protein complex.

The effects of the KCl concentration (Azuma and Watanabe, 1965) on the viscosities of tropomyosin preparations of this study are presented in Figure 14. As shown in the graphs, a sharp decrease of viscosity is evident. This indicates the presence of Bailey tropomyosin (or tropomyosin B) in these preparations.

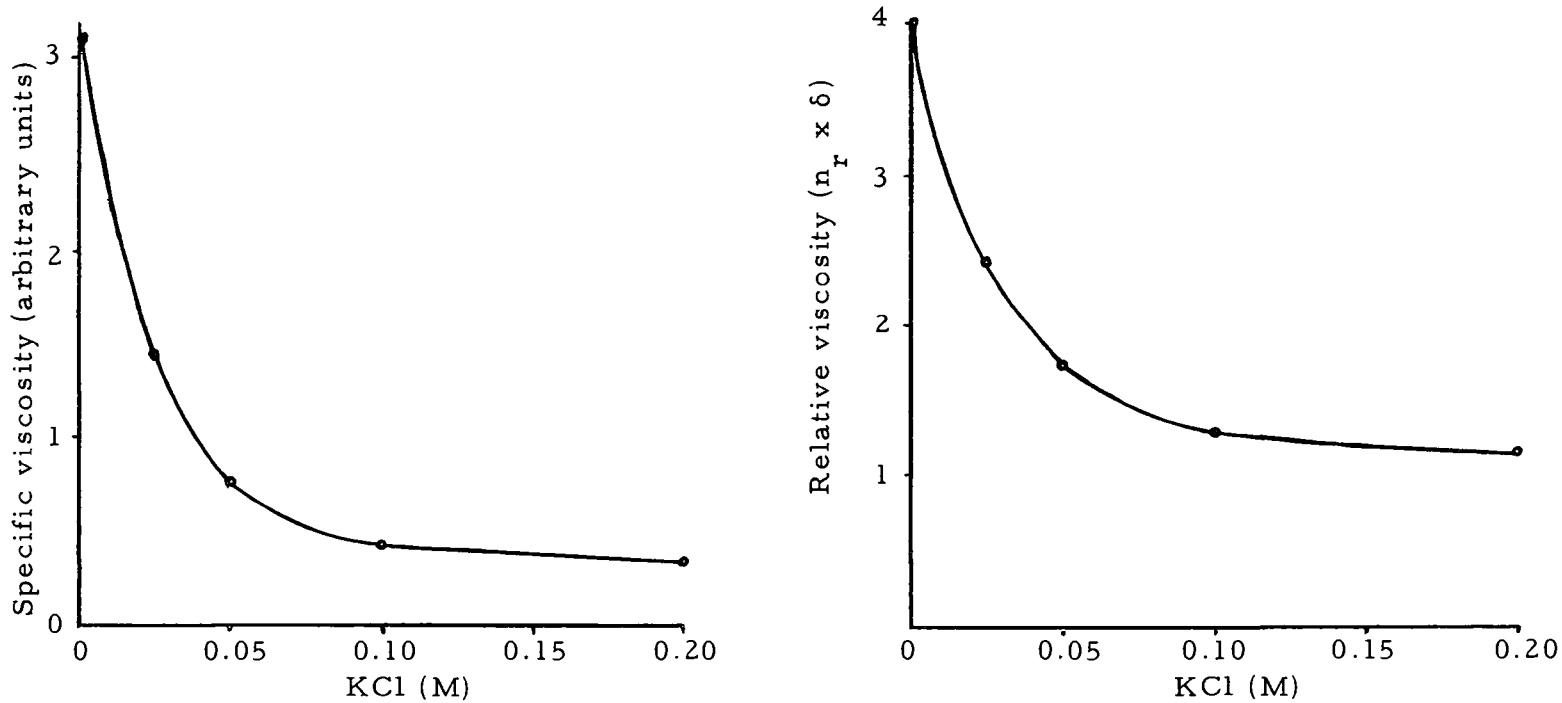


Figure 14. Effect of the KCl concentration on the viscosities of tropomyosin B prepared by isoelectric precipitation (pH 4.6). Specific viscosity is in arbitrary units corresponding to $t/t_o \delta - 1$. Relative viscosity actually corresponds to $n_r \times \delta$. The factor $\delta = d_o/d$ is considered constant.

SUMMARY AND CONCLUSIONS

The effect of aging upon certain protein components of bovine psoas major muscle was investigated in two parts. In the first part, low molecular weight nitrogen compounds were studied in terms of detection and their possible accumulation during an aging period of 12 days. The second part involved the investigation of the possible hydrolysis or alteration of extra protein complex during aging.

Electrophoretic studies of soluble proteins either before or after gel filtration on Sephadex G-25 columns indicated that the soluble proteins were not altered or changed during a 12-day aging period.

Data of absorbance measurements (280 and 265 nm) of the soluble protein extracts separated by gel filtration showed that the low molecular weight nonprotein nitrogen compounds increased during aging. Results of nitrogen determinations of the gel filtered extracts also supported this finding. Moreover, data of ultraviolet scanning, vertical gel electrophoresis and two-dimensional paper chromatography-high voltage electrophoresis of gel filtration fractions suggested that the low molecular weight nonprotein nitrogen compounds were mostly nucleic acids. Whether the latter components are inherently associated with muscle proteins and/or are released from nucleoproteins during aging is not known.

No evidence was obtained during the course of this study to

indicate the presence of polypeptides in either fresh (0-day) or aged (12-day) samples. Even though some polypeptides may be formed during aging as a result of proteolysis, the amounts that may have accumulated were not sufficient for detection by the analytical methods employed.

Evidence was obtained in the second part of this study which indicated that the extra protein was altered during 12 days of aging. Vertical gel electrophoretic patterns of the 0-day extra protein samples showed one more band than patterns of the 12-day samples. The disappearance of this band during aging might be the result of proteolysis or an alteration in the ability of the protein constituting this band to bind with other muscle proteins. The binding ability of this protein may be weakened during aging which would allow for it to be solubilized and removed during the extraction procedure of extra protein.

Verification of the presence of tropomyosin and nucleoproteins in the extra protein complex was obtained by ultraviolet absorbance measurements and viscometric analysis of the fractions of extra protein separated by vertical gel electrophoresis and collected by the elution convection procedure. Viscometric changes in the tropomyosin part of the extra protein complex were not evident with aging. This would indicate that tropomyosin is not hydrolyzed during aging.

In conclusion, this is the first time that evidence has been

obtained which shows an electrophoretic change occurring in a myofibrillar protein during aging. An extra band appearing in the electrophoretic pattern of the 0-day sample but not in the 12-day pattern can be interpreted that either this part of the extra protein was degraded during aging or its ability to bind to other proteins had been altered. This may be an important finding when considering that changes in the binding and anchoring of proteins to the Z-line have been suggested by several workers as a partial explanation of the tenderization of muscle during aging.

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