STUDIES ON BOVINE ACTIN

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CHAPTER I

INTRODUCTION

The most striking and characteristic properties of actin, which have great biological significance and relevance to the use of muscle as a food, are its capacity for polymerization and its interaction with myosin. In the live animal, when muscle contraction takes place, actin combines with myosin to form actomyosin. In the dead animal, when rigor mortis develops, it is mainly due to actomyosin formation, too. Both muscle contraction and rigor mortis are important factors influencing the market quality of block beef. It would seem that if methods or treatments could be devised to control the formation of actomyosin, such meat quality factors as tenderness, juiciness, et cetera, might be enhanced.

Actin, one of the major muscle proteins, can exist in two forms, G-actin which consists of relatively small globular units having a molecular weight of about 60,000, and F-actin, in which these globular units are aggregated end to end to form a double chain. G-actin polymerizes into F-actin in the presence of salts. It is 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. Thus it would seem that if the polymerization of G-actin to F-actin could be inhibited or prevented, one might be able to control or influence actomyosin formation. Before we can examine various possible mechanisms to control muscle contraction and rigor mortis, we need more fundamental

knowledge of the physical chemistry of bovine actin than we now possess.

The study described below has been designed to meet, in part, this need and includes the following objectives: (1) To establish procedures for the isolation and purification of bovine G-actin and F-actin. (2) To determine certain physico-chemical characteristics of bovine G- and F-actin. (3) To determine the effect of certain chemical treatments, as well as reaction conditions, on the G- to F-actin transformation.

CHAPTER II

REVIEW OF LITERATURE

Muscle Proteins

The general chemical composition of mammalian striated muscle on a wet tissue weight basis is as follows: water 74.4%, protein 20%, lipid 3%, carbohydrate 0.6%, organic extractives 1% and inorganic constituents 1% (West and Todd, 1962). Thus, excluding water, protein is the most abundant, and probably the most important, cell constituent. Muscle protein may be further sub-divided into the myofibrillar proteins, sarcoplasmic proteins and stroma proteins.

Myofibrillar Proteins

This group of proteins, contained in the myofibrils of striated muscle, is responsible for muscle contraction. Three well-identified proteins have been isolated from the myofibril of rabbit skeletal muscle: actin, myosin and tropomyosin B. In addition, some lesser known, and as yet poorly defined, components have been detected. S. V. Perry (1965) has reported the approximate protein composition of the isolated rabbit myofibril to be 50-55% myosin, 20-25% actin, 10-15% tropomyosin B and 5-10% other components. For ease in presenting the chemical and physical properties of each, the three primary myofibrillar proteins will be considered separately.

G-actin

Isolation and Purification. Although the basic characteristics of actin are undisturbed, reevaluation of some quantitative aspects may be in order as a consequence of the recent, widespread concern for the purity of G-actin preparations. Ulbrecht et al. (1960) examined the effect of KCl or Mg^{++} polymerization and isoelectric precipitation methods on the protein and nucleotide compositions of actin solutions, noting that while both procedures eliminate nucleotide impurities, the latter method does not remove protein impurities. By far the greatest concern has arisen over a widespread interpretation of the Mommaerts' procedure (1952) as involving extraction of the acetone powder at room temperature, for it has been observed that G-actin prepared in this way may contain 10 to 25 % tropomyosin (Laki et al., 1959; anonymous, 1962; Martonosi, 1962; Drabikowski et al., 1962). While regarded as a misinterpretation by Carsten and Mommaerts (1963), reappraisal of the method indicates that extraction in the cold greatly reduces or eliminates the tropomyosin content (Drabikowski et al., 1962; Carsten et al., 1963). It has also been observed that partial polymerization of actin by low concentrations of Mg^{++} will eliminate the tropomyosin contamination (Martonosi, 1962a; Laki et al., 1962); however, the yield of G-actin is considerably reduced. With the development of gel filtration, it has been possible to develop a completely new approach to the preparation of G-actin (Adelstein et al., 1963). With Sephadex G-200, it has been possible to prepare G-actin free of tropomyosin as well as the common enzymatic impurities. The procedure also has the virtue that any partially polymerized actin in the initial extract is depolymerized on the column.

Molecular Weight and Structure. Past estimates of the molecular weight of G-actin have placed the value for this parameter at approximately 60,000. Working primarily with KI solutions of actin, Kay (1960) has obtained values of 66,000 from light scattering and 62,000 from a combination of the sedimentation constant and intrinsic viscosity. Estimates of the molecular weight from the nucleotide content of G-actin have favored 62,000 (Ulbrecht et al., 1960). Recently, Lewis et al. (1963), working with tropomyosin-free G-actin and using the recently developed variants on the sedimentation-equilibrium method, obtained a value of 57,000 in dilute ATP solution. Although KI has been used as a solvent for G-actin since it favors dissociation of any F-actin in the preparation, Lewis et al. have observed that when G-actin was kept for prolonged times in this solvent, G-actin was gradually inactivated, forming aggregates, which have a molecular weight between G-actin and F-actin. Inactivation of G-actin by treatment with EDTA leads to some aggregation of G-actin, the molecular weight suggesting a dimer. Raising the ionic strength then leads to formation of massive aggregates of F-actin. From a consideration of the hydrodynamic data on G-actin and the electron-microscopic and X-ray evidence on the structure of F-actin, Lewis, et al. (1963) postulated that the G-actin molecule consists of a rigid portion, with a diameter of about 56 Å, and another flexible and penetrable realm which is responsible for the apparently large effective volume which they estimated. Upon denaturation, this flexible portion collapses, becoming impenetrable.

Ooi (1961) from light-scattering measurements obtained a molecular weight of 117,000 for what he regarded as a dimer and hence the active G-actin unit. Preparation and solvent conditions employed by him do not

appear to differ significantly, however, from those used by a number of individuals giving G-actin with a molecular weight near 60,000.

Measurement of the intrinsic viscosity of highly purified G-actin has yielded a value of 0.04 ± 0.01 dl/g (Cohen, 1966). This value, unlike the higher ones reported earlier (Lewis et al., 1963; Drabikowski et al., 1962), is consistent with the electron microscope images showing that the molecule is globular, with an axial ratio of 3 or less (Hanson et al., 1963; Rowe, 1964). The apparent discrepancy (Hayashi, 1967) between the previously accepted values for the sedimentation constant (3.25S) and molecular weight (58,000) of G-actin (Lewis et al., 1963; Krans et al., 1965) may have been resolved by the discovery that the earlier preparations of actin contained an appreciable amount of inactive dimer. After removal of this dimer by filtration through G-200 Sephadex, the purified actin solutions yield a substantially lower molecular weight of about 46,000 (Rees et al., 1967).

Electrophoretic Behavior. Starch-gel electrophoresis of G-actin gives a single component when the preparative method avoids tropomyosin contamination and loss of SH groups (Carsten et al., 1963). In contrast, Krans et al. (1962) observed four components on starch-gel electrophoresis of G-actin. On the other hand, reduced and alkylated actin gave only a single component--a fact which suggests that the multicomponent pattern arose from intermolecular disulfide bridges and emphasizes some of the hazards in physical characterization of actin.

A preliminary report on the behavior of actin in guanidinium chloride solution suggests that the G-actin molecule may consist of small subunits (Adelstein et al., 1963).

Optical Properties. Considerable variation in the optical rotatory properties of G-actin has been reported in the data of Standaert and Laki (1962). From the extensive and thorough studies by Nagy and Jencks (1962) on the optical rotatory dispersion of G-actin, it seems probable that $[\alpha]_D = 44^\circ$, λ_c of the Drude equation = 252 mμ, and the constant b_o of Moffitts equation = -184 represent the best values available for these parameters. From the standpoint of optical rotatory properties, these authors identified three structural states, that of active G-actin, a less ordered state after removal of bound nucleotide in which all the rotatory values are lowered, and the random coil observed in urea or guanidinium chloride.

Standaert and Laki found that G-actin prepared by polymerization with dilute Mg^{++} and examined following depolymerization, although completely polymerizable, showed optical rotatory parameters somewhat lower than those observed after a subsequent polymerization-depolymerization cycle with KCl. In addition, the inorganic phosphate liberation with this material was only 40 to 70 % of the expected value (Nagy et al., 1962). One is led to question whether this reaction is caused by incomplete depolymerization leaving polymers too small to be removed by ultracentrifugation or otherwise detected. Standaert and Laki (1962) suggested that the variations in optical rotatory parameters observed with their preparations might be caused by variable contamination by F-actin; and it seems possible that the reported variability in these values might be explained by contamination with either F-actin or inactive actin, which was unpolymerizable G-actin in 0.1 M KCl solution.

Amino Acid Composition. Demonstrations that G-actin preparations may contain considerable tropomyosin made it probable that the published

(Poglazov et al., 1961; Kominz et al., 1962; Katz et al., 1962) amino acid composition was in error. An analysis of tropomyosin-free actin (Carsten, 1963) is in agreement with the older values if the latter are corrected for the estimated tropomyosin contamination. While these analyses indicate 6.7 half-cystines per molecule, the number of these present as cysteine has been uncertain, until recently. The older literature has suggested four or five, but it is not apparent that all of these half-cystines are present in the reduced form (Poglazov et al., 1961; Katz et al., 1962; Tonomura et al., 1962).

Of the six cysteine residues per molecule, it has been determined that two react readily with N-ethylmaleimide and that this reaction does not alter the activity of the G-actin (Katz et al., 1962; Tonomura et al., 1962). Two other groups react with silver ions or p-chloromercuribenzoate without loss of polymerization. Two groups reacting slowly with the latter two reagents appear to be essential for the polymerization reaction (Katz et al., 1962; Barany, 1956). In contrast to G-actin in which all six groups respond to amperometric silver titration or reaction with salyrgan, two groups in F-actin appear to be resistant to modification (Katz et al., 1962; Barany, 1956). Complete titration of G-actin SH by p-CMB leads to a decrease in the optical rotatory properties comparable to the transition from active to inactive G-actin (Tonomura et al., 1962), a fact which is not surprising since this titration is accompanied by a loss of bound ATP (Tonomura et al., 1962; Barany et al., 1961; Martonosi et al., 1961a; Strohman et al., 1962; Drabikowski et al., 1963a).

In addition to the requirement of unmodified cysteine residues for

the structural integrity and reactivity of G-actin, Tonomura et al. (1962a; Tokura et al., 1963) have presented evidence that a specific lysine residue to G-actin is required for the myosin-actin interaction, although the polymerization reaction is apparently unaffected. In contrast, Martonosi and Gouvea (1961) have observed that photo-oxidation, which indicates a histidine residue, leads to a loss of polymerizability of G-actin and depolymerization of F-actin, without loss of the ability of the modified actin to endow myosin ATPase with Mg^{++} activation, a property usually associated with F-actin. That polymerizability and capacity to modify characteristically the ATPase activity of myosin are independent properties of G-actin has been further demonstrated by Ulbrecht et al. (1960) who observed that G-actin which had lost its ability to polymerize, through exposure to X-ray or thorough isoelectric precipitation in the absence of ATP, still retained the latter property. In addition to the functional groups discussed above, it has been observed that the loss of one tyrosine residue per G-actin molecule through coupling with diazotized sulfanilic acid leads to complete inhibition (Martonosi et al., 1961). Only slight reaction of histidine residues was observed.

The N-terminal portion of the G-actin molecule has been isolated from pronase digests and shown to consist of N-acetyl-Asp-Glu-Thr (Alving et al., 1966; Gaetjens et al., 1966). The uncommon amino acid previously reported in actin hydrolysates (Kominz et al., 1962) has now been identified as 3-methylhistidine (Asatoor et al., 1967; Johnson et al., 1967). The content of 3-methylhistidine corresponds to 1 mole per 47,000 gm (Johnson et al., 1967), supporting the revised lower value for the molecular weight. There is now agreement that the G-actin molecule with a molecular weight near 60,000 consists of a single polypeptide chain (Krans et al., 1965; Rees et al., 1967; Gaetjens et al., 1966), and the

earlier report of two subunits (Adelstein et al., 1963) has been with drawn (Rees et al., 1967).

Sulfhydryl Groups. On complete labeling of SH groups of G-actin with ^{14}C-NEM in 4M guanidine HCl (Martonosi, 1965; Groschel-Stewart et al., 1963) followed by tryptic hydrolysis, eight distinct labeled peptides can be separated by the fingerprint technique. As pointed out by Martonosi (1965), the number of distinct peptides agrees well with the number of SH groups per 6 x 10^5 gm (Drabikowski, et al., 1964). Thus, either subunits (Adelstein, et al., 1963) do not exist in G-actin; or if they do, they must be chemically different. The apparent reaction of two moles of NEM (N-ethylmaleimide) per mole of G-actin is due to a stoichiometric reaction with one of the SH-peptides, and a weak reaction with several others (Martonosi, 1965), similar to the situation with myosin (Sekin, et al., 1962). Unfortunately, the reaction of the SH group with NEM does not lead to functional alterations, nor does this seem to be the case with other covalently reacting thiol reagents (Drabikowski et al., 1964). Although mercurials have made it possible to distinguish between SH groups involved in polymerization and in nucleotide binding (Drabikowski et al., 1963a), these reagents do not lend themselves to the marking of the corresponding peptides because of the labile linkage between the reagent and the SH group, and the possibility of subsequent reactions with originally unreacted SH groups. Katz (1964) reported an increased reactivity of SH groups with mercurials in G-ADP-actin as compared with G-ATP-actin. While the increased reactivity does not appear to be correlated with indications of conformational change (Higashi et al., 1965), the stabilizing effect of free nucleotides on SH groups (Katz, 1965) seems to be in line with the apparent conforma-

tional change produced in those G-actin molecules which are devoid of the nucleotide (Hagashi, et al., 1965).

Dowben et al., (1965) was able to attach an azomercurial dye, 4-(p-hydroxybenzeneazo) phenylmercuriacetate to SH groups of G-actin. The interest in this compound lies in the fact that the pK of the attached dye depends on the state of polymerization or denaturation of G-actin. Although these changes cannot be interpreted unequivocally, they may reflect changes in the immediate surroundings of the bound dye. Dowben and his colleagues suggest that changes in the structural water that surround the proteins may accompany the polymerization or denaturation of G-actin, causing a shift in the pK of dye (Katz, 1964).

Nucleotides. The protecting effect of nucleotides on SH groups (Katz, 1964) appears to be correlated with the ability of these nucleotides to combine with G-actin as first shown by Martonosi and Gouvea (1961; 1961a) and recently more quantitatively evaluated by Iyengar and Weber (1964). The relative affinities, taking that of ATP = 1, are as follows: ITP, 0.1; UTP and ADP, 0.02; CTP, GTP, and IDP, 0.002. A similar sequence was obtained by Kahol and Weber (1965) on the basis of the exchange with the bound ADP of F-actin subjected to ultrasound. In the case of every NTP, the exchange step was accompanied by hydrolysis of the phosphate residue.

While the importance of the bound nucleotide in polymerization and in the stabilization of G-actin has been sufficiently documented, recent studies in Oosawa's laboratory (Kasai et al., 1965) have demonstrated that under rather special condition (50% sucrose), it is possible to prepare G-actin that is free of nucleotides, as well as of divalent cations, and that this nucleotide-free G-actin can be readily polymerized by salt concentration lower than that required for native G-actin. This

would suggest that polymerization depends neither on the presence of the nucleotide nor on the clevage of the triphosphate present in conventional G-actin preparations, a fact also shown by the polymerization of G-ADP preparation (Hayashi et al., 1962; Hayashi et al., 1964). At pH 6.5, in 0.3 mM $MgCl_2$ (Tsuboi et al., 1965), dephosphorylation of bound ATP takes place without a change in viscosity, while further acidification results in polymerization and increased viscosity, facts suggesting that hydrolysis of ATP is not directly coupled to polymerization. The study of the polymerization of G-actin in $H_2{}^{18}O$ (Barany et al., 1964), showing the appearance of the label in the phosphate moiety, essentially rules out the existence of an active ADP complex as an intermediate in polymerization. Kahol and Weber (1965) stress the role of steric effects as being responsible for the tight binding of nucleotides to F-actin. Depue et al. (1965) found that the steric hindrance preventing exchange is also removed by heating F-actin ($60^{\circ}C$) without a change in physico-chemical properties (viscosity, DRF). The exchange produced by heating, which is reduced by free divalent cations, is accompanied by the hydrolysis of ATP.

Divalent Cations. The classes of binding sites can be distinguished in G-actin. The first has one member per 60,000 gm with an affinity constant of about $10^5 M^{-1}$ and corresponds to the tightly bound Ca^{++} or Mg^{++} found in G-actin preparations. Seven to ten sites appear in the second class with a constant of about $10^4 M^{-1}$, with no significant difference between the binding of Mn^{++}, Ca^{++}, and Mg^{++}, and divalent cation binding at these sites goes hand in hand with polymerization (Martonosi et al., 1964). Although removal of the tightly bound Ca^{++} of G-actin in

aqueous solution renders it unpolymerizable (Martonosi et al., 1961a), it seems that dialysis can remove Ca^{++} from F-actin without a corresponding depolymerization (Oosawa et al., 1965) and that the calcium- and nucleotide-free G-actin prepared in 50% sucrose appears to show normal polymerizability (Kasai et al., 1965). Similar to the case of nucleotides discussed above, it does not appear that calcium is an essential component in holding together the structure of F-actin, but is mainly required for maintaining the structural integrity of G-actin prior to polymerization. Ca^{++} binding to F-actin is stabilized both by free ATP and by added Ca^{++} or Mg^{++} (Oosawa et al., 1965). These findings suggest interactions with ATP and metal ions at several points, possibly resulting in conformational changes.

The tightly bound calcium of G-actin can be replaced if Mg^{++} is added in concentrations too low to initiate polymerization, e.g., 10^{-4}M (Oosawa et al., 1964; Drabikowshi et al., 1963). On the addition of salt this so-called Mg^{++}-G-actin polymerizes much faster than Ca^{++}-G-actin. However, after the initial exchange, further addition of either Ca^{++} or Mg^{++} equally accelerates the rate of polymerization (Mihashi et al., 1965).

F-Actin

Molecular Structure. The most characteristic properties of actin, which are of great biological concern and of pertinence to the use of muscle as a food, are its capacity for polymerization and its interaction with myosin. The transformation of G- to F-actin appears to take place by the polymerization of a globular molecule into a linear aggregate which may have a molecular weight of many million (Mommaerts, 1952 and 1966). The polymers consist of a double helix, each helix consisting

of globular monomers of actin, about 55 Å in diameter. The strands are intertwined in a regular manner so that the cross-over points occur at regular intervals. These intersections are spaced at 350 Å intervals along the filament. The over-all diameter of the filament is about 80 Å, because the subunits of opposite strands are displaced relative to one another by ½ of their diameter. There are approximately 13 to 15 globular subunits per turn of the helix. Recent work by Depue and Rice (1965) shows that the strands are in a right-handed helix. The 40 to 50 residues of proline per 1000 total amino acid residues in F-actin are consistent with the smaller level of helical structure (30%) considered to occur in this protein, compared to 56% in myosin and 95% in tropomyosin (Hanson and Lowy, 1964).

Polymerization. The polymerization (reaction = $n(G\text{-}ATP) \rightarrow (G\text{-}ADP)_n + nPi$) of G-actin starts upon the addition of neutral salts with a maximum velocity at a KCl or NaCl concentration of 0.1 - 0.15M. As polymerization occurs, the viscosity increases markedly. This change in the aggregation state of the protein is thought to be partly due to a decrease in the negative charge of the G-actin molecules in the presence of salts--a condition which results in the suppression of electrostatic repulsion (Poglazov, 1966).

The polymerization of G- to F-actin also results in the dephosphorylation of ATP to ADP, although the dephosphorylation is not an absolute requirement for polymerization. The ADP of F-actin is well protected from enzymatic attack and does not exchange with free ATP or ADP of the medium. This polymerization is evidently accompanied by conformational changes in the protein molecules which lead to masking of bound ADP and of Ca^{++} (Mommaerts, 1966). The thixotropic properties of F-actin thus

formed are of utmost importance to muscle biology and function and are of key importance to many aspects of the use of muscle as food. It has been pointed out that the conversion of F- to G-actin in tissue would possibly be of great importance to the use of muscle for food (Davies, 1966). ATP, ultrasound, shearing and freezing offer possibilities for converting F- to G-actin.

The question also arises as to forces involved in holding G-actin monomers together in the double-helical structure of F-actin. Divalent cations and nucleotides do not seem to play a critical role in the structure of F-actin once polymerization has taken place. Nagy and Jencks (1962) studied the effect of a number of salts and organic compounds, often regarded as denaturing agents, on the depolymerization of F-actin. On the basis of these latter studies it was possible to exclude covalent bonds, the binding of ions to charged groups, hydrophobic forces and alterations in the structure of water.

On the basis of the extensive studies of the Nagoya group on the kinetics and mechanism of the actin polymerization process (Ooi, 1961; Kasai et al., 1960; Anonymous, 1962; Anonymous, 1962a; Oosawa, et al., 1961; Asakura et al., 1960; Anonymous, 1960a), Oosawa and Kasai have proposed a theory of linear and helical aggregation of macromolecules (Oosawa et al., 1961) in which the equilibrium distribution is treated as a condensation phenomenon.

Biological Function. Actin, along with myosin, forms the contractible component of muscle. Actin represents about 13% of the total muscle protein or is present in muscle with respect to myosin in a ratio of 1:3. It is the F-actin which combines with myosin to produce the contractible actomyosin of active or pre-rigor muscle and the inex-

tensible actomyosin of muscle in rigor mortis (Lawrie, 1966).

The generally accepted theory of muscle contraction is Huxley's sliding filament theory. Huxley (1965) describes the structural framework in which the chemical reactions and physical actions producing muscle contraction occur. It would seem that a knowledge of the mechanism occurring during muscle contraction and relaxation, in vivo, however, has by no means been precisely elucidated.

Perry (1965) suggests a possible scheme of chemical reactions in which actin could be associated with the sliding filament theory of contraction. The following is a summary of Perry's proposal. (1) Resting muscle: Ca^{++} is bound to the sarcoplasmic reticulum. ATP concentration between the A-filament (myosin) and I-filament (F-actin) is high. Since the Mg^{++} concentration is high, myosin ATPase activity is inhibited. (2) Activation: Upon stimulation of the muscle, Ca^{++} is released from the sarcoplasmic reticulum and the calcium concentration in the cell is increased ($pCa > 6$). As F-actin comes into close proximity of myosin, it confers Mg^{++} activation characteristics to the enzyme center of myosin. This actomyosin type ATPase activity results in a local hydrolysis of ATP at the site of interaction between actin and myosin. As a result of this local depletion of ATP, a link is formed between the actin combining center of the HMM (heavy meromyosin) portion of the myosin molecule and the myosin combing center of the spherical monomer (G-actin) of F-actin. (3) Contraction: As a result of these links between F-actin monomers and myosin, the I-filament is pulled into the A-band. (4) Relaxation: Ca^{++} is "pumped" out of the cell and is again bound to the sarcoplasmic reticulum. The local ATP levels are restored primarily by the creatine phosphokinase system, and the myosin-actin links are broken. Thus, shortening occurs by the simulation of a series of make and break pro-

cesses, each in itself causing a small movement. This scheme of chemical events does not answer all questions concerning the biological function of actin in muscle contraction, however.

Myosin

Myosin, contained in the A-filament of the sarcomere, is an asymmetric molecule of average dimensions of 25 x 1600 Å (Perry, 1965); and this asymmetry is further indicated by its intrinsic viscosity of 2.0 (Portzehl et al., 1950). Values for the molecular weight of myosin have varied from 400,000 to 850,000, but a more recent assessment has given a value of 524,000 (Muller, 1964). In solution, myosin may exist as a fairly stable monomer and in a dimer form. The formation of the dimer is facilitated by short exposure to moderate temperatures (Holtzer, 1956), but dimerization does not result in a loss in capacity to combine with actin or in ATPase activity. Spontaneous aggregation in solution is further accelerated by higher temperatures resulting in the formation of particles of varying size (Szent-Gyorgyi, 1960). This point is important in the use of ultracentrifuge sedimentation patterns for determining molecular weight, as well as for a tool in evaluating the homogeniety of particle size in an isolated myosin preparation. Since the viscosity of actin-free myosin preparations is not changed by the addition of ATP, pyrophosphate or Mg^{++} ions, a comparison of the viscosity before and after the addition of ATP serves as a method for detecting the presence of actin (Szent-Gyorgyi, 1960). Ribonucleic acid contamination in myosin preparations can be removed by ribonuclease digestion without impairment of the activity of myosin (Mihalyi et al., 1957). These techniques serve as physical measures of purity in isolated myosin pre-

parations in the development of myosin standards.

Under mild conditions of tryptic digestion, myosin was found to be composed of two major fragments, light meromyosin (LMM) and heavy meromyosin (HMM) (Szent-Gyorgyi, 1951). By the use of electron microscopy, the LMM was observed to be rod-shaped, and the HMM appeared tadpole-shaped (Rice, 1964). It is this globular end of the HMM portion of the myosin molecule that possesses biological activity (i.e. ATPase and actin combining centers).

Electrophoretic mobility of myosin is greatly influenced by the presence of various ions. Thus, an understanding of the interaction of myosin with various ions is important. An isoionic point of myosin of pH 7.4 was reported by Mihalyi (1950). Electrophoretic mobility studies indicate an isoelectric point of pH 5.4 in potassium chloride solutions. Yet, in the presence of magnesium or calcium ions, the electrophoretic mobility of myosin is greatly altered, and the isoelectric point is shifted in the alkaline direction. In 0.1 M $CaCl_2$, the isoelectric point of myosin is at pH 9.6 (Erdos and Snellman, 1948).

Myosin ATPase activity is profoundly affected by ions, and in the presence of actin these ionic effects are modified. The literature concerning these effects is extensive and has been reviewed by Perry (1955) and Szent-Gyougyi (1960). Ca^{++} ions are potent activators of myosin ATPase activity. Potassium chloride also activates enzymatic activity both in the presence and absence of Ca^{++}, but to a lesser extent when Ca^{++} is absent. Moreover, the Ca^{++} activated ATPase activity is greatest when the ionic strength of KCl does not exceed 0.2. Thus, it appears that myosin possesses greater ATPase activity in the gel rather than in the solid state. Na^{+} decreases the activity of K^{+} activated

ATPase, but the concentration of Na^+ required is higher than the Na^+ concentrations in muscle. Mg^{++} ions inhibit the ATPase activity of pure myosin. This inhibition occurs either in the presence or absence of other salts and effectively counteracts the activating effect of Ca^{++} when both are present.

When actin is added to myosin to form actomyosin, the picture changes. In contrast to myosin ATPase, actomyosin ATPase is activated by Mg^{++} at low ionic strengths. However, above ionic strengths of about 0.2, the addition of ATP to an actomyosin solution leads to a dissociation of the actomyosin. The ATPase characteristics of the free myosin in this solution will then appear (Hasselbach, 1952). Both myosin ATPase and actomyosin ATPase are activated by Ca^{++}.

To understand the problem of inhibiting myosin ATPase activity and hence preventing the formation of actomyosin, it would be beneficial to review some of the results obtained with the various inhibitors. Under certain ionic and temperature conditions, ethylenediaminetetraactic acid (EDTA) may be a powerful activator of myosin ATPase activity (Friess, 1954). The activating effect of EDTA depends upon the presence of monovalent cations. With K^+ the activation is considerable; however, NH_3^+ is the most potent activator. Na^+ and Li^+ have no effect (Kielley et al., 1956). The effect of EDTA and other chelating agents may be due to some change in the binding or orientation of the nucleotides in order to facilitate hydrolysis. Since free-sulfhydryl groups of myosin are necessary for ATPase activity, various sulfhydryl reagents have been used. Treatment with p-chloromercuribenzoate (p-CMB) abolishes both ATPase activity and the capacity to bind with actin (Bailey and Perry, 1947). Partial reaction with p-CMB or N-ethylmaleimide (NEM) increases the rate of the ATP hydrolysis up to the point when about half of the

free-sulfhydryl groups of myosin have reacted (Kielley and Bradley, 1956). This indicates that not all of the sulfhydryl groups are necessary for the hydrolysis of ATP. Phenylmercuric acetate and 2,4-dinitrophenol (DNP) activate at low concentration and inactivate at high concentration. Various oxidizing agents such as iodosobenzoate, hydrogen peroxide and iodine readily bring about inhibition (Perry, 1955). Another factor that must be considered is the temperature at which the experiments are performed. Activation of myosin ATPase by EDTA, p-CMB, zinc ions, and DNP occurs at 25°C, but the same reagents will inhibit at 0°C, even at low concentrations. These results led to the proposal that activation consists of the reversal of the myosin aggregation which occurs at moderate temperatures (Szent-Gyorgyi, 1960).

Tropomyosin

Tropomyosin is the only fibrous protein which forms true crystals (Bailey, 1947). The molecular weight has been reported in the range of 54,000 to 60,000 (Gergely, 1966) and the isoelectric point at pH 5.1 (Szent-Gyorgyi, 1960). That no tryptophane and very little proline are contained in tropomyosin, is an indication of the purity of a given preparation (Bailey, 1947).

There are two types of tropomyosins, designated as A and B. Tropomyosin A has been isolated only from invertebrate smooth muscle, and tropomyosin B is the type isolated from vertebrate skeletal muscle (Perry, 1965). Although tropomyosin B is a quantitatively significant component of the myofibril, its exact function in the contraction mechanism is still unknown. It has been hypothesized by Huxley (1965)

that tropomyosin B is involved in the attachment of the I-filament (actin) to the Z-line (primarily made up of tropomyosin B).

CHAPTER III

MATERIALS AND METHODS

Muscle Source

Five hereford steers were obtained from the Oklahoma Agriculture Experiment Station herd. The steers in each experiment were of similar breeding, age, and weight (approximately 860 kg when slaughtered). The steers were delivered to the Meat Laboratory prior to slaughter. Feed and water were withheld overnight. Slaughtering was according to the procedures established at the Meat Laboratory and consistent with methods and practices currently used in the industry. The longissimus dorsi muscles were excised immediately post mortem, chilled in ice and were held in a $0^{o}C$ cooler of approximately 70% relative humidity. They were freed of surrounding fat and connective tissue, then minced twice in a grinder through 3 mm plate.

Preparation of Muscle Powder

A 500 gm aliquot of the minced muscle was extracted immediately with 3 volumes (1500 ml) of cold 0.3 M KCl + 0.09 M KH_2PO_4 + 0.06 M K_2HPO_4 (pH 6.5) in a $0^{o}C$ cooler. After having been stirred for 15 minutes, the mixture was centrifuged for 10 minutes at 10,000 x g. The supernatant was discarded and the residue was washed, in the cold, by gentle stirring in 4 volumes of 0.05 M $NaHCO_3$ (a pH of about 7 resulted) for 20 minutes,

strained through 6 layers of cheese cloth, resuspended in 10 volumes of water, and stirred for 25 minutes. It was again filtered through 6 layers of cheese cloth and pressed by hand as dry as possible. The residue was extracted twice with 2 volumes of ice-cold n-butanol and twice with 2 volumes of acetone in the $0^{\circ}C$ temperature; each time the mixture was briefly disintegrated in a Waring blender (3 times, each of 3 seconds duration), then left suspended for 20 minutes, and filtered through 6 layers of cheese cloth and pressed as dry as possible. After the last extraction, the material was spread out on a filter paper and left to dry in the cold. It can be stored in a jar, but even at low temperature, such as $-10^{\circ}C$, the material usually deteriorates within a few weeks.

Preparation of Crude Actin

A 50 gm dry muscle powder was suspended in 1 liter of 2×10^{-4} M ATP solution at pH 8.0 - 8.2. The pH of the suspension was adjusted by NaOH or HCl whenever it was necessary. The dry muscle material, after butanol-acetone treatment, did not wet easily; and the mixture had to be stirred to assure wetting. After 30 minutes extraction in the cold, the suspension was then filtered through 16 layers of cheese cloth and was re-extracted with 300 ml of ATP solution. The combined extracts, 600 - 900 ml, were clarified by centrifugation for 1 hour at 10,000 x g; the supernatant was filtered through 8 layers of cheese cloth. The solution so prepared containing globular G-actin was designated as crude G-actin, usually of about 30 to 50% purity and having a protein concentration of about 5 mg per ml.

Preparation of Pure Actin

A 20 ml solution of 2 M KCl was added to the crude G-actin extract; and polymerization was allowed to proceed for several hours, at least 3 hours (best overnight), in a 0^{o}C cold room. The progress of polymerization was best tested by observing the increase in viscosity, which was so obvious that it could be seen from the slow rotation of the fluid set in motion by swirling and from the slow rise of air bubbles trapped by the moving solution.

After polymerization, the F-actin was collected by centrifugation in a Spinco model L preparative ultracentrifuge, No. 30 rotor, for 2 hours at 59,000 x g. The extract was centrifuged in two batches, the second sample having been sedimented on top of the pellets from the first run. The supernatants were discarded, and the final sediment was rinsed with a small amount of water to remove any foam. The pellets were dissolved in the cold, in 400 ml of 2×10^{-4} M ATP solution at pH 8.2, by rapid stirring (without foaming) for 2 hours; 10 ml of 2 M KCl was added to reverse any depolymerization during the dissolution process, and the fibrous actin, after standing for 30 minutes or longer, was again sedimented by centrifugation for 3 hours at 59,000 x g.

The sediment was dissolved in 200 ml of 4×10^{-4} M ATP solution by stirring. This solution was dialyzed with continuous slow stirring for 2 days in the cold (some toluene was added) against 2 changes of 10^{-4} M ATP solution at pH 8.2. If required, dialysis was continued. The solution was filtered through 4 layers of cheese cloth, and was centrifuged for 2 hours at 59,000 x g. The residue was discarded and the supernatant was G-actin. The average yield was about 1000 mg pure G-actin.

Estimation of Protein Concentration

The concentration of protein in the diluted G-actin solutions was determined by Layne et al. (1957) biuret method. Crystallized bovine serum albumin was used as the standard. The optical density at 550 mμ was measured in a Bausch and Lomb Spectronic 20.

Polymerization of Actin

Polymerization was induced by the addition of KCl to a final concentration of 0.1 M at a specific pH. Polymerization was allowed to proceed for two hours at a constant temperature of 24°C, except as otherwise stated.

Viscosity Determination

The viscosity of the protein aliquots were determined by the use of a Cannon-Fenske viscometer, F88, size 100, capacity 7.3 ml. Viscosity measurements were conducted at a specific constant temperature of 24°C, except where otherwise stated. The time in seconds required for 5 ml liquid to flow through the viscometer capillary tube was recorded. The final reading for each sample was obtained by averaging the two determinations that agreed within one second.

Statistical Analysis

An analysis of variance and F-test (Steel and Torrie, 1960) were used to determine the effect of treatments on the polymerization of bovine G-actin. An IBM computer (System 360, Model 30) was used for statistical analysis.

CHAPTER IV

ISOLATION AND PURIFICATION OF BOVINE G-ACTIN

For the study of the polymerization of G-actin to F-actin, which is intimately involved in developing the rigid state in beef muscle during the initial post mortem phase, a pure system of actin must be obtained. All isolation work conducted was with pre-rigor muscle, since a greater yield of pure actin can be obtained at this time.

The purification of actin is not based on the traditional sequence of preparation or adsorption procedures, but on a highly specific property of this protein: the ability to be transformed into a polymer, F-actin, by the addition of neutral salt. The polymerized protein is separated from the unpolymerized impurities by preparative ultracentrifugation and is reconverted into the monomer.

The method starts with the preparation of an acetone-dried muscle powder as described by Straub (1943), discoverer of actin, to which some modifications have been proposed (Tsao and Bailey, 1953). The difficulties involved in the actin extraction from bovine muscle, which contains considerable quantities of lipid material and tropomysin, have been resolved by the treatment of the muscle with acetone and n-butanol at low temperature ($0^{o}C$). After the dry muscle powder is obtained, it can be stored in a jar over silica gel, but even at low temperature ($-10^{o}C$), the material usually deteriorates within a few weeks.

The purification procedure used in this experiment is a modified

version of that of Mommaerts (1952a). The techniques used by Mommaerts to extract actin from rabbit muscle involved treatment with ATP solution, which has first been freed of air, with a current of nitrogen or helium; this step has been omitted in this experiment, although anaerobic conditions are maintained during the extraction as far as feasible. The suspension is filtered through several layers of cheese cloth in the entire procedure instead of filtered through paper by suction. Although the modifications are relatively minor, they are important from the standpoint of yield, degree of purity and biological activity.

Experimental Procedure

Analytical Ultracentrifuge Analysis

The ultracentrifugation was performed using a Beckman Model E analytical ultracentrifuge equipped with electronic speed control, automatic temperature control and adjustable optical components. The AN-D rotor, operated at 59,780 rpm and 20°C, contained two 12 mm cells with Kel-F centerpieces, one having a plain quartz upper window, the other having a 2° positive wedge quartz upper window. The schlieren patterns were obtained with automatic 16 minutes photographic sequence and 10 seconds exposure. The diaphragm angle was 65°. In this analysis G-actin was dissolved in 4×10^{-4} M ATP solution; the concentration of crude and pure G-actin was 4.18 mg/ml and 4.5 mg/ml, respectively.

Bio-Rad P-100 Gel Filtration

Bio-Rad P-100 gel with a mesh range between 100 and 200 was used for the column chromatography. These porous polyacrylamide beads were commercially available from Bio-Rad Laboratories, Richmond, California.

Two sizes of columns were employed. The dimensions of the column gel bed were 1.5 cm x 8.5 cm and 2.5 x 16 cm for the small and large column, respectively. The lower ends of the column were nearly flat and were equipped with 2 mm Leur joints to allow the fitting of a syringe needle. This needle was in turn connected to canular tubing which carried the effluent from the column to the fraction collector. Gels were retained in the column by a disc of Whatman #1 filter paper and 1 cm glass wool. Protein solutions were placed on the column by layering under the eluant. Metal free water was used as elution solvent. Effluent from the small and large columns was collected in 5 ml and 3 ml fractions, respectively.

Polyacrylamide Gel Disc Electrophoresis

Aliquots of the protein samples were electrophoresed (ca. 45 min.) against a Tris-glycine buffer (pH 8.4; ionic strength 0.1) in the Canalco Disc electrophoresis unit. Current was maintained at 5 ma. per tube and voltage was 250 V per tube. All fractions were migrated towards the anode. The use of a tracking dye (1% Bromophenol Blue) permitted the electrophoresing of all samples to a common end-point.

Gels were stained overnight in amido black stain (400 ml dist. H_2O; 400 ml methyl alcohol; 100 ml glacial acetic acid; 1 gm amido black dye). Gels were then destained, for about 90 minutes in the Canalco unit in a $4^{\circ}C$ cold room.

Gels were scanned and plotted via a Densicord Recording Electrophoresis Densitometer Model 542 and the fractions (area under each peak on the plot) integrated via an Integraph Automatic Integrator Model 49. Both pieces of equipment are manufactured by the Photovolt Corporation.

To permit accurate scanning, plotting and integration the above

equipment was modified as follows: (a) aperture slit was reduced to 2.5 mm in length and 0.1 mm in width; (b) chart speed was reduced to 0.75 inches per minute; (c) plexiglass chart guide was routed directly above integration count line to prevent smearing of the chart ink.

Results and Discussion

Criteria of Purity

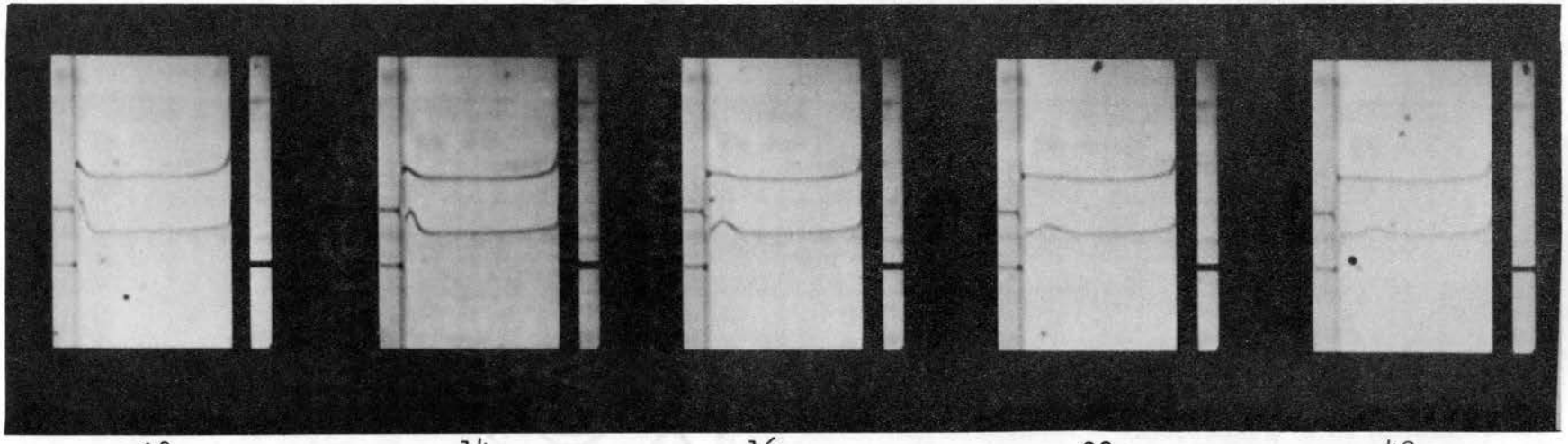
Three criteria were used to test the purity of actin isolated. They were ultracentrifuge sedimentation pattern, column chromatography on Bio-Rad P-100 gel and polyacrylamide gel disc electrophoresis.

Analytical Ultracentrifuge Analysis. The purification of actin by ultracentrifugal isolation of its polymer (Mommaerts, 1952, 1952a), followed by depolymerization, has become widely accepted. However, the judgment as to the purity of the products rests on somewhat limited criteria: the complete polymerizability of the product upon the addition of salt, and the homogeneous appearance of the sedimenting boundary of the monomer in the ultracentrifuge. The first criterion is the more valuable, but it might not detect moderate amounts of impurity associated with the sedimenting polymer; the second criterion was not used quantitatively since the sedimentation in a medium without electrolyte other than a small excess of ATP might be a complex theoretical problem (Mommaerts, 1952a).

Two sedimentation pictures are shown in Figure 1. The bottom curve represents the ultracentrifuge sedimentation pattern of crude G-actin. It shows that the sedimentation is not completely symmetrical on both sides, as several zigzag minor components are discernible in the leading

Figure 1. Ultracentrifuge Sedimentation of Bovine Crude Actin.

The sedimentation patterns of crude G-actin (shown in bottom curves) and crude F-actin (shown in top curves) at a concentration of 4.18 mg/ml in 4×10^{-4} M ATP solution, pH 8.2, in a synthetic boundary cell. Photographs were taken at the time indicated after the full rotor speed of 59,780 rpm was attained. The temperature was $20^{\circ}C$, and the phase plate angle was 65°. Sedimentation is from left to right. $S_{20,w} = 3.215$.



10 14 16 32 48

Time in Minutes

slope. This indicates that the crude actin preparations were heterogeneous with one major component and several minor components. The top curve represents the crude F-actin obtained by the polymerization of the crude G-actin in 0.1 M KCl solution. The curve shows that F-actin was sedimented quickly to the right, in less than 10 minutes at the speed of 59,780 rpm; yet there are still some unpolymerizable materials indicated in the sedimentation pattern.

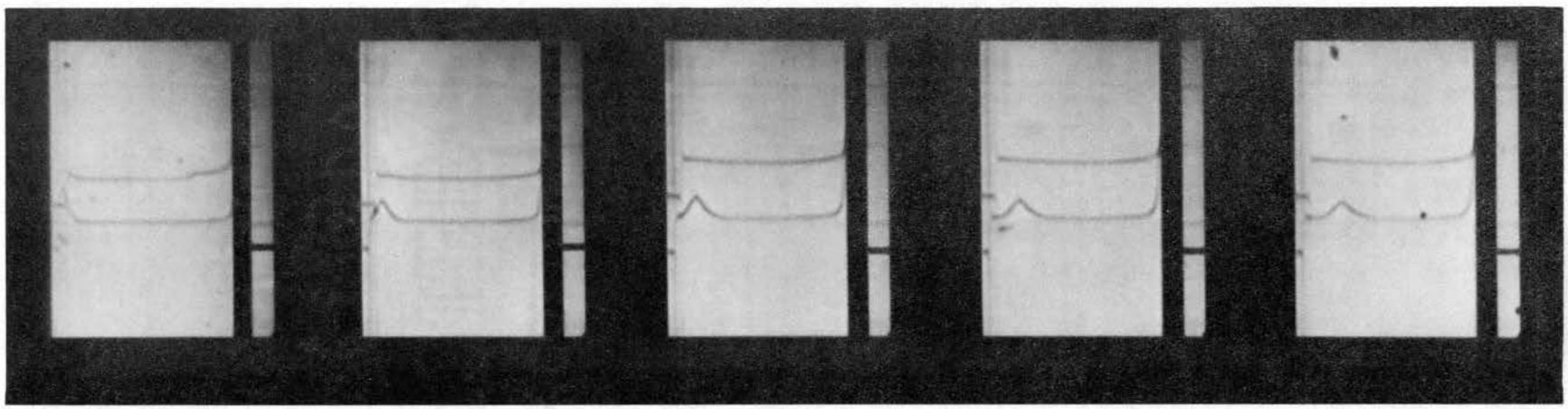
After crude G-actin was purified by the polymerization-depolymerization cycle, pure G-actin was obtained. Its sedimentation pattern is shown in Figure 2. A single, almost symmetrical, boundary can be observed indicating a high degree of purity of the preparation.

The F-actin obtained by the polymerization of pure G-actin was shown in the top of the same figure. The F-actin sedimentation was visible at 14 minutes; but at 16 minutes after the centrifuge reached the speed of 59,780 rpm, the peak completely disappeared. The straight and smooth curve provided a second criterion of purity in this particular case, since it permitted a test of the completeness of polymerization after the addition of salt.

The Schlieren pattern of the protein isolated with pH 6.6 ATP solution is shown in Figure 3. Although a high degree of purity of the preparation is indicated by the single fraction, close examination reveals that this pattern is not exactly homogeneous. The small "shoulder" on the leading slope might be attributed to partial polymerization of the G-actin and the formation of low polymers, such as dimers or trimers of G-actin. Meanwhile, there are considerable amounts of heterogeneous materials which sedimented in the first 15 minutes (Figure 3). However, when the G-actin was isolated with pH 8.2 ATP solution, the

Figure 2. Ultracentrifuge Sedimentation of Bovine Pure Actin

The sedimentation patterns of pure G-actin (shown in bottom curves) and pure F-actin (shown in top curves) at a concentration of 4.5 mg/ml in 4 x 10^{-4} M ATP solution, pH 8.2, in a synthetic boundary cell. Photographs were taken at the time indicated after the full rotor speed of 59,780 rpm was attained. The temperature was $20^{o}C$, and the phase plate angle was 65^{o}. Sedimentation is from left to right. $S_{20,w} = 3.266$.

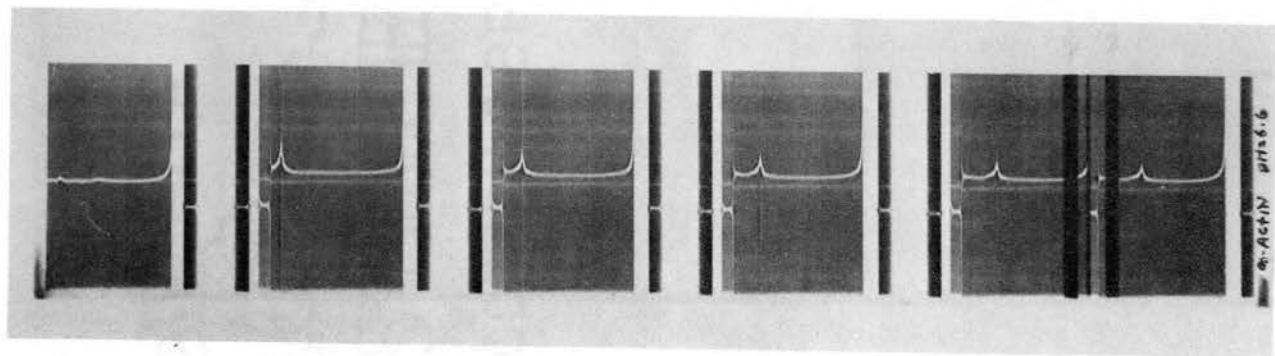


14 16 32 48 64

Time in Minutes

Figure 3. Ultracentrifuge Sedimentation of Bovine G-actin Isolated at pH 6.6

The sedimentation patterns of bovine pure G-actin (isolated with pH 6.6 ATP solution) at a concentration of 3.922 mg/ml in 4 x 10^{-4} M ATP solution, pH 6.6, in a synthetic boundary cell. Photographs were taken at the time indicated after the full rotor speed of 59,780 rpm was attained. The temperature was 20°C, and the phase plate angle was 65°. Sedimentation is from left to right. $S_{20,w}$ = 3.715.



15 16 32 48 54 80

Time in Minutes

Schlieren pattern (Figure 2) showed a single sharp peak, indicative of purity.

According to the techniques for characterization of proteins (Schachman, 1968), the sedimentation coefficient of bovine G-actin was determined. It was found that pure G-actin isolated at pH 8.2 had a $S_{20,w} = 3.266$. This value was in close agreement with those published for rabbit G-actin. Lewis, et al. (1963) reported a value of 3.25; and Krans, et al. (1965) reported values of 3.7 and 2.6, respectively, for non-reduced and reduced G-actin isolated from rabbit muscle. However, the sedimentation coefficient of pure bovine G-actin isolated at pH 6.6 ($S_{20,w} = 3.715$) was higher than it was at pH 8.2. This might be due to the effect of the solvent since the sedimentation coefficient was a function both of the molecular weight of the solute and of the frictional resistance which the molecules or particles experience as they move through the solvent. It also was determined that crude G-actin at pH 8.2 had a $S_{20,w} = 3.215$ which was slightly lower than the pure ones at the same pH.

Bio-Rad P-100 Gel Filtration. Results from the chromatographic separation of G-actin preparation are presented in Figures 4 and 5. In Figure 4 a smooth and symmetrical peak was observed, indicative of purity. However, when the small column was replaced by the more critical, larger one, a small peak can be noted in the ascending slope of the curve (Figure 5), indicating that the G-actin preparation contained small amounts of higher molecular weight materials.

Polyacrylamide Gel Disc Electrophoresis. The electropherograms obtained on the G- and F-actin preparation are shown in Figure 6. The

Figure 4. Chromatographic Separation of Bovine G-Actin Preparation on Bio-Rad P-100 Gel (8.5 cm x 1.5 cm Small Column)

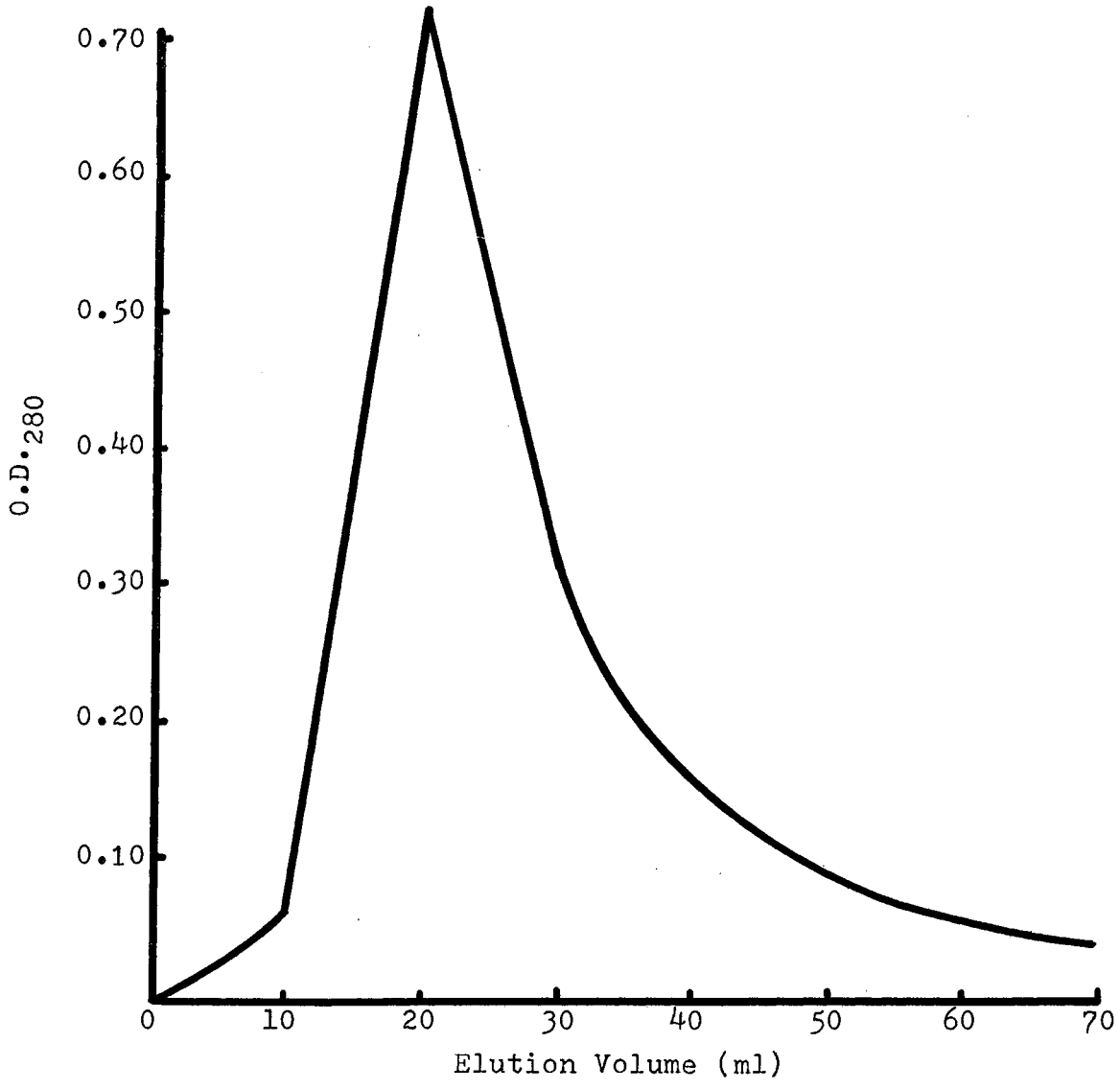
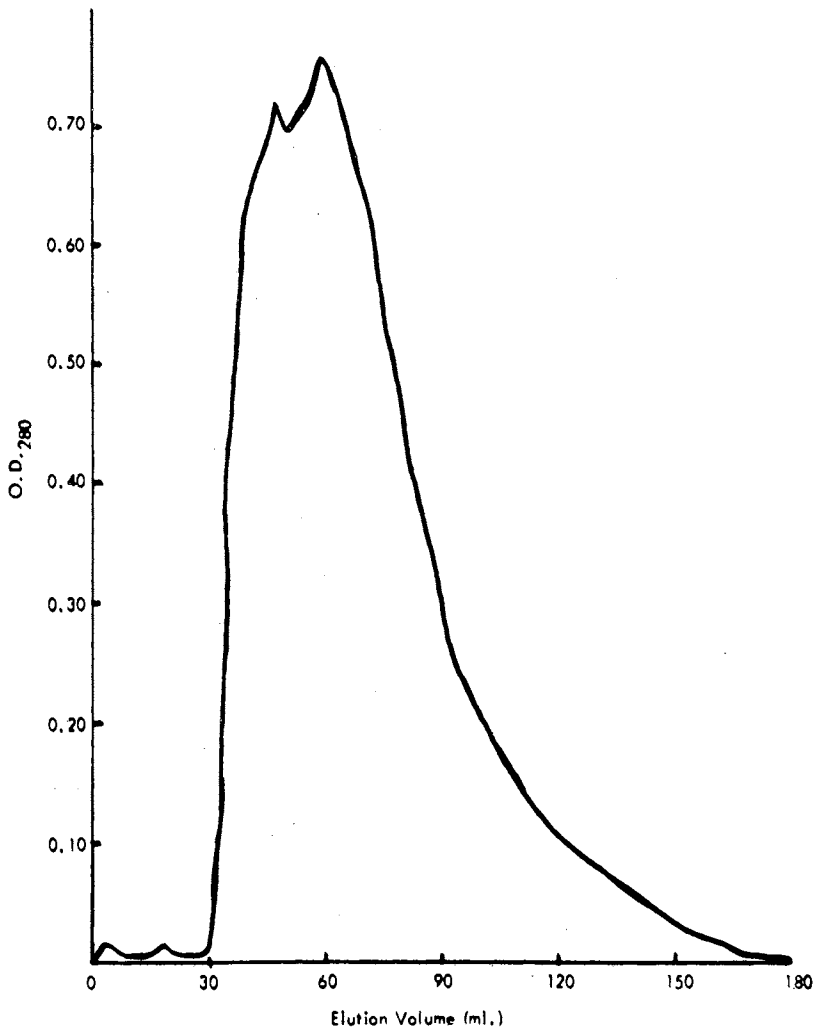

0.70
0.60
0.50
0.40
0.30
0.20
0.10
O.D.280
0
10
20
30
40
50
60
70
Elution Volume (ml)

Figure 5. Chromatographic Separation of Bovine G-Actin Preparation on Bio-Rad P-100 Gel (16.0 cm x 2.5 cm Large Column)


0.70
0.60
0.50
0.40
0.30
0.20
0.10
O.D.280
0
30
60
90
120
150
180
Elution Volume (ml.)

patterns for these two proteins are quite similar with respect to overall mobility and location of nine and eight major fractions, respectively. It can be seen, however, that the G-actin pattern contained an additional very mobile component.

In comparing the electrophoretic patterns obtained from the gels and scanning chart (Figure 7), it was noted that, in all cases, eight to nine distinct and easy to quantitate bands were obtained. In addition, these bands appeared to exhibit similar mobility from sample to sample.

Electrophoretic patterns of the chromatographed bovine G-actin preparation, obtained from the large column, are shown in Figure 8. Five protein bands were noted. This deterioration of the pattern might be ascribed to a higher purity of bovine G-actin obtained by filtration through Bio-Rad P-100 gel.

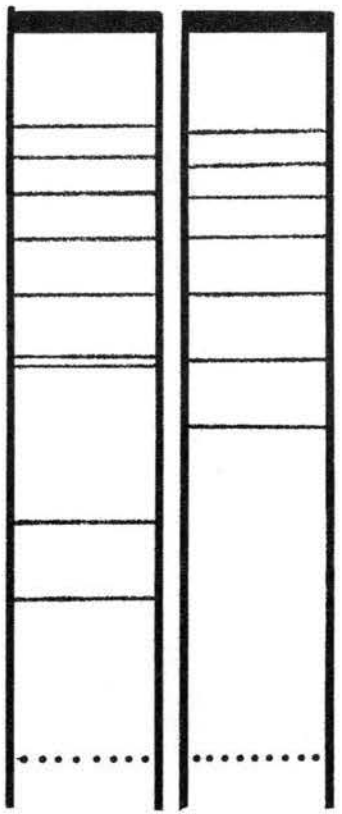
It must be pointed out, however, that much sharpness is lost during photography and many of the smaller fractions, though well resolved on the actual gel, are obscure on the photograph.

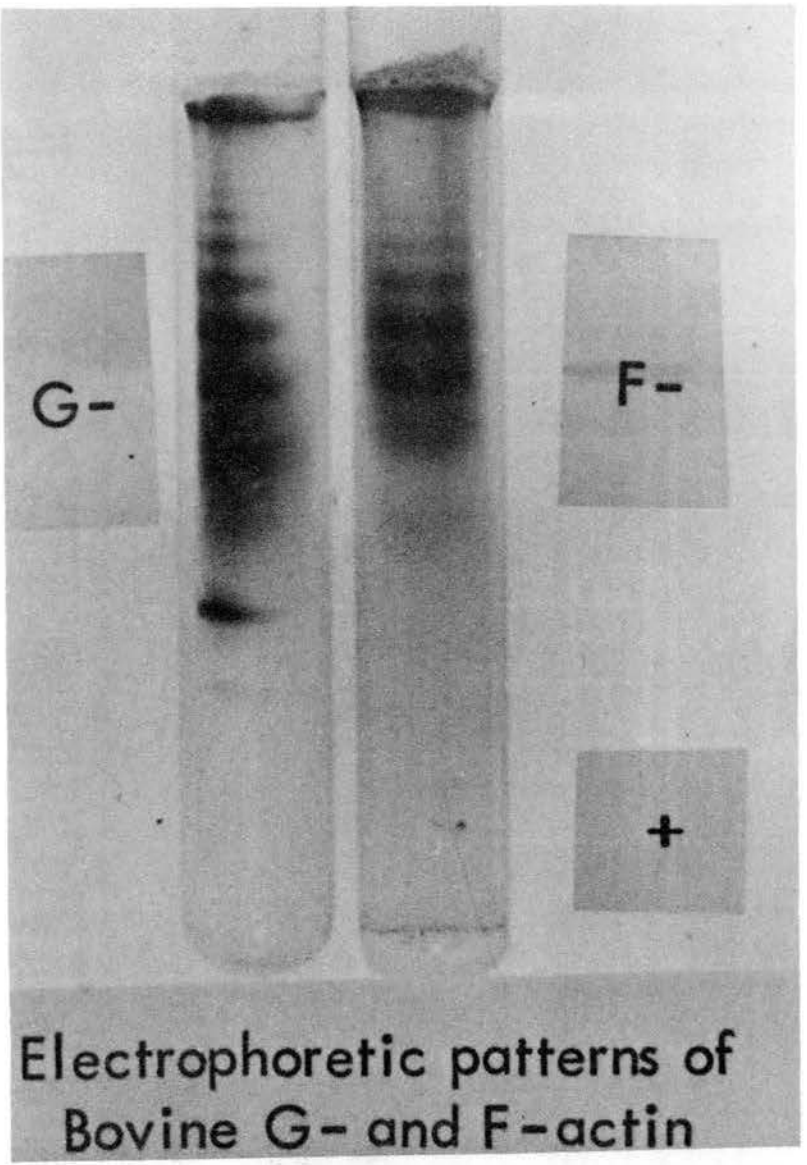
Krans, et al. (1965) conducted starch-gel electrophoresis studies with G-actin prepared from skeletal muscle of the rabbit. Four bands were always present (occasionally one or two very faint additional bands nearer to the origin, could be observed), indicating that this protein is heterogeneous. Identical starch-gel diagrams were also obtained by Krans, et al. (1965) for skeletal muscle of the rabbit, the breast muscles of the pigeon, and skeletal muscle of the ox. This suggests that the heterogeneity is a general property of G-actin.

Figure 6. Electrophoretic Patterns of Bovine G- and F-Actin

Electrophoresis was conducted in 7% separating polyacrylamide gel. The cathode end of the gels was oriented toward the top; the anode end was toward the bottom. In the diagrams, solid lines refer to the protein bands; broken lines refer to the dye fronts.

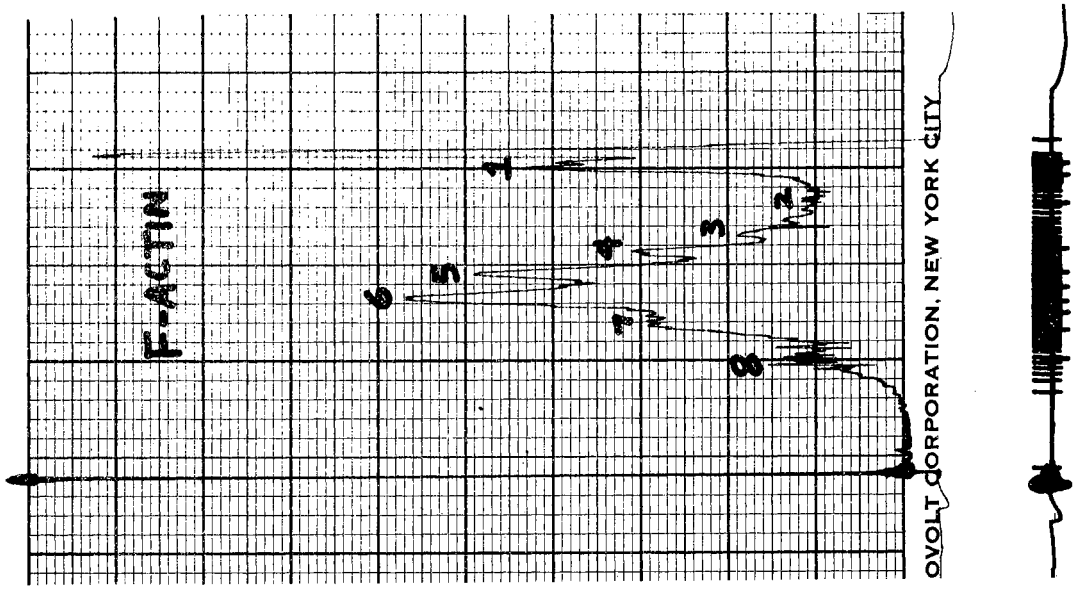
1. G-actin
2. F-actin





Electrophoretic patterns of
Bovine G- and F-actin

Figure 7. Densitometer Plots of Disc Gel Electrophoresis Patterns of Bovine G- and F-Actin


F-ACTIN
OVOLT CORPORATION, NEW YORK CITY

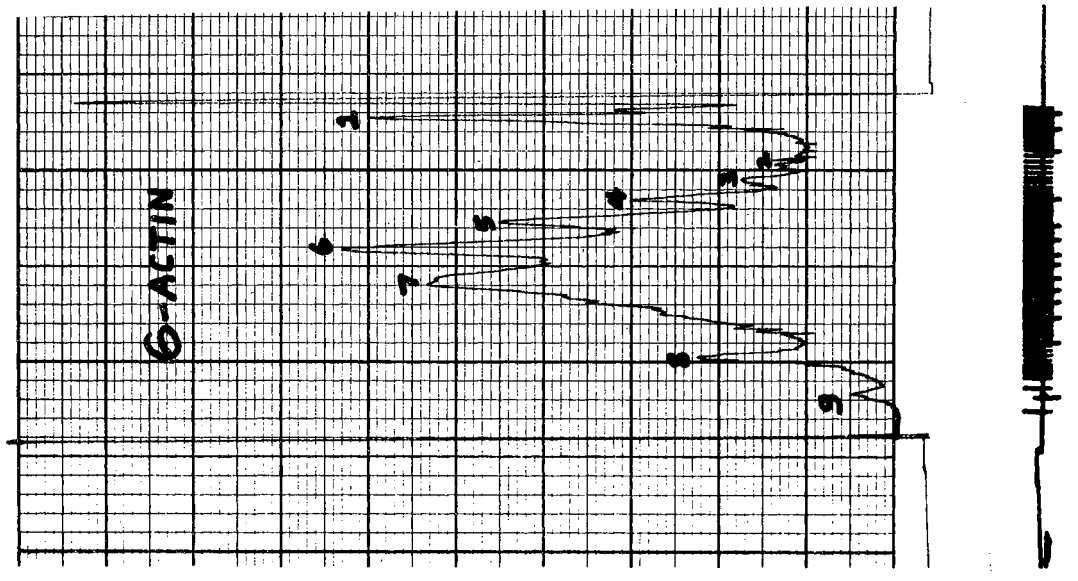
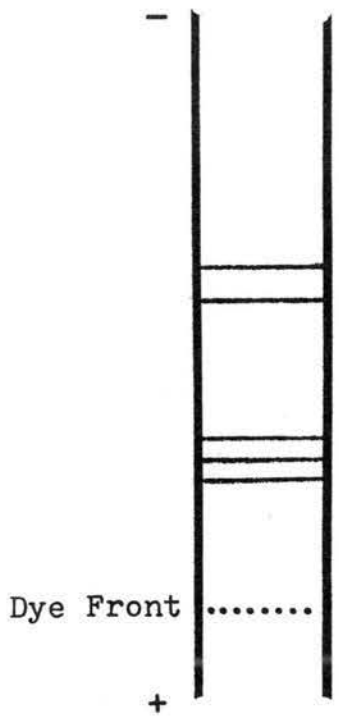
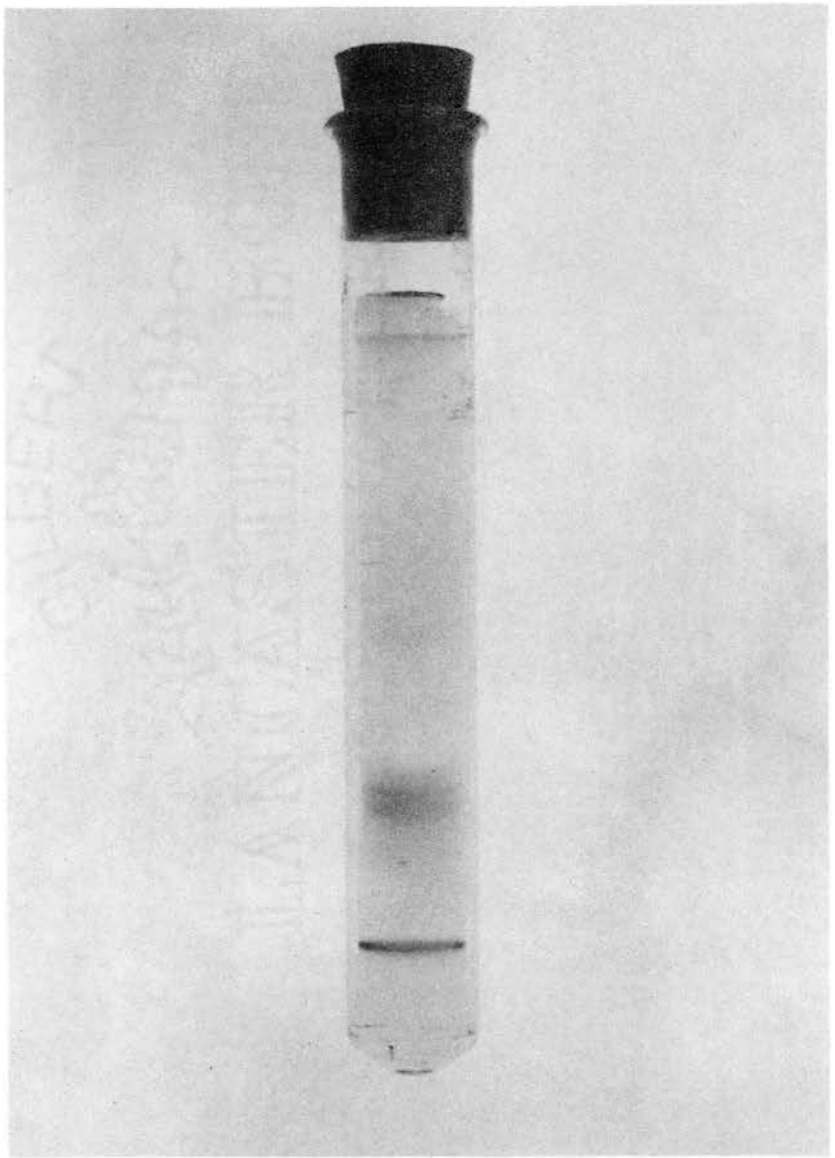

G-ACTIN

Figure 8. Electrophoretic Separation of the Chromatographed Bovine G-Actin Preparation Obtained from the Large Column.

Electrophoresis was conducted in 7% separating polyacrylamide gel. The cathode end of the gels was oriented toward the top; the anode end was toward the bottom. In the diagram, solid lines refer to the protein bands; the broken line refers to the dye front.


−
Dye Front
+



Important Factors Affecting the Isolation

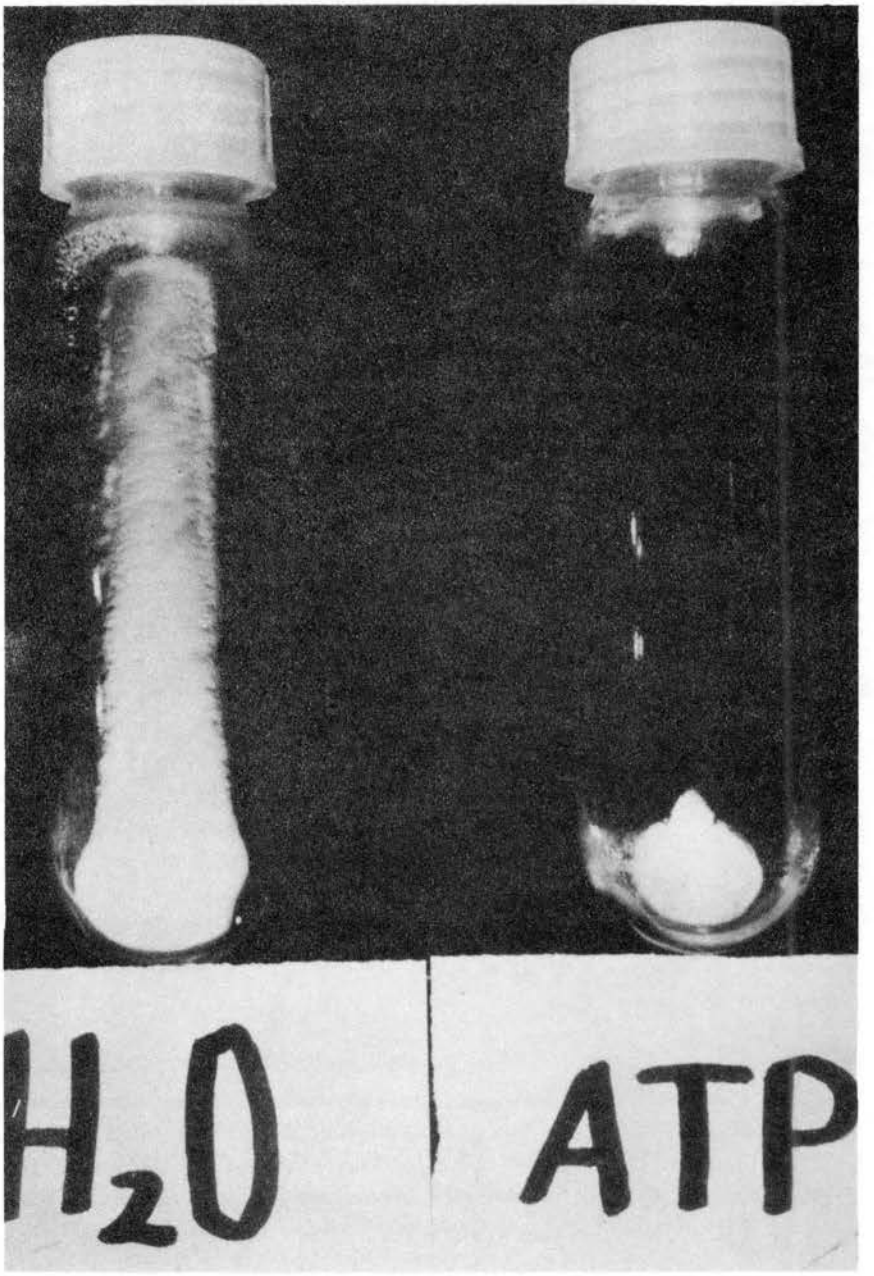
There are two major factors which will affect the yield and biological activity of bovine G-actin preparation. These factors involve the presence of ATP and the maintenance of the proper pH (8.2) of the extraction solution through the entire isolation and purification procedure.

Effect of ATP on the Depolymerization of F-Actin. In the last step of purification of bovine actin, the same volume of metal free distilled H_2O was used to replace the 200 ml of 4×10^{-4} M ATP solution for depolymerization and dialysis. The dialyzed sample was centrifuged for 2 hours at 59,000 x g as described in the section of preparation of pure actin. The supernatant, containing the pure G-actin, was quantitatively determined, and the precipitate in the centrifuge tube was photographed.

Figure 9 shows that the residue obtained after depolymerization of F-actin in distilled H_2O was much more than in ATP solution. Since more F-actin was obtained in the residue, less G-actin would appear in the solution. This suggests that ATP should be used throughout the isolation and purification procedure if one is interested in obtaining a high yield of bovine G-actin.

Comparison of the Polymerization of G-Actin Isolated with pH 8.2 and 6.6 ATP Solution. G-actin was isolated from pre-rigor bovine longissimus dorsi muscle according to the procedure reported previously. However, pH 8.2 and pH 6.2 ATP solutions were used in the preparation. F-actin was induced from the polymerization of G-actin in dilute KCl salt solution. The viscosity of each 5 ml actin solution was measured at $25^{o}C$ with a Cannon-Fenske Viscometer, F88, size 100.

Figure 9. Inactive Actin Precipitate Residue Derived from the Depolymerization of F-Actin in H_2O and in ATP Solution


H_2O
ATP

The biological polymerization activity in terms of viscosity reading for G-actin isolated at pH 6.6 and pH 8.2 and of their respective F-actin polymers was presented in Table I and Figure 10. The net increase in the F-actin viscosity readings over their respective G-actin readings are plotted in Figure 11.

These results show that while the viscosity readings of the G-actin prepared at pH 6.6 were significantly higher than those of the G-actin prepared at pH 8.2, when these protein preparations were polymerized to F-actin, the converse was true (Figure 10). From the net increase values plotted in Figure 11 one can calculate the relative polymerization efficiencies of the G-actin preparations as 12.5% versus 100%, respectively, for G-actin prepared at pH 6.6 and pH 8.2. It would appear then, that the pH of the ATP solution used in isolating G-actin is very critical, if one is interested in assessing subsequent F-actin formation.

From the values obtained in each curve, the relationship between viscosity and protein concentration can be expressed in the following equation:

pH = 6.6 G-actin

$$\text{Viscosity} = 10^{1.79338}\ 10^{0.00079\ (\text{concentration})}$$

pH = 6.6 F-actin

$$\text{Viscosity} = 10^{1.79697}\ 10^{0.01033\ (\text{concentration})}$$

pH = 8.2 G-actin

$$\text{Viscosity} = 10^{1.79645}\ 10^{0.00267\ (\text{concentration})}$$

pH = 8.2 F-actin

$$\text{Viscosity} = 60.93332 + 4.62553\ (\text{concentration})$$

The net increase in F-actin viscosity readings over their respective G-actin readings also can be expressed as follows:

TABLE I

COMPARISON OF THE POLYMERIZATION OF G-ACTIN ISOLATED WITH pH 8.2 AND 6.6 ATP SOLUTIONS

pH of ATP	G-Actin Concentration (mg/5ml)	Viscosity Reading (sec)[1]		
		G-Actin	F-Actin	NI
6.6	0.0000	62.45	62.75	0.30
	3.8359	66.55	68.05	1.50
	7.6718	71.22	75.25	4.03
	11.5077	76.65	83.10	6.45
	15.3436	82.37	90.65	8.25
	19.1795	88.65	98.00	9.35
8.2	0.0000	62.75	62.12	-0.63
	3.0937	63.70	74.85	11.15
	6.1874	64.90	88.50	23.60
	9.2811	66.00	103.65	37.65
	12.3748	67.60	117.50	49.90
	15.4685	69.00	133.67	64.67

[1]Each value is an average of two viscosity readings.

NI Refers to the net increase in viscosity reading from G-actin to F-actin.

Figure 10. Comparison of the Polymerization of G-Actin Isolated with pH 8.2 and 6.6 ATP Solution

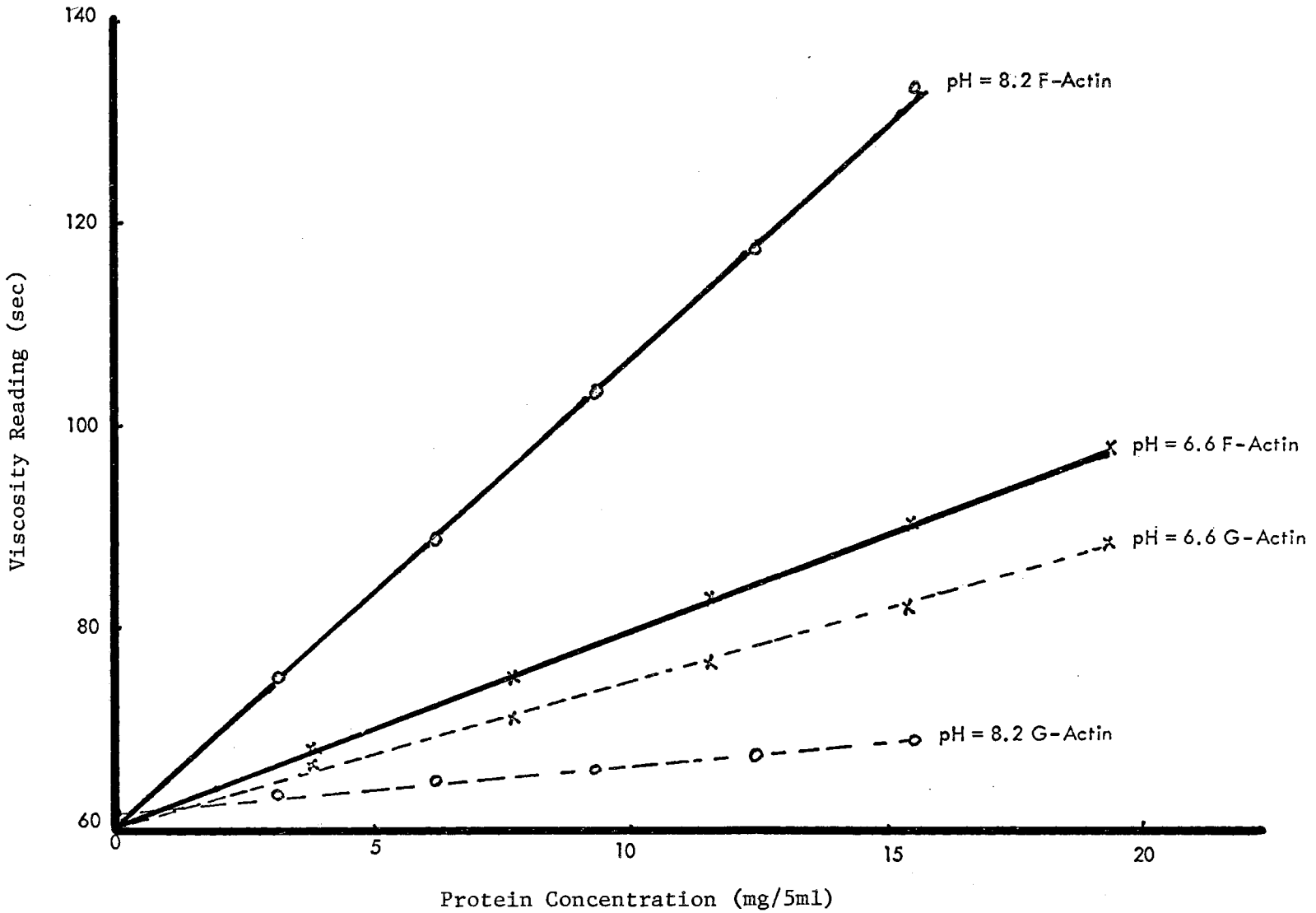
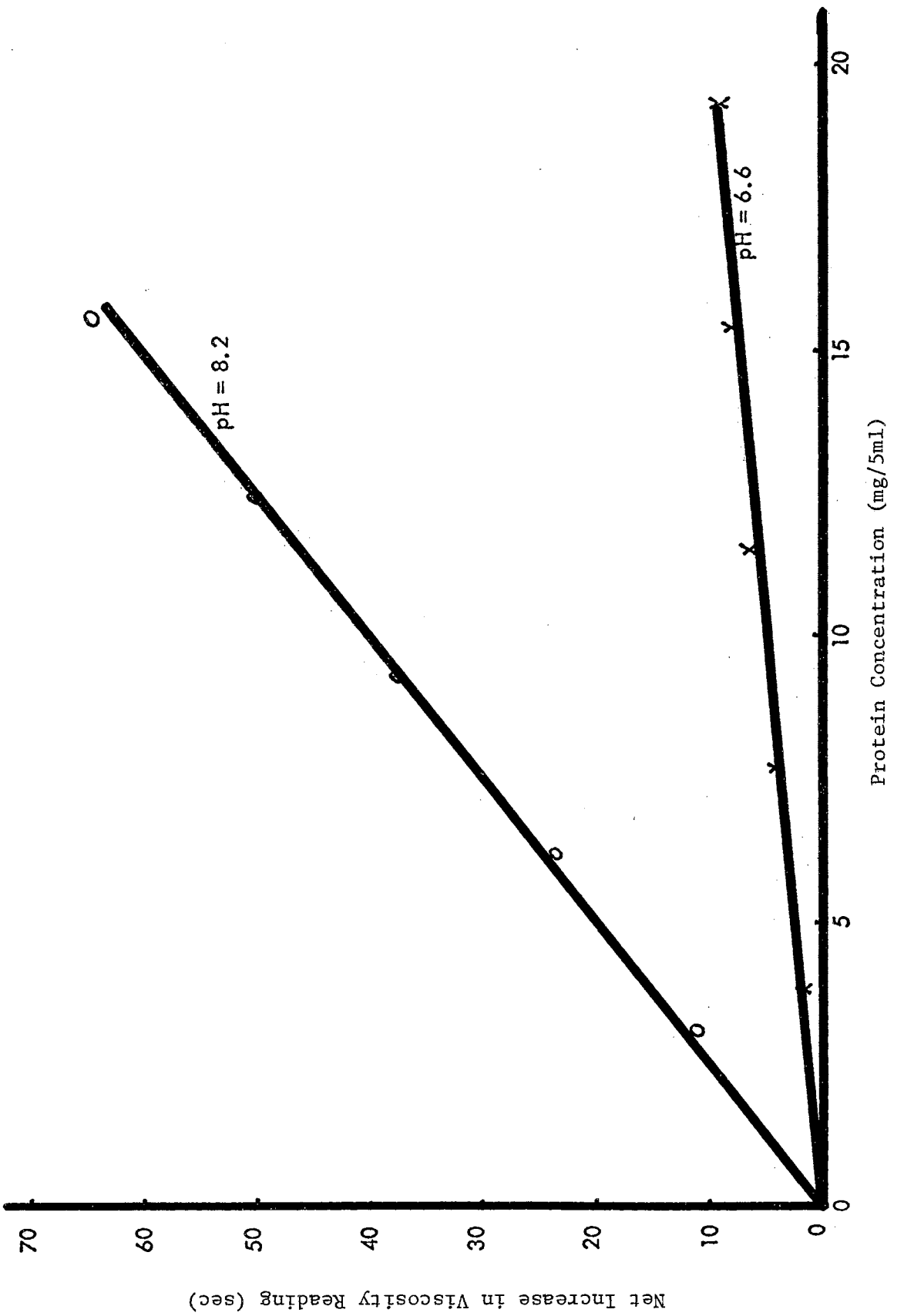

140
120
100
80
60
Viscosity Reading (sec)
0
5
10
15
20
Protein Concentration (mg/5ml)
pH = 8.2 F-Actin
pH = 6.6 F-Actin
pH = 6.6 G-Actin
pH = 8.2 G-Actin

Figure 11. Net Increases in F-Actin Viscosity Readings over Their Respective G-Actin Readings for the Actin Isolated with pH 8.2 and 6.6 ATP Solution


Net Increase in Viscosity Reading (sec)
70
60
50
40
30
20
10
0
0
5
10
15
20
Protein Concentration (mg/5ml)
pH = 8.2
pH = 6.6

F-G at pH = 6.6

$$\text{Viscosity} = 0.59522\ (\text{concentration}) - 0.61085$$

F-G at pH = 8.2

$$\text{Viscosity} = 10^{0.34542}\ 10^{0.13924\ (\text{concentration})}$$

From the above equations, if the protein concentration is known, it is possible to predict its viscosity in particular systems.

In summary, the procedure outlined above yielded a G-actin preparation of high purity, an essential pre-requisite to the study of its polymerization characteristics.

CHAPTER V

AMINO ACID COMPOSITION OF BOVINE G-ACTIN

Experimental Procedure

Pure G-actin was prepared according to the methods described in Chapter III. Protein concentrations were determined by the biuret assay. Amino acid analyses were carried out on a Beckman model 120 C automatic amino acid analyzer according to the method of Moore and Stein (1963). G-actin samples (4.8305 mg/ml) were acid hydrolyzed at 110°C for 12, 24 and 72 hours in sealed, evacuated tubes.

The values for threonine, serine and half-cystine were determined by extrapolation of the data to zero time of hydrolysis, since these amino acids were partially destroyed when the time of acid hydrolysis was prolonged. The value for ammonia was obtained by subtracting losses in serine, thereonine, and half-cystine after 24 hours hydrolysis, from the observed ammonia value at 24 hours.

Results and Discussion

The data from the amino acid analyses of the isolated pure G-actin after different periods of hydrolysis are shown in Tables II through IV. Statistical analysis is shown in Table V.

Results indicated that after 12, 24 and 72 hours acid hydrolysis the amino acid recovery percents were 96.35, 97.44 and 89.92, respectively (Table II). From the analysis of variance (Table V), it was found

TABLE II

AMINO ACID RECOVERIES AFTER ACID HYDROLYSIS

Amino Acid	mg/4.8305 mg G-actin		
	12 hr hydrolysis	24 hr hydrolysis	72 hr hydrolysis
Lysine	0.22141	0.24601	0.23898
Histidine	0.10550	0.13188	0.12412
Ammonia	0.08743	0.08045	0.06889
Arginine	0.26828	0.28396	0.27176
Aspartic Acid	0.46718	0.47117	0.44508
Threonine	0.31442	0.30132	0.26392
Serine	0.21858	0.20282	0.15385
Glutamic Acid	0.63707	0.67091	0.59205
Proline	0.20953	0.20147	0.09249
Glycine	0.22070	0.21770	0.21019
Alanine	0.28508	0.27439	0.26156
Half-cystine	0.10572	0.08169	0.06343
Valine	0.20852	0.23312	0.23804
Methionine	0.09549	0.08206	0.07281
Isoleucine	0.29250	0.30562	0.31375
Leucine	0.35022	0.36858	0.34419
Tyrosine	0.27359	0.27359	0.22032
Phenylalanine	0.18666	0.20153	0.18104
Unknown	0.10624	0.07864	0.08720
	4.65412	4.70691	4.34367
Recovery %	96.35%	97.44%	89.92%

TABLE III

NUMBERS OF AMINO ACID RESIDUES PER
MOLECULAR WEIGHT = 60,000 G-ACTIN

Amino Acid	12 hr hydrolysis	24 hr hydrolysis	72 hr hydrolysis
Lysine	23.4758	26.0842	25.3389
Histidine	8.4463	10.5579	9.9368
Ammonia	63.8443	58.7516	50.3053
Arginine	19.1284	20.2463	19.3768
Aspartic Acid	43.5979	43.9706	41.5360
Threonine	32.7916	31.4253	27.5251
Serine	25.8358	23.9726	18.1844
Glutamic Acid	53.7832	56.6400	49.9824
Proline	22.6063	21.7368	20.7680
Glycine	36.5179	36.0211	34.7790
Alanine	39.7474	38.2569	36.4682
Half-cystine	5.4652	4.2231	3.2791
Valine	22.1095	24.7179	25.2396
Methionine	7.9494	6.8315	6.0614
Isoleucine	27.6989	28.9411	29.7112
Leucine	33.1642	34.9032	32.5928
Tyrosine	18.7558	18.7558	15.1040
Phenylalanine	14.0358	15.1537	13.6134
Unknown	9.5642	7.0800	7.8501

TABLE IV

AMINO ACID COMPOSITION OF BOVINE G-ACTIN

Amino Acid	Residues[1] per molecule[2]	gm per molecule[2]	Residue % (moles)	Weight % (gm)
Lysine	26.0842	3055.7640	5.13	5.12
Histidine	10.5579	1638.1637	2.08	2.75
Ammonia	49.8034[3]	847.2408	9.80	1.42
Arginine	20.2463	3527.1079	3.98	5.92
Aspartic Acid	43.9706	5852.4868	8.65	9.82
Threonine	34.1579[4]	4068.2058	6.72	6.82
Serine	27.6990[4]	2910.3879	5.45	4.88
Glutamic Acid	56.6400	8333.4432	11.14	13.93
Proline	21.7368	2502.5577	4.28	4.20
Glycine	36.0211	2704.1039	7.09	4.53
Alanine	38.2569	3408.3072	7.53	5.72
Half-cystine	6.7073[4]	1611.6971	1.32	2.70
Valine	24.7179	2895.7019	4.86	4.86
Methionine	6.8315	1019.3281	1.34	1.71
Isoleucine	28.9411	3796.2040	5.69	6.37
Leucine	34.0932	4573.2527	6.87	7.68
Tyrosine	18.7558	3398.3634	3.69	5.70
Phenylalanine	15.1537	2503.2397	2.98	4.20
Unknown	7.0800	976.8984	1.39	1.64
	508.2696	59627.4541		

[1]Values taken at 24 hours hydrolysis.

[2]Assumes M. Wt. of 60,000.

[3]The value for ammonia was obtained by subtracting losses in serine, threonine, and half-cystine after 24 hours hydrolysis, from the ammonia value at 24 hours.

[4]Extrapolated value.

TABLE V

ANALYSIS OF VARIANCE OF AMINO ACID RECOVERIES AFTER ACID HYDROLYSIS[1]

Source	df	SS	MS
Total	56	1.04775	-----
Time	2	0.00406	0.00203***
A.A.	18	1.03444	0.05746***
Error (Time x A.A.)	36	0.00925	0.00025

***P < 0.001

[1]See Table II

TABLE VI

THE ESTIMATION OF MOLECULAR WEIGHT OF BOVINE G-ACTIN

	Minimum molecular weight for G-actin	Assumed No. of residues/ molecule	Molecular weight
Methionine	8782.72727	6.8315*	59,999.201
		7	61,479.683
Tyrosine	3199.00662	13.7558*	59,999.928
		9	60,781.125

*See Table III.

that there was a significant difference among total amino acid residues for 12, 24 and 72 hours hydrolysis. However, Table II showed that hydrolysis for 72 hours resulted in a reduction in total amino acid recovery, indicating that several of the amino acids were destroyed when acid hydrolysis was prolonged.

The values for threonine, serine and half-cystine were determined by extrapolation of the data to zero time of hydrolysis, since significant change with time in the values for these amino acids was observed (Table II).

At the 24 hour hydrolysis period, 57 glutamic acid and 44 aspartic acid residues per mole were found. This indicates that the isoelectric point of bovine G-action was on the acidic side. The amino acid of smallest quantity in this protein was methionine which has only 7 residues per molecule. Excluding the high amount of acidic amino acids, bovine G-actin also contained considerable quantity of neutral amino acids such as alanine (38 residues/molecule), glycine (36 residues/molecule), and leucine (34 residues/molecule)(Table III). These values were found to agree excellently with G-actin isolated from skeletal muscle of rabbit (Krans, et al., 1965; Carsten, et al., 1963; Laki, et al., 1962). However, rabbit G-actin contained a much higher amount of sulfur containing amino acids. For example, there were 7 and 20 methionine residues per mole of bovine and rabbit G-actin, respectively.

In view of the specie differences noted in these functional amino acids, it appeared that further characterization of this protein, isolated from bovine muscle would be in order.

The minimal molecular weight of the bovine G-actin was calculated from its amino acid composition, assuming one mole of methionine per

mole of protein (Table VI). This gave a value of 8,782.727. If seven methionine residues were present per molecule of G-actin, the molecular weight would then be about 61,479. If the molecular weight is computed in terms of tyrosine residues, then the molecular weight thus obtained was 60,781. These results are in reasonable agreement with the values estimated from other methods (Mommaerts, 1952; Ulbrecht, et al., 1960; Lewis, et al., 1963; Krans, et al., 1965).

CHAPTER VI

ELECTROPHORETIC STUDIES ON BOVINE G- AND F-ACTIN

Experimental Procedure

G-actin and F-actin were prepared as reported previously in Chapter III. Electrophoretic separations were accomplished via the polycrylamide gel disc electrophoretic procedure outlined in Chapter IV.

Results and Discussion

Effect of Reducing Agent on the Electrophoretic Pattern of G-Actin

Figure 12_2 shows that when a 2 µg of thioglycolate was added between the sample gel and spacing gel, the number of bands of G-actin was not altered from Figure 12_1 which received no thioglycolate treatment. However, the sharpness of the band was greatly reduced. When the reducing agent (thioglycolate) was added to the electrophoresis upper buffer, the bands were decreased from nine to four as shown in Figure 12_1 and 12_3, respectively.

When the acrylamide content of the separating gel was decreased from 7% (Figure $12_{1,2,3}$) to 3.5% (Figure $12_{4,5,6}$), the entire electropherogram was changed as shown in Figure $12_{4,5,6}$. Both the pure G-actin without any reducing agent treatment (Figure 12_4) and the one which had received a 2 µg reducing agent addition between the sample and spacing

Figure 12. Effect of Reducing Agent on the Electrophoretic Pattern of G-Actin

Electrophoresis was conducted in 7% (gels 1-3) and 3.5% (gels 4-6) separating polyacrylamide gels. The cathode end of the gels was oriented toward the top; the anode end was toward the bottom. In the diagrams, solid lines refer to the protein bands; broken lines refer to the dye fronts.

1. Using 7% separating gel, no reducing agent (thioglycate) added.

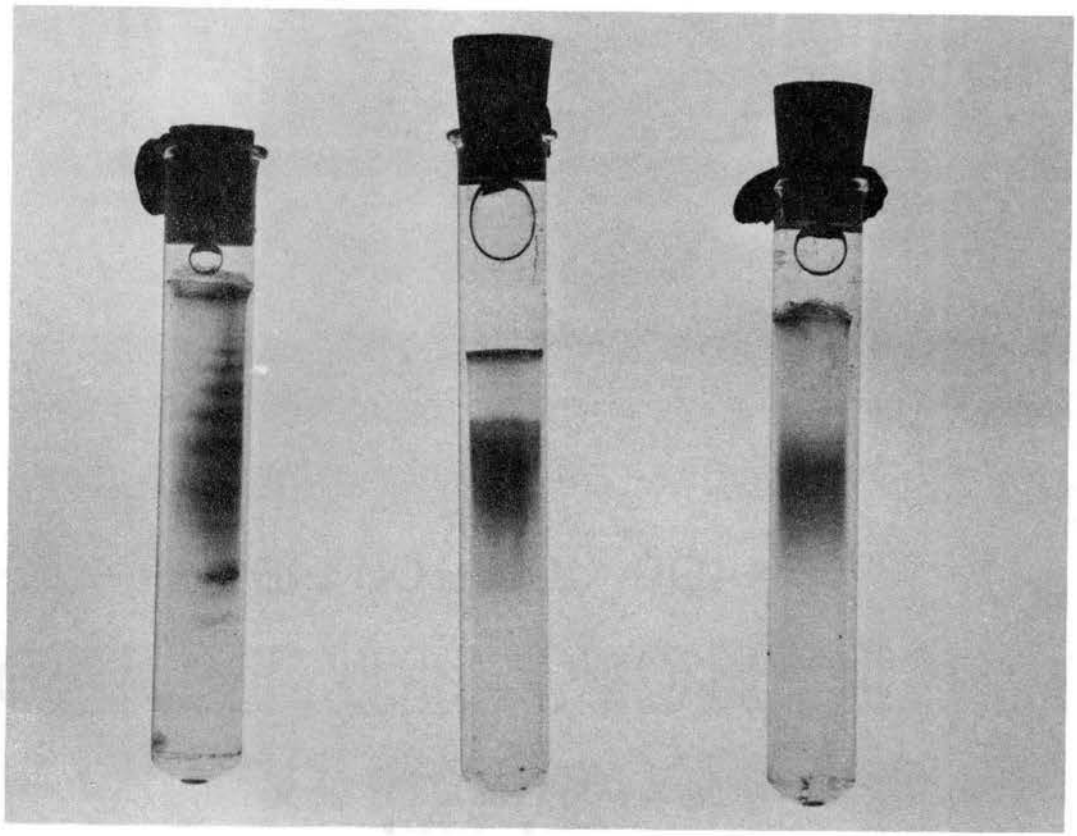
2. Using 7% separating gel, reducing agent (2 µg thioglycate) added between the sample and spacing gel.

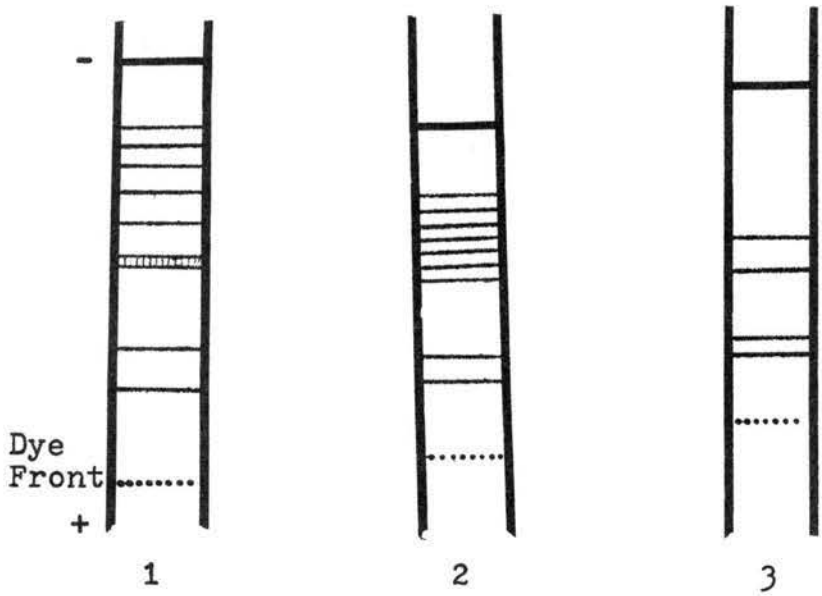
3. Using 7% separating gel, reducing agent added in the upper electrophoretic buffer solution (0.2 µg thioglycolate/ml buffer).

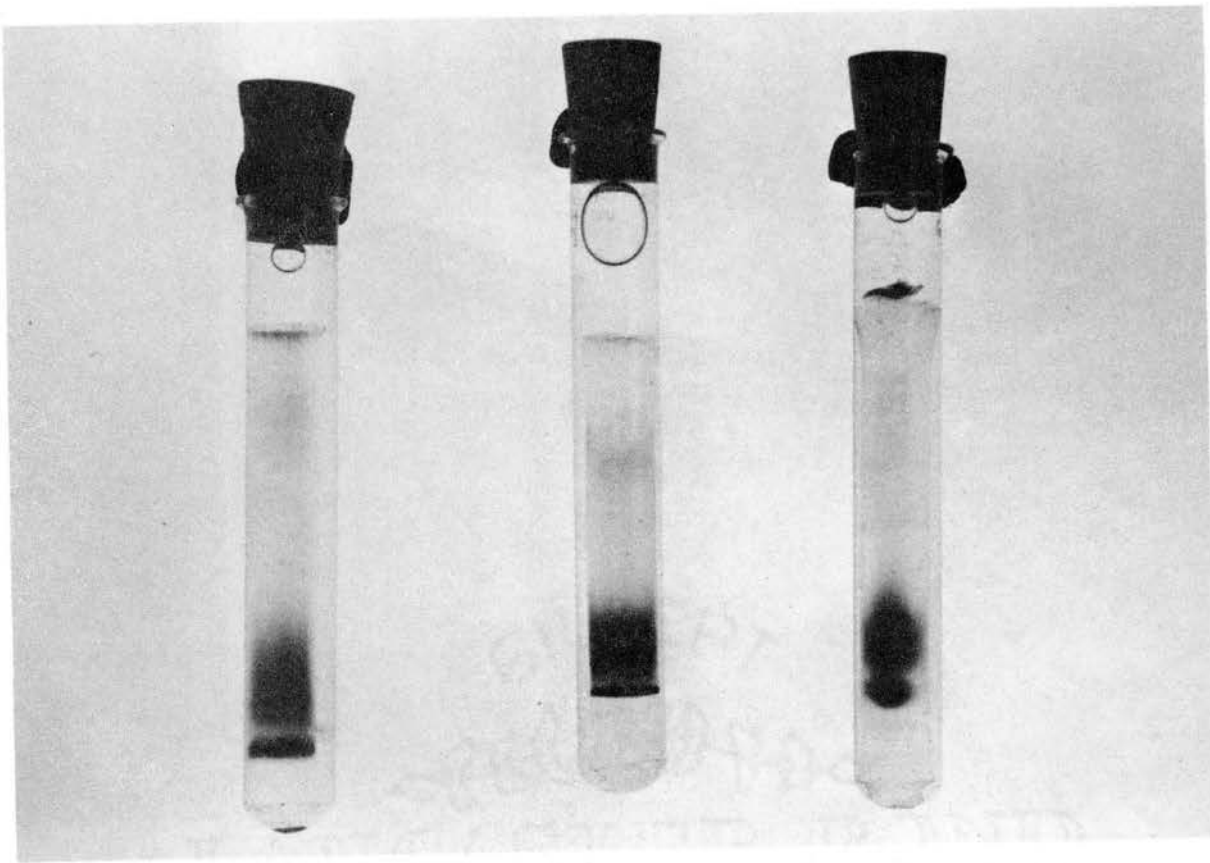
4. Same as gel 1 except by using 3.5% separating gel.

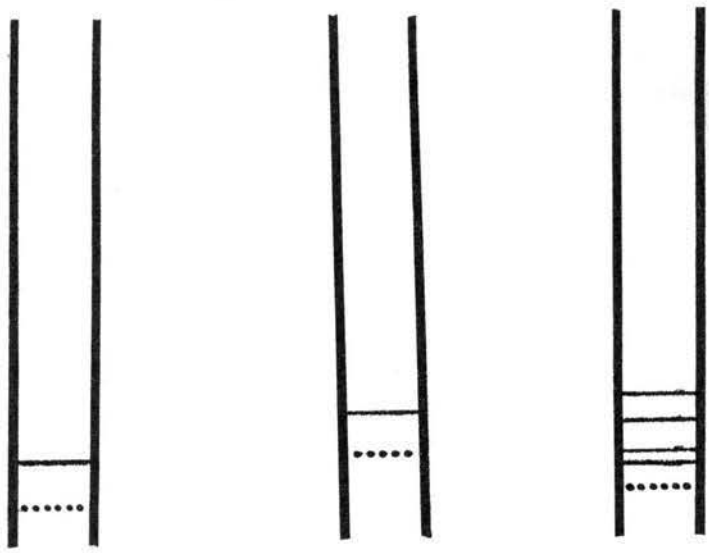
5. Same as gel 2 except by using 3.5% separating gel.

6. Same as gel 3 except by using 3.5% separating gel.




-
Dye
Front
+
1
2
3





4 5 6

gel (Figure 12_5) showed a single, large, diffused band. This was not entirely unexpected since the higher crosslinkage of the polyacrylamide gel will separate the same protein into more bands than the lower one during the electrophoresis. However, when the reducing agent was added into the upper buffer, four small bands in two diffused locations were observed (Figure 12_6). This indicated that the oxidation state of the upper buffer solution was important in dictating the electrophoretic pattern of G-actin. However, no explanation could be given to describe the mechanism involved.

Effect of Reducing Agent on the Electrophoretic Pattern of F-Actin

Figure 13 shows that the effect of the thioglycolate on the electrophoretic behavior of F-actin was quite similar to that of G-actin (Figure 12). There are no changes in total number of bands when the reducing agent was added between the sample and spacing gel (Figure $13_{1,2}$). However, when the electrophoresis upper buffer contained thioglycolate, the bands decreased from eight (Figure 13_1) to six (Figure 13_3).

When the acrylamide content of the separating gel was decreased from 7% (Figure $13_{1,2,3}$) to 3.5% (Figure $13_{4,5,6}$) the entire electropherogram was changed as shown in Figure $13_{4,5,6}$. Both the pure F-actin without any reducing agent treatment (Figure 13_4) and the one which had received a 2 μg thioglycolate addition between the sample and spacing gel (Figure 13_5) showed a single, large, diffused band. However, when the reducing agent was added to the upper buffer solution, three bands were observed in the gel (Figure 13_6). The mechanism involved in the

Figure 13. Effect of Reducing Agent on the Electrophoretic Pattern of F-Actin

Electrophoresis was conducted in 7% (gels 1-3) and 3.5% (gels 4-6) separating polyacrylamide gels. The cathode end of the gels was oriented toward the top; the anode end was toward the bottom. In the diagrams, solid lines refer to the protein bands; broken lines refer to the dye fronts.

1. Using 7% separating gel, no reducing agent (thioglycolate) added.

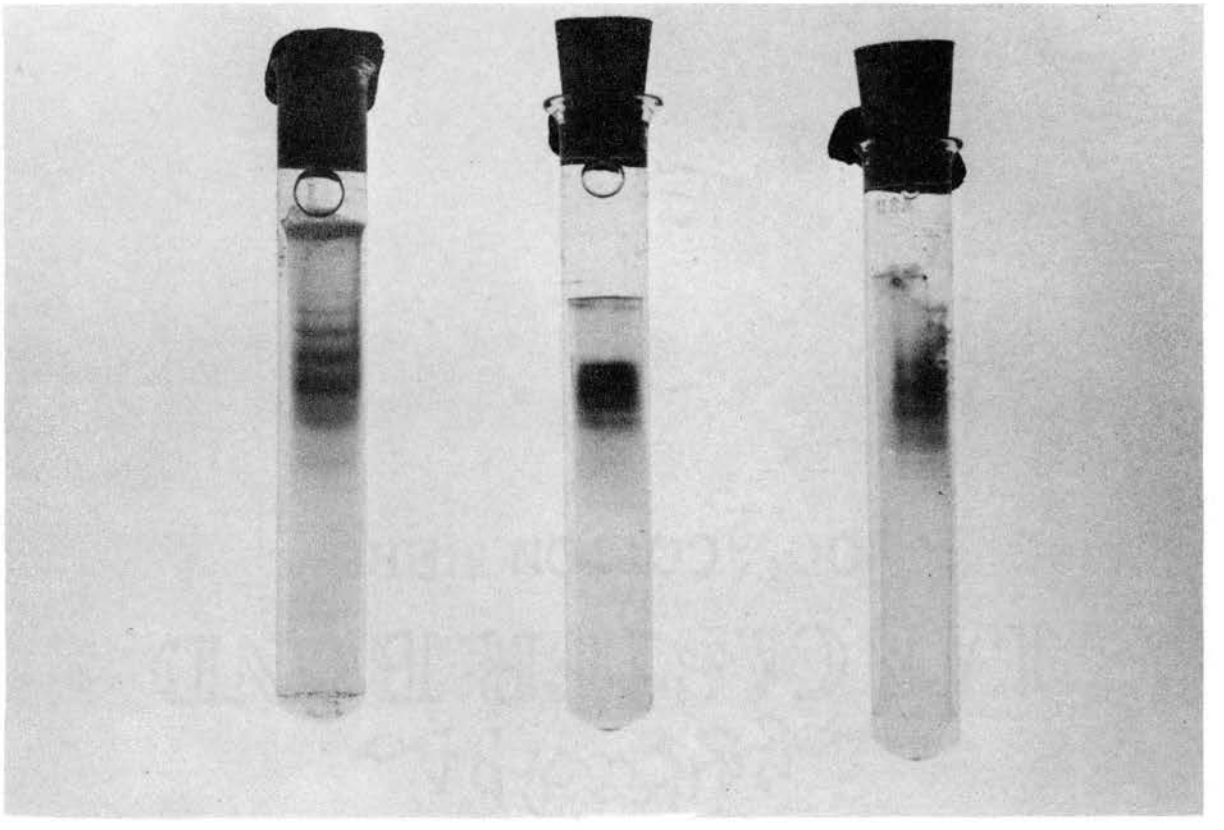
2. Using 7% separating gel, reducing agent (2 μg thioglycolate) added between the sample and spacing gel.

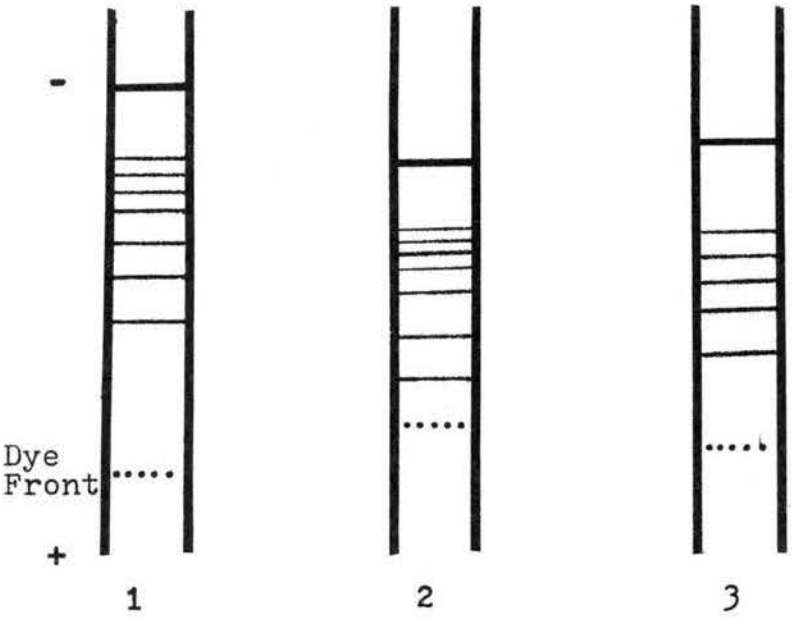
3. Using 7% separating gel, reducing agent added in the upper electrophoretic buffer solution (0.2 μg thioglycolate/ml buffer).

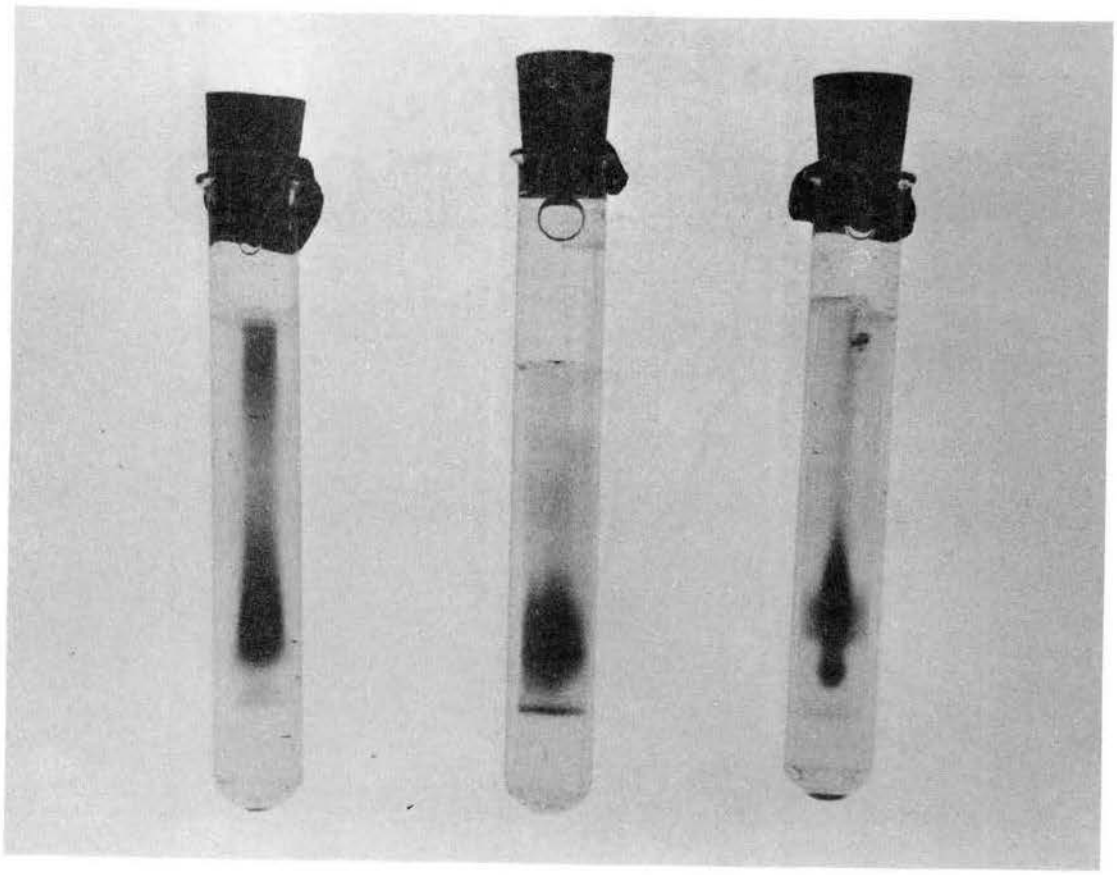
4. Same as gel 1 except by using 3.5% separating gel.

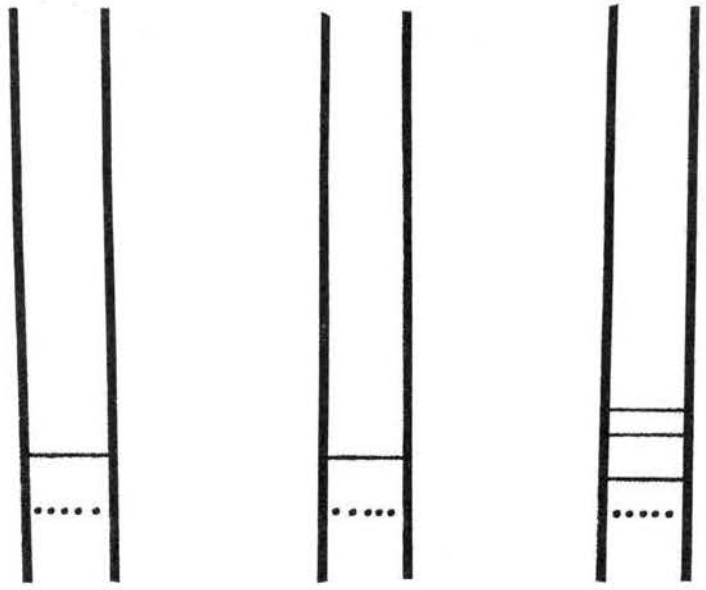
5. Same as gel 2 except by using 3.5% separating gel.

6. Same as gel 3 except by using 3.5% separating gel.




-
Dye
Front
+
1
2
3





4 5 6

alternation of the electrophoretic pattern by the effect of a reducing agent, thioglycolate, could not be explained.

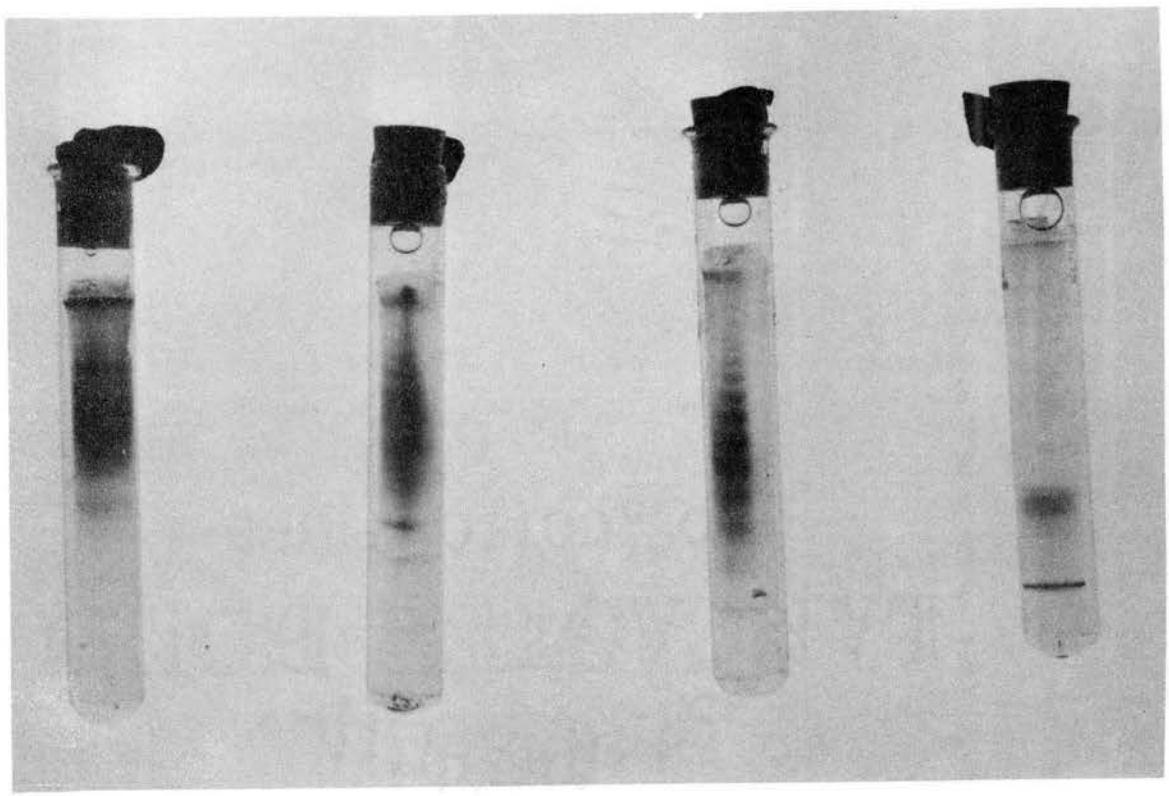
Electrophoretic Separation of Lyophilized and Chromatographed G-Actin

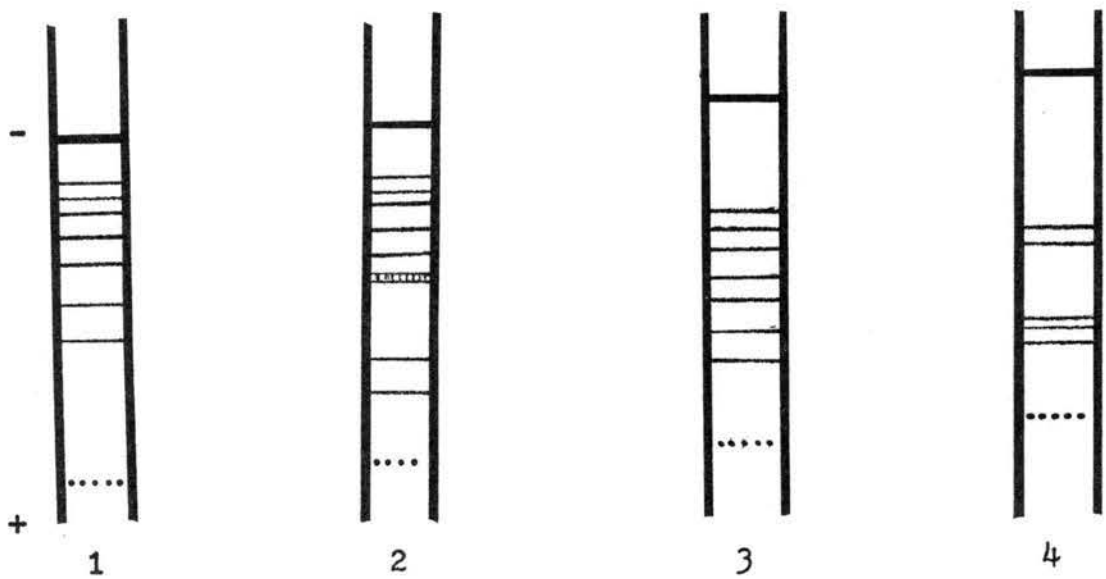
When G-actin was chromatographed through Bio-Rad P-100 column, lyophilized and redissolved in dilute ATP solution, the electrophoretic pattern (Figure 14_4) showed five distinct bands plus an immobile one. This suggests that higher purity of G-actin was obtained through the chromatogram. Lyophilization seemed to have no dramatic influence on the overall patterns (Figure 14_3) when compared to the un-lyophilized one (Figure 14_2).

Figure 14. Electrophoretic Separation of Lyophilized and Chromatographed G-Actin

Electrophoresis was conducted in 7% separating polyacrylamide gel. The cathode end of the gels was oriented toward the top; the anode end was toward the bottom. In the diagrams, solid lines refer to the protein bands; broken lines refer to the dye fronts.

1. F-actin
2. G-actin
3. G-actin, lyophilized and redissolved in 2×10^{-4} M ATP solution.
4. G-actin, chromatographed, lyophilized and redissolved in 2×10^{-4} M ATP solution.




-
+
1
2
3
4

CHAPTER VII

FACTORS AFFECTING THE G- TO F-ACTIN TRANSFORMATION

Experimental Procedure

G-actin was prepared according to the method described in Chapter III. Polymerization was induced by addition of KCl to a final concentration of approximately 0.1 M. At a pH of 6.20, the reaction was allowed to proceed for 2 hours at a constant temperature of 24°C, except where otherwise stated. The total volume of each polymerization system was 7.0 ml. G-actin concentration was 1 mg/ml. The viscosity of a 5.0 ml protein aliquot from each sample was determined by the use of a Cannon-Fenske viscometer, F88, size 100, as described in Chapter III. KCl and NaOH or HCl were used to adjust the ionic strength and pH, respectively, in the test where applicable.

Results and Discussion

Effect of Ionic Strength on the Polymerization of G-Actin

A highly significant difference ($P < 0.005$) was found in the polymerization of G-actin due to the effect of ionic strength (Tables VII and VIII). Results in Figure 15 show that polymerization increased rapidly as the ionic strength was raised from 0 to 0.057. Optimum polymerization occurred in the 0.0857 - 0.257 ionic strength range. This

TABLE VII

EFFECT OF IONIC STRENGTH ON THE POLYMERIZATION OF G-ACTIN

Ionic Strength ($x10^{-3}$)	Viscosity Reading[1] (sec)
0.00	62.75
28.57	78.57
57.14	80.15
85.71	80.60
114.28	80.65
142.85	81.00
171.42	81.20
200.00	81.32
228.57	82.05
257.14	80.60
285.71	79.65
342.85	79.55
400.00	79.75
457.14	75.50
514.28	75.02
571.42	74.10

[1]Each value is an average of two viscosity readings.

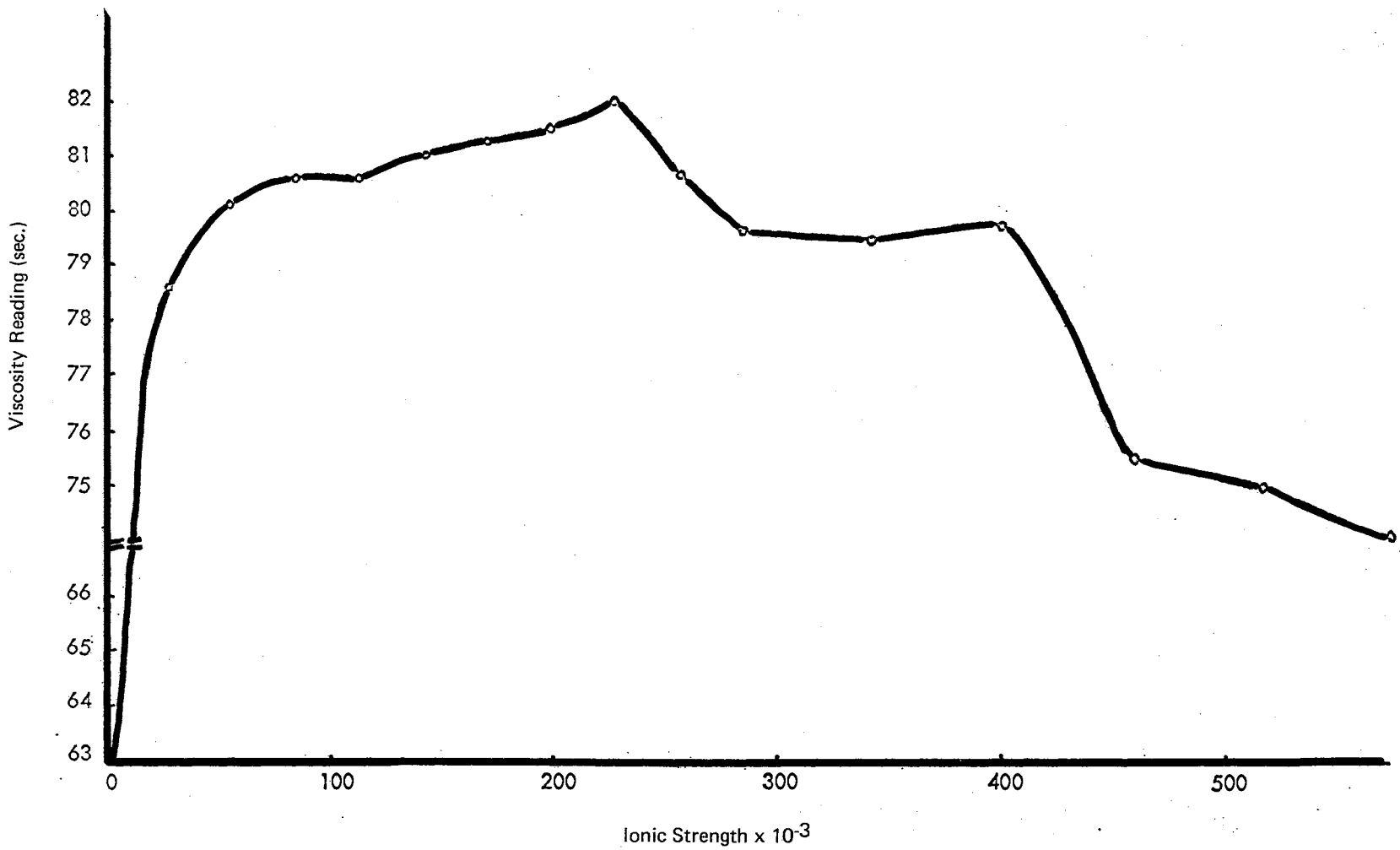
TABLE VIII

ANALYSIS OF VARIANCE FOR THE EFFECT OF IONIC STRENGTH ON THE POLYMERIZATION OF G-ACTIN

Source	df	SS	MS	F
Total	31	687.8693	----	----
Ionic Strength	15	684.0055	45.6003	188.8370***
Error (Readings)	16	3.8637	0.2414	----

***$P < 0.005$

Figure 15. Effect of Ionic Strength on the Polymerization of G-Actin


Viscosity Reading (sec.)
82
81
80
79
78
77
76
75
66
65
64
63
0
100
200
300
400
500
Ionic Strength x 10^{-3}

was a wider range than the value of 0.1 - 0.15 which was obtained from the skeletal muscle of rabbit by Poglazov (1966). Maximum polymerization for bovine G-actin was at an ionic strength of 0.228. As the ionic strength was increased beyond 0.400, polymerization was gradually decreased.

It has been shown (Mommaerts, 1952a) that actin molecules in solution repel each other owing to their electrostatic charges, and it was found that a distance of shortest approach of about 250 Å limits the molecular movements. This value is of a statistical nature, some repulsion being operative at larger distances and increasing strongly at the indicated value. These forces are eliminated by the addition of salt; and hence collisions, leading to intermolecular reactions, now become possible.

Effect of pH on the Polymerization of G-Actin

Among the primary distinguishing properties of actin is its characteristic change in viscosity accompanying the polymerization of the protein monomer. Although this parameter of actin is a highly pH-dependent process (Tsuboi et al., 1965; Mommaerts, 1952a), little systematic investigation of the relationship has been reported.

The effect of pH on the polymerization of G-actin was shown in Table IX and Figure 16. A highly significant difference ($P < 0.005$) was found among the viscosity readings due to the pH effect (Table X). The optimum pH for polymerization was 5.8 (Table IX). However, a plateau (Figure 16) over a pH range of 6.00 - 6.40 was observed. As pH was either increased or decreased from the optima, polymerization was greatly inhibited (Figure 16).

TABLE IX

EFFECT OF pH ON THE POLYMERIZATION OF G-ACTIN

pH	Viscosity Reading (sec)[1]
13.25	63.75
11.75	62.35
9.25	67.55
6.55	108.35
6.42	113.15
6.41	113.95
6.40	114.65
6.30	114.95
6.22	115.10
6.20	115.00
6.18	113.75
6.13	115.30
6.05	114.75
6.00	115.05
5.90	115.30
5.85	198.50
3.45	62.00
2.50	62.00
1.75	62.00

[1]Each value is an average of two viscosity readings.

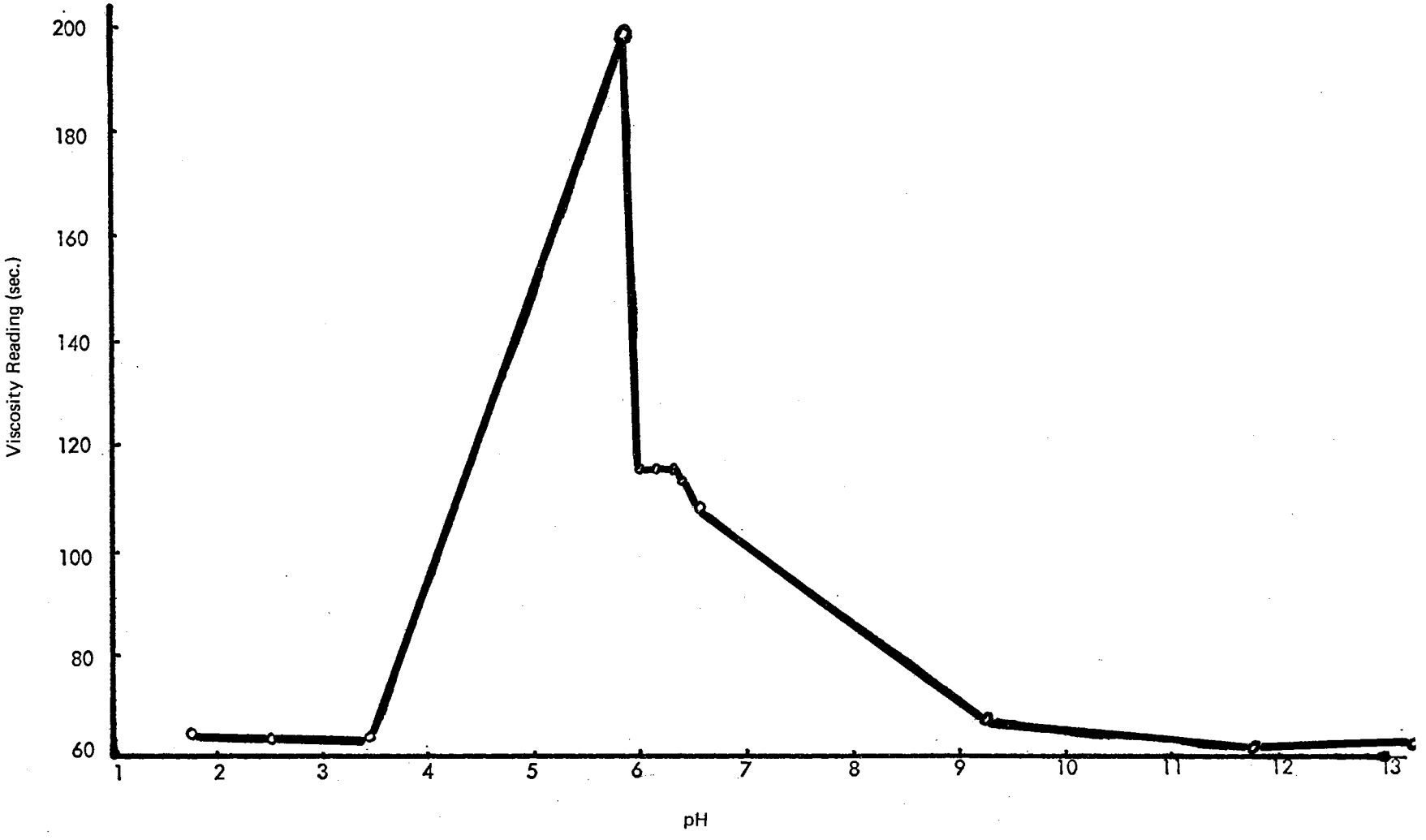
TABLE X

ANALYSIS OF VARIANCE FOR THE EFFECT OF pH ON THE POLYMERIZATION OF G-ACTIN

Source	df	SS	MS	F
Total	37	40295.5234	-----	-----
pH	18	40292.8484	2238.4915	15900.6362***
Error (Readings)	19	2.6750	0.1407	-----

***P < 0.005

Figure 16. Effect of pH on the Polymerization of G-Actin


Viscosity Reading (sec.)
200
180
160
140
120
100
80
60
1
2
3
4
5
6
7
8
9
10
11
12
13
pH

Polymerization is thought to be partly due to a decrease in the negative charge of the G-actin molecules in the presence of salts, which results in the suppression of electrostatic repulsion (Poglazov, 1966). Therefore, factors which increase such charge repulsions, such as raising the pH, give rise to depolymerization of F-actin, whereas factors leading to a decrease in repulsion, such as lowering the pH, accelerate polymerization (Tsuboi, et al., 1965).

Effect of Temperature on the Time Course of G-Actin Polymerization

Polymerization temperatures studied were 1°C, 10°C and 24°C.

Results are presented in Figure 17 and Table XI. As expected, temperature and time had a significant effect ($P < 0.005$) on the time course of polymerization (Table XII). Maximum polymerization at 24°C occurred at 10 minutes. When the reaction time was prolonged, there was very little change in the viscosity readings. At the temperature of 10°C, the polymerization increased gradually in the first 80 minutes; however, beyond this point the viscosity was slightly increased. The polymerization of bovine G-actin at 0°C was very slow. This might be due to the fact that biological activity was always low when it was near the freezing point. Of the temperatures studied, the polymerization temperature of 24°C would be most advantageous, from the standpoint of maximum sensitivity and minimum time.

Effect of Protein Concentration and Temperature on the Polymerization of G-Actin

Results in Figure 18 show that the extent of polymerization was proportionate to G-actin concentration except at extremely high protein con-

Figure 17. Effect of Temperature on the Time Course of G-Actin Polymerization.

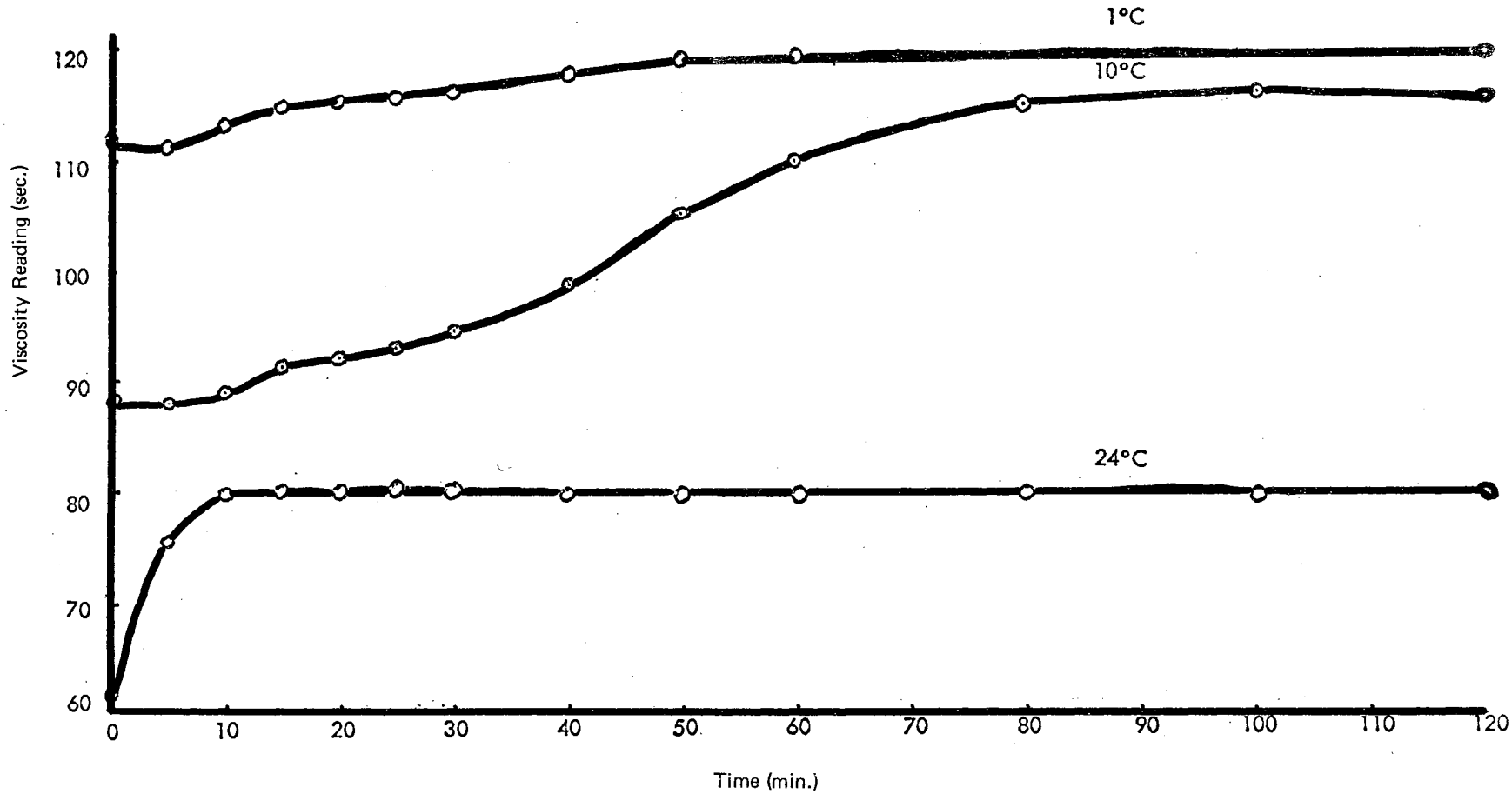

1°C
10°C
24°C
120
110
100
90
80
70
60
Viscosity Reading (sec.)
0
10
20
30
40
50
60
70
80
90
100
110
120
Time (min.)

TABLE XI

EFFECT OF TEMPERATURE ON THE TIME COURSE OF G-ACTIN POLYMERIZATION

Time (min)	Viscosity Reading (sec)[1]			Relative to Control		
	24°C	10°C	1°C	24°C	10°C	1°C
0	61.70	88.45	111.90	1.0000	1.0000	1.0000
5	75.40	88.00	111.20	1.2220	0.9949	0.9937
10	79.80	89.30	113.10	1.2933	1.0096	1.0107
15	80.00	91.60	114.40	1.2965	1.0356	1.0223
20	80.00	91.80	115.20	1.2965	1.0378	1.0294
25	80.40	93.10	115.40	1.3030	1.0525	1.0312
30	80.10	94.60	116.10	1.2982	1.0695	1.0375
40	79.25	98.90	117.50	1.2844	1.1181	1.0500
50	79.40	105.20	119.00	1.2868	1.1893	1.0634
60	79.40	110.00	119.17	1.2868	1.2436	1.0649
80	79.40	114.90	119.28	1.2868	1.2990	1.0659
100	79.40	115.75	119.39	1.2868	1.3086	1.0669
120	79.40	116.10	119.51	1.2868	1.3126	1.0680
300	81.70	117.20	119.50	1.3241	1.3250	1.0679

[1]Each value represents one determination.

TABLE XII

ANALYSIS OF VARIANCE FOR THE EFFECT OF TEMPERATURE ON THE TIME COURSE OF G-ACTIN POLYMERIZATION

Source	df	SS	MS	F
Total	41	12560.4531	------	------
Temperature	2	10357.1250	5178.5625	144.7082***
Time	13	1272.8886	97.9145	2.7360**
Error (Temp. x Time)	26	930.4421	35.7862	------

***P < 0.005

**P < 0.025

centrations, i.e., 3.3 mg/ml. As expected, the viscosity readings increased as temperature was decreased. Nevertheless, the general course of the curves was similar for all temperatures. The interaction between actin concentration and temperature was significant (Tables XIII and XIV).

Effect of Heating Time and Heating Temperature on the Polymerization Ability of G-Actin

G-actin was incubated for various time intervals (5, 10, 20 and 40 minutes) at each of the following temperatures: 25, 35, 45 and 55°C. Results in Figure 19 indicate that G-actin's ability to polymerize decreased rapidly as the incubation temperature was elevated beyond 35°C. A complete loss in its polymerizability was observed when G-actin was heated at 55°C for 10 minutes. This loss in the ability to polymerize was more pronounced at 55°C than at the lower temperatures, 35°C or 45°C, as indicated in Figure 20 and in Tables XV and XVI.

Figure 18. Effect of Protein Concentration and Temperature on the Polymerization of G-Actin

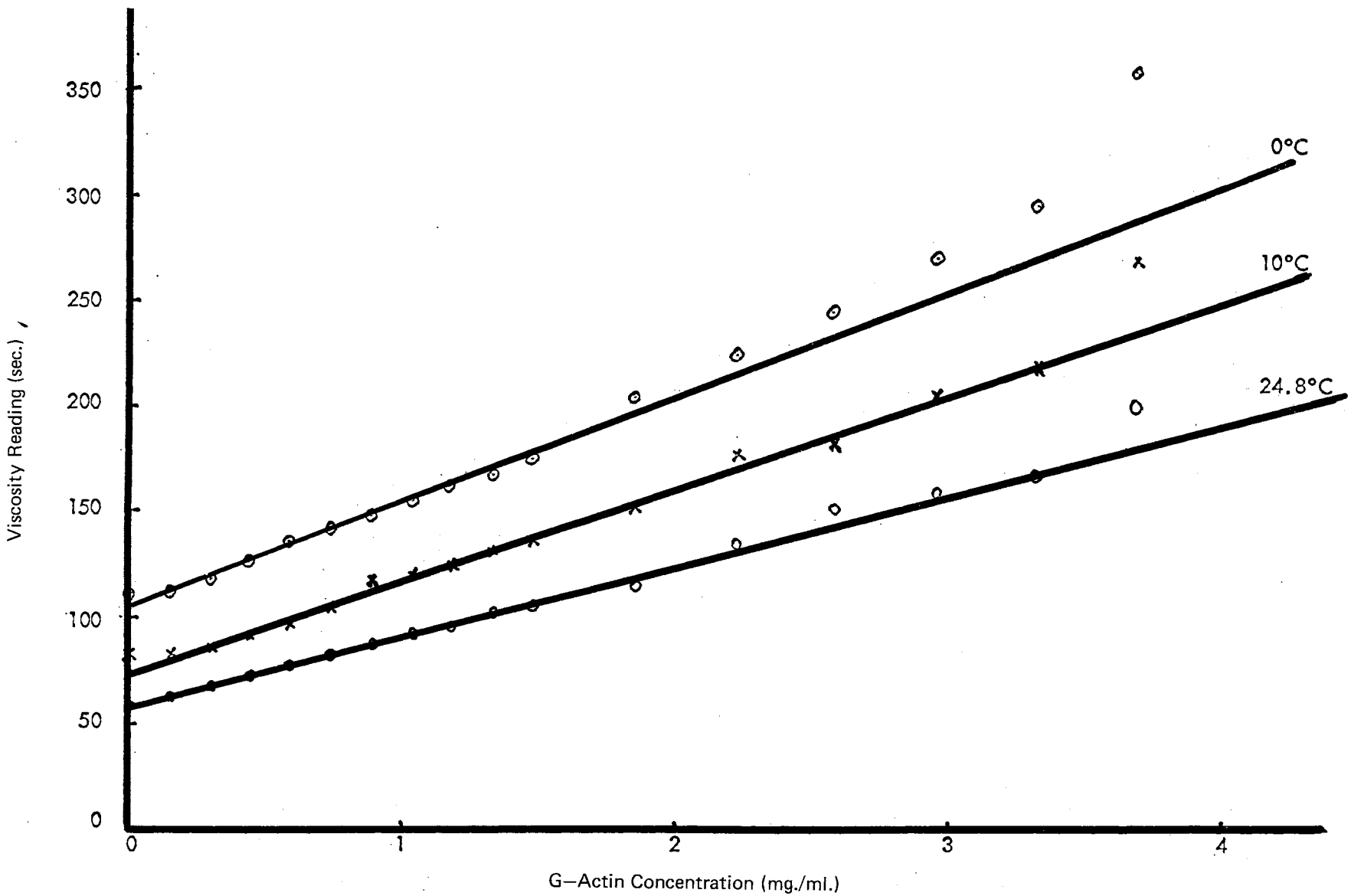

Viscosity Reading (sec.)
350
300
250
200
150
100
50
0
0
1
2
3
4
G—Actin Concentration (mg./ml.)
0°C
10°C
24.8°C

TABLE XIII

EFFECT OF PROTEIN CONCENTRATION AND TEMPERATURE ON THE POLYMERIZATION OF G-ACTIN

Actin Conc. (mg/ml)	Viscosity Reading (sec)[1]			Relative to Control		
	24°C	10°C	1°C	24°C	10°C	1°C
0.0000	58.75	82.95	111.85	1.0000	1.0000	1.0000
0.1476	62.87	82.95	112.95	1.0702	1.0000	1.0098
0.2953	67.95	84.90	118.35	1.1565	1.0235	1.0581
0.4430	74.12	91.05	126.15	1.2617	1.0976	1.1278
0.5907	79.95	96.85	136.45	1.3608	1.1675	1.2199
0.7384	82.40	104.65	141.20	1.4025	1.2616	1.2624
0.8861	87.60	117.75	147.20	1.4910	1.4195	1.3160
1.0338	92.05	119.50	155.15	1.5668	1.4406	1.3871
1.1814	95.30	125.60	162.85	1.6221	1.5141	1.4559
1.3291	102.35	131.65	168.05	1.7421	1.5871	1.5024
1.4768	105.10	136.55	174.62	1.7889	1.6461	1.5612
1.8460	116.80	151.75	202.00	1.9880	1.8294	1.8059
2.2152	135.35	175.60	223.60	2.3038	2.1169	1.9991
2.5845	152.35	183.40	245.95	2.5931	2.2109	2.1989
2.9537	158.80	205.35	270.35	2.7029	2.4755	2.4170
3.3229	168.40	218.85	294.70	2.8663	2.6383	2.6347
3.6921	200.65	269.10	353.05	3.4153	3.2441	3.2011
4.4305	267.80	382.90	497.80	4.5582	4.6160	4.4506

[1]Each value is an average of two viscosity readings.

TABLE XIV

ANALYSIS OF VARIANCE FOR THE EFFECT OF PROTEIN CONCENTRATION AND TEMPERATURE ON THE POLYMERIZATION OF G-ACTIN

Source	df	SS	MS	F
Total	107	87.89380	------	------
Treatment	53	87.83987	------	------
Concentration	17	86.95119	5.11477	5166.43434***
Temperature	2	0.66955	0.33477	338.15151***
Concentration x Temperature	34	0.22913	0.00673	6.79797***
Error (Readings)	54	0.05393	0.00099	------

***$P < 0.005$

Figure 19. Effect of Heating Temperature on the Polymerization Ability of G-Actin

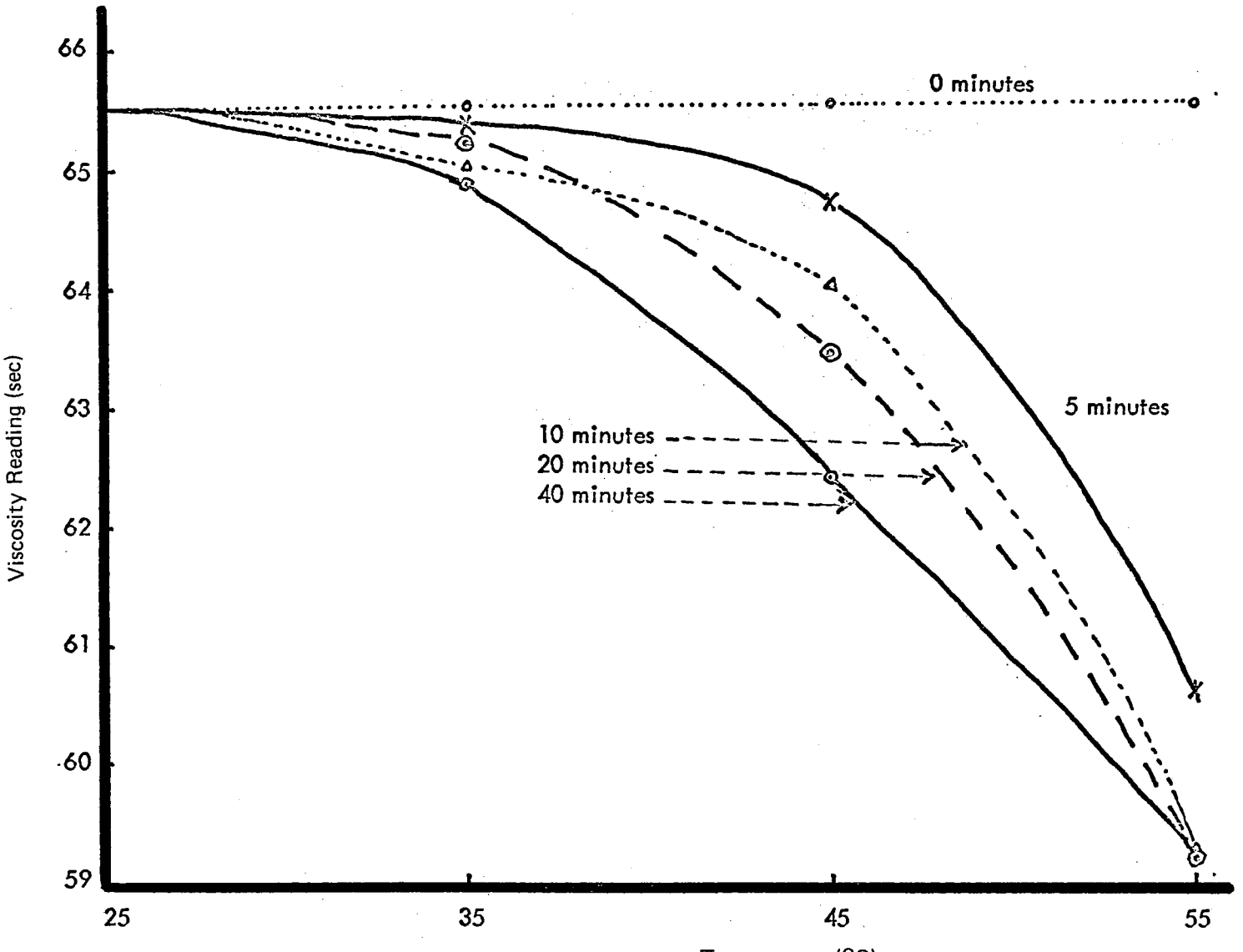

66
65
64
63
62
61
60
59
Viscosity Reading (sec)
0 minutes
5 minutes
10 minutes
20 minutes
40 minutes
25
35
45
55
Temperature (°C)

Figure 20. Effect of Heating Time on the Polymerization Ability of G-Actin

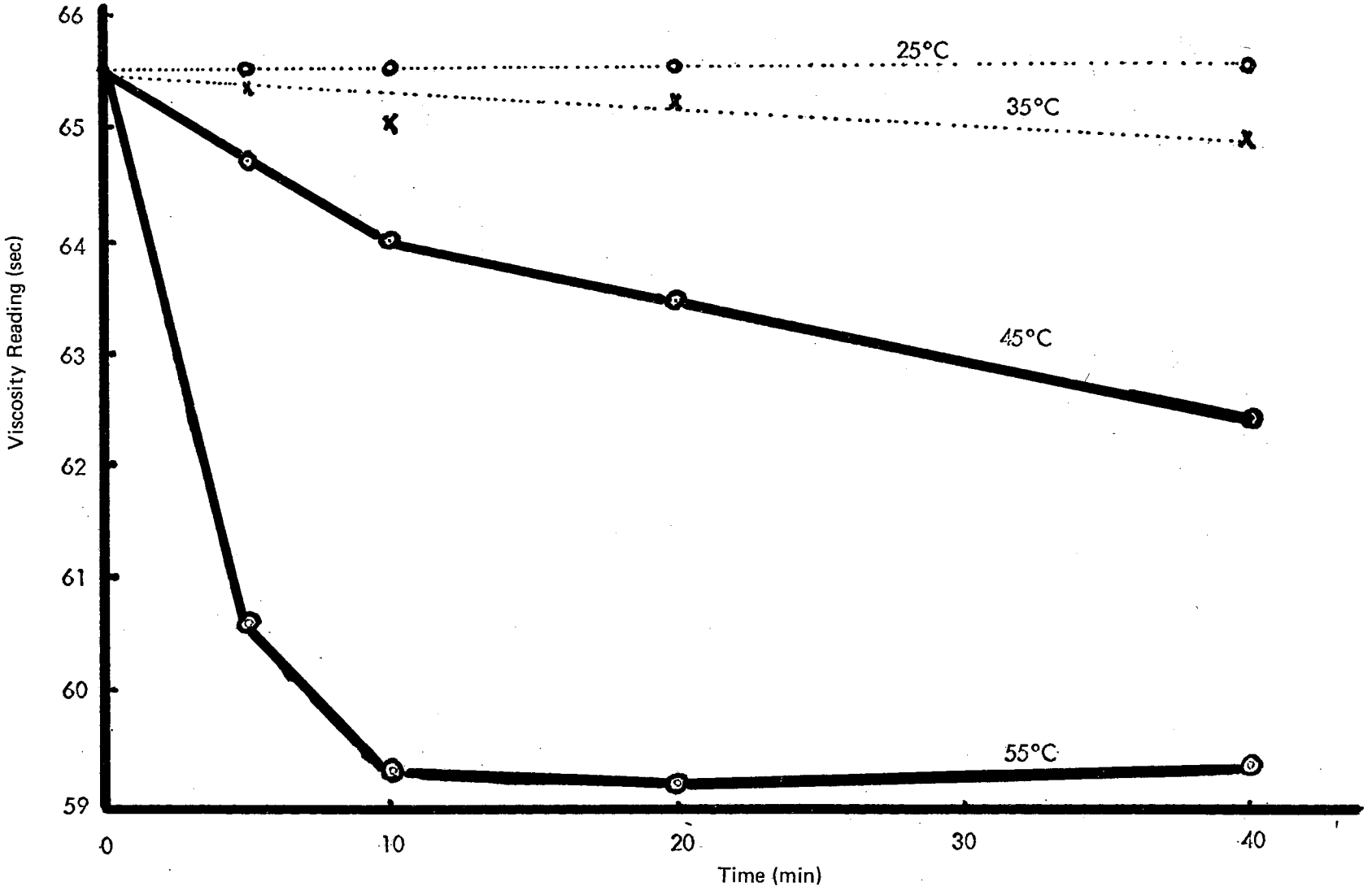

66
65
64
63
62
61
60
59
Viscosity Reading (sec)
25°C
35°C
45°C
55°C
0
10
20
30
40
Time (min)

TABLE XV

EFFECT OF HEATING TIME AND HEATING TEMPERATUARE ON THE POLYMERIZATION ABILITY OF G-ACTIN[1]

Heating Temperature (°C)	Heating Time (min)				
	0	5	10	20	40
25	65.65	65.57	65.40	65.37	65.32
35	65.45	65.35	65.00	65.18	64.85
45	65.40	64.72	64.02	63.45	62.41
55	63.37	60.58	59.27	59.22	59.35

[1]Each value is an average of four determinations which were obtained by making two readings on each of the duplicate samples.

TABLE XVI

ANALYSIS OF VARIANCE FOR THE EFFECT OF HEATING TIME AND HEATING TEMPERATURE ON THE POLYMERIZATION ABILITY OF G-ACTIN

Source	df	SS	MS	F
Total	79	413.3522	------	-------
Treatment	19	411.7190	------	-------
Time	4	61.8589	15.4647	578.9865***
Temperature	3	277.9685	92.6561	3468.9700***
Time x Temperature	12	71.8915	5.9909	224.2965***
Error	60	1.6331	0.0272	-------
Duplicates	20	0.5343	0.0267	-------
Readings	40	1.0987	0.0274	-------

***$P < 0.005$

CHAPTER VIII

EFFECT OF CERTAIN CHEMICAL REAGENTS ON THE G- TO F-ACTIN TRANSFORMATION

Experimental Procedure

The procedures used here were the same as in Chapter VII. A 0.7 mg/ml of G-actin was used in the polymerization system for the test of $MgCl_2$, H_2O_2, KNO_3, KNO_2, K_2CrO_4, KI, NaI, $HgCl_2$, EDTA and urea. A 1.4 mg/ml of G-actin was used for the test of $CaCl_2$, NEM, IAA, IAAM, p-CMB, thioglycolate, cysteine and ascorbic acid.

Results and Discussion

Effect of $MgCl_2$ and $CaCl_2$ on the Polymerization of G-Actin

In general, results show that when $MgCl_2$ was added to the reaction mixture to a concentration of about 8 to 9 mM, polymerization was enhanced. However, as the concentration of $MgCl_2$ was increased beyond this level, polymerization was suppressed (Figure 21 and Table XVII). These results were similar to those obtained with $CaCl_2$ which gave maximum polymerization at a concentration of approximately 14 mM (Figure 21 and Table XVII). The strong activation effect of Mg^{++}, as well as Ca^{++}, on the polymerization of bovine G-actin may contribute to the consequence of the preferential binding of cations to the negatively charged protein. This would lead to a decrease of net negative charge and electrostatic repulsion, consequently resulting in polymerization as has been earlier suggested by Mommaerts (1952a) and Mihashi *et al.* (1965).

Figure 21. Effect of $MgCl_2$ and $CaCl_2$ on the Polymerization of G-Actin

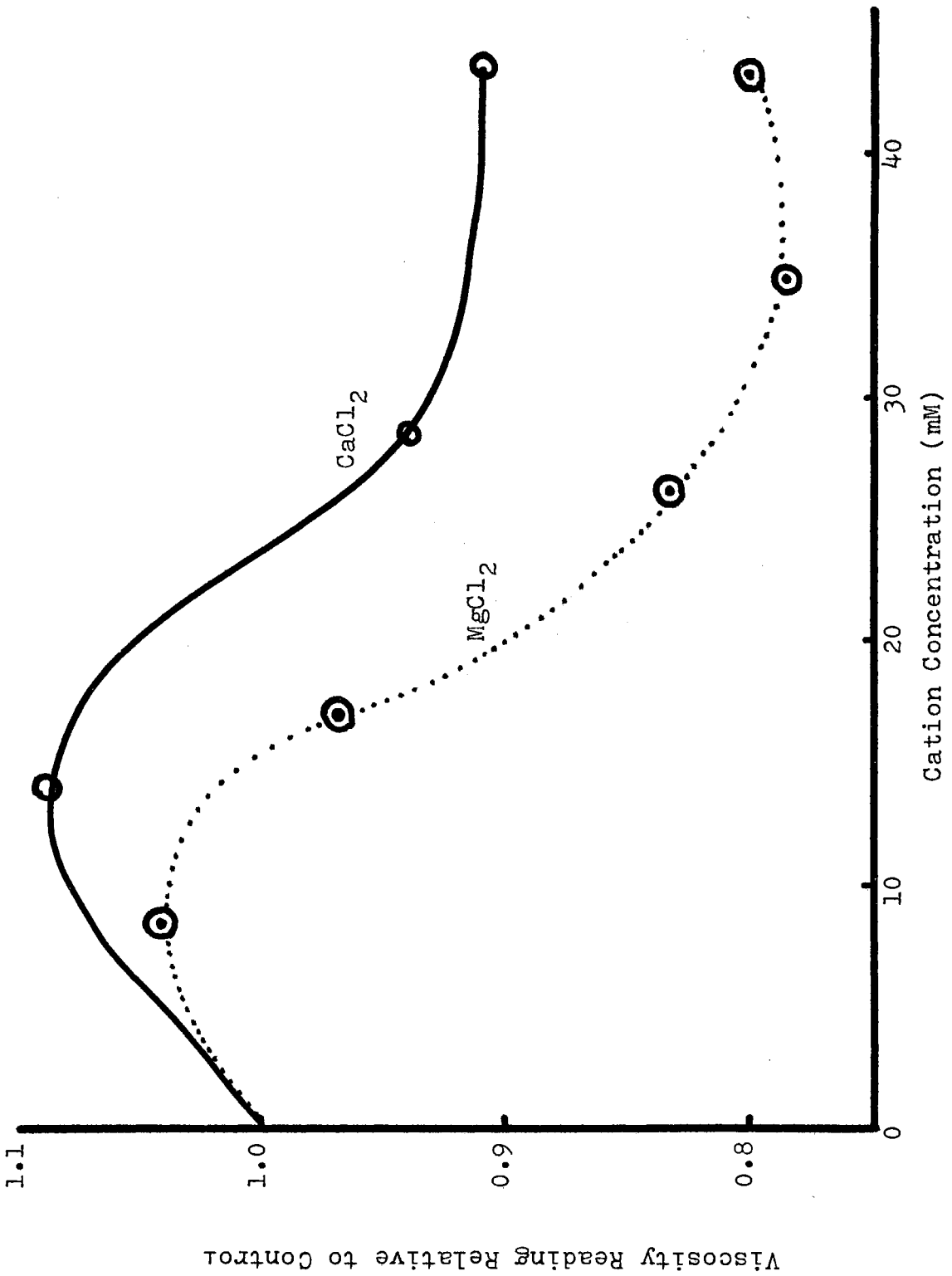

Viscosity Reading Relative to Control
1.1
1.0
0.9
0.8
0
10
20
30
40
Cation Concentration (mM)
$CaCl_2$
$MgCl_2$

TABLE XVII

EFFECT OF $MgCl_2$ AND $CaCl_2$ ON THE POLYMERIZATION OF G-ACTIN

Factor	Cation Conc. (mM)	VR[1,3]	RTC[2]
$CaCl_2$	0.00	81.00	1.0000
	14.28	88.15	1.0882
	28.57	76.10	0.9395
	42.85	73.50	0.9074
$MgCl_2$	0.00	96.20	1.0000
	8.57	100.25	1.0420
	17.14	93.55	0.9724
	25.71	79.95	0.8310
	34.71	75.65	0.7863
	42.85	76.95	0.7998

[1]VR refers to the viscosity reading in seconds.

[2]RTC refers to the viscosity readings relative to control.

[3]Each value is an average of two viscosity readings.

TABLE XVIII

ANALYSIS OF VARIANCE FOR THE EFFECT OF $MgCl_2$ AND $CaCl_2$ ON THE POLYMERIZATION OF G-ACTIN

Source	$CaCl_2$		$MgCl_2$	
	df	MS	df	MS
Total	7	-----	11	------
Concentration	3	82.9945***	5	233.0328***
Error (Readings)	4	0.1912	6	0.0741

***$P < 0.005$

Martonosi, et al. (1964) indicated that there is a definite relationship between the physico-chemical properties of actin and the number of bound cations. When the amount of bound Mg^{++} or Ca^{++} reaches 4 to 5 moles per mole of actin, the concentration of F-actin rapidly increases. Half-maximal polymerization is reached when 5 to 6 cations are bound, and the polymerization becomes complete when 7 to 8 cations are bound per actin molecule. Binding of 10 moles of Mg^{++} or Ca^{++} causes isoelectric precipitation at pH 6.8 to 7.

Martonosi (1962) also indicated that the relationship between the concentration of Mg^{++} or Ca^{++} and the extent of polymerization depends on the concentration of ATP in the system. The inhibition of high $CaCl_2$ and $MgCl_2$ concentration on polymerization was presumably due to the formation of Ca-ATP or Mg-ATP complex. The presence of ATP in the G-actin molecule and a sufficient amount of ATP in the solvent are indispensable in stabilizing the structural integrity of G-actin prior to polymerization (Oosawa, et al., 1965; Kasai, et al., 1965).

Effect of H_2O_2, KNO_3, KNO_2 and K_2CrO_4 on the Polymerization of G-Actin

The graphs in Figure 22 represent the influence of various chemical agents on the polymerization of G-actin. The concentration of H_2O_2 needed to inhibit polymerization was very high. If the viscosity of the control was assumed as 100%, it required 9.26 M H_2O_2 in order to achieve an 80% inhibition (Table XIX).

The data in Table XIX show that KNO_3 was a more powerful inhibitor than KNO_2; this could be attributed to the fact that the former was a stronger oxidation reagent than the latter. A 91% inhibition would be

Figure 22. Effect of H_2O_2, KNO_3, KNO_2, and K_2CrO_4 on the Polymerization of G-Actin

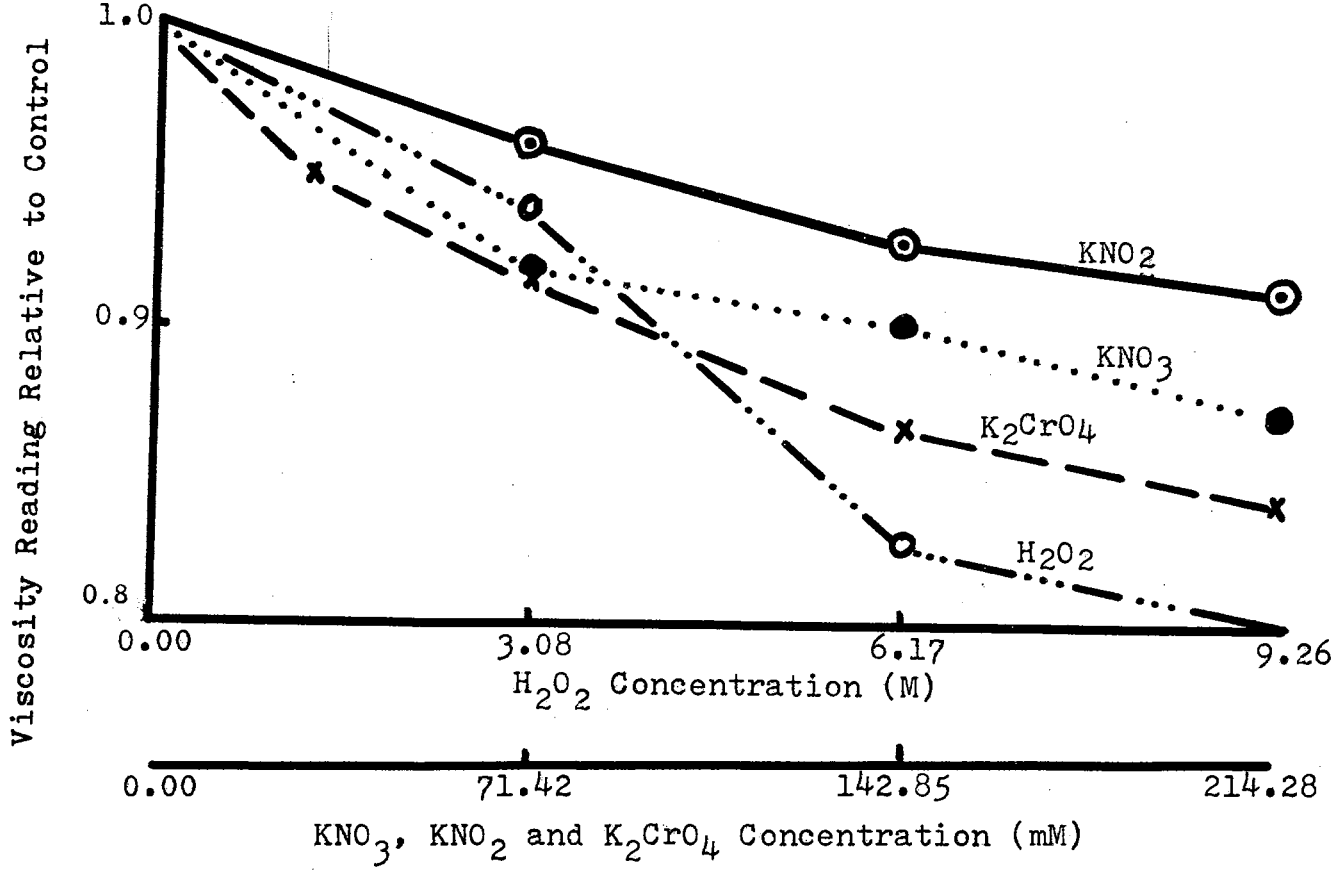

Viscosity Reading Relative to Control
1.0
0.9
0.8
KNO2
KNO3
K2CrO4
H2O2
0.00
3.08
6.17
9.26
H2O2 Concentration (M)
0.00
71.42
142.85
214.28
KNO3, KNO2 and K2CrO4 Concentration (mM)

TABLE XIX

EFFECT OF H_2O_2, KNO_3, KNO_2 AND K_2CrO_4 ON THE POLYMERIZATION OF G-ACTIN

Factor	Concentration (mM)	VR[1,3]	RTC[2]
H_2O_2	0.00	72.50	1.0000
	3088.30	68.20	0.9406
	6176.60	59.75	0.8241
	9264.90	58.05	0.8006
KNO_3	0.00	82.25	1.0000
	71.42	75.60	0.9191
	142.85	74.10	0.9009
	214.28	71.70	0.8717
KNO_2	0.00	83.25	1.0000
	71.42	78.75	0.9574
	142.85	76.00	0.9240
	214.28	75.15	0.9136
K_2CrO_4	0.00	76.85	1.0000
	28.57	73.20	0.9525
	71.42	70.35	0.9154
	142.85	66.40	0.8640
	214.28	64.80	0.8432

[1]VR refers to the viscosity reading in seconds.

[2]RTC refers to viscosity readings relative to control.

[3]Each value is an average of two viscosity readings.

TABLE XX

ANALYSIS OF VARIANCE FOR THE EFFECT OF H_2O_2, KNO_3, KNO_2 AND K_2CrO_4 ON THE POLYMERIZATION OF G-ACTIN

	H_2O_2		KNO_3		KNO_2		K_2CrO_4	
Source	df	MS	df	MS	df	MS	df	MS
Total	7	------	7	------	7	------	9	------
Concentration	3	94.5716***	3	40.8612***	3	21.4945***	4	48.3865***
Error (Readings)	4	0.0325	4	0.0562	4	0.0037	5	0.0060

***$P < 0.005$

achieved by 71.42 mM KNO_3; however, 214 mM KNO_2 was needed in order to obtain the same degree of inhibition.

When the concentration of K_2CrO_4 was increased from 0 to 214 mM, the viscosity was decreased from 100% to 84%. Consequently, it seems that K_2CrO_4 was the most effective inhibitor of the reagents studied. In the order of total effect, these chemicals might be ranked as K_2CrO_4 > KNO_3 > KNO_2 > H_2O_2.

The effect of these oxidizing reagents on the polymerization might be pertinent to the oxidation of G-actin. This is in line with the results of Carsten, et al. (1963) and Mommaerts, (1952a) that the oxidizing reagents enhance time-dependent inactivation of G-actin; thus a progressive failure to polymerize was observed.

Effect of KI, NaI and $HgCl_2$ on the Polymerization of G-Actin

The effects of KI, NaI and $HgCl_2$ on the polymerization of G-actin are presented in Figure 23 and Table XXI. Of the reagents studied, mercuric chloride had the largest inhibitory influence when inhibitor concentration was considered. It was observed that there were no significant differences among the concentrations of 0.35, 1.71, 2.57, 3.42, and 4.28 mM $HgCl_2$ used to inhibit the polymerization of G-actin.

The effect of potassium iodide and sodium iodide was indicated by its inhibition at all concentrations. The initial concentration necessary for inhibition, however, was quite high. From the polymerization curve (Figure 23) it can be noted that KI was a slightly stronger inhibitor than NaI.

Figure 23. Effect of KI, NaI and $HgCl_2$ on the Polymerization of G-Actin

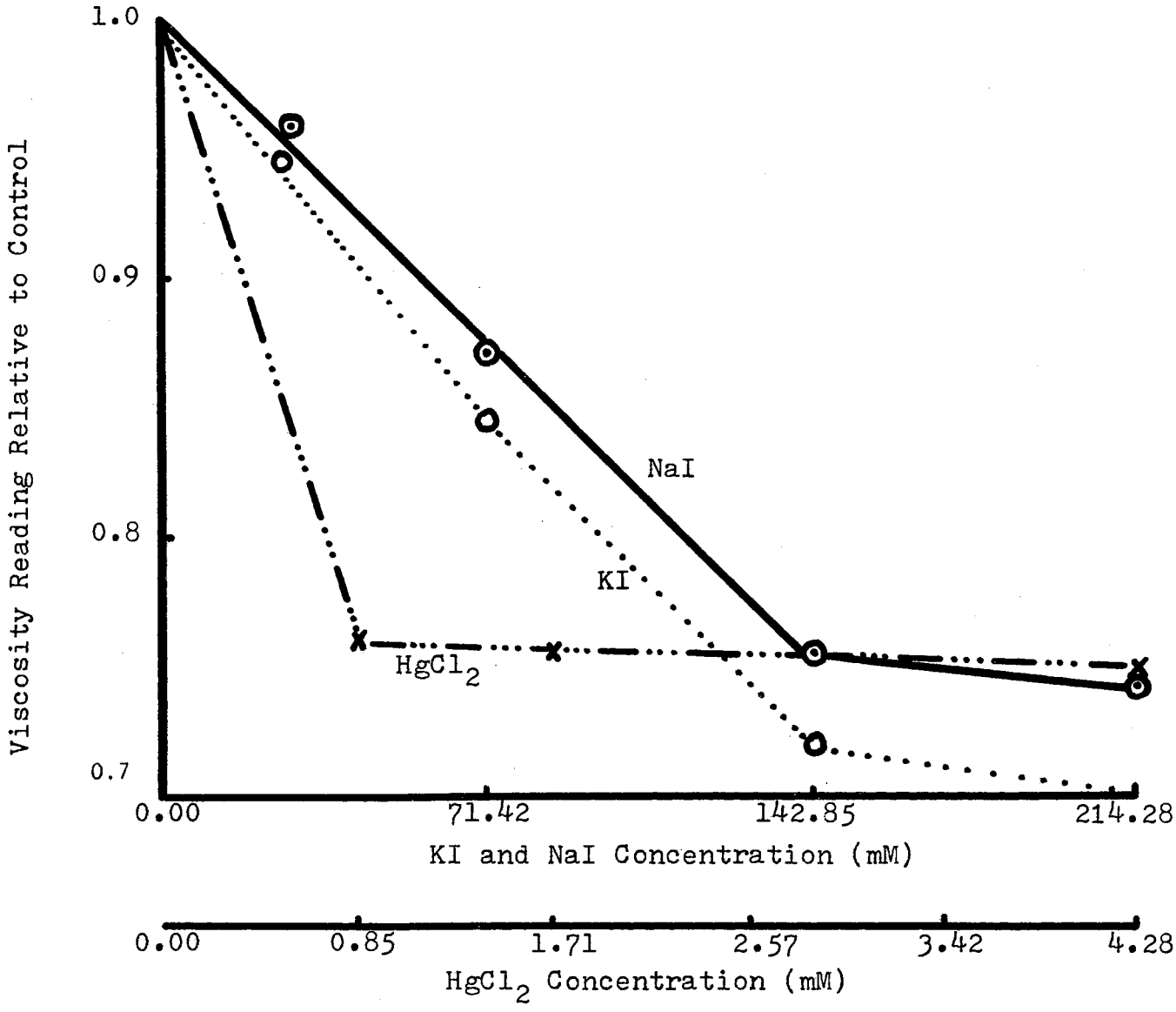

1.0
0.9
0.8
0.7
Viscosity Reading Relative to Control
NaI
KI
HgCl2
0.00
71.42
142.85
214.28
KI and NaI Concentration (mM)
0.00
0.85
1.71
2.57
3.42
4.28
HgCl2 Concentration (mM)

TABLE XXI

EFFECT OF KI, NaI AND $HgCl_2$ ON THE POLYMERIZATION OF G-ACTIN

Factor	Concentration (mM)	VR[1,3]	RTC[2]
KI	0.00	78.75	1.0000
	28.57	73.95	0.9390
	71.42	66.70	0.8469
	142.85	56.95	0.7231
	214.28	55.45	0.7041
NaI	0.00	77.75	1.0000
	28.57	74.80	0.9620
	71.42	67.65	0.8700
	142.85	58.75	0.7556
	214.28	57.90	0.7446
$HgCl_2$	0.00	80.30	1.0000
	0.85	61.15	0.7615
	1.71	60.65	0.7552
	2.57	60.45	0.7528
	3.42	60.40	0.7521
	4.28	60.40	0.7521

[1]VR refers to the viscosity reading in seconds.

[2]RTC refers to viscosity readings relative to control.

[3]Each value is an average of two viscosity readings.

TABLE XXII

ANALYSIS OF VARIANCE FOR THE EFFECT OF KI, NaI AND $HgCl_2$ ON THE POLYMERIZATION OF G-ACTIN

Source	KI		NaI		$HgCl_2$	
	df	MS	df	MS	df	MS
Total	9	------	9	------	11	------
Concentration	4	209.4060***	4	163.5065***	5	129.3948***
Error (Readings)	5	0.1120	5	0.0910	6	0.0491

***$P < 0.005$

The inhibitory mechanism of these reagents on the polymerization of G-actin might be attributed to the fact that they act by blocking and/or destroying the sulfhydryl group in G-actin, thus preventing the formation of the necessary disulfide cross-linkage to form the polymer (Oosawa, et al., 1961; Nagy, et al., 1962).

Effect of N-Ethylmaleimide (NEM), Iodoacetic Acid (IAA), Iodoacetamide (IAAM) and p-Chloromercuribenzoate (p-CMB) on the Polymerization of G-Actin

When the concentrations of N-ethylmaleimide (NEM) were at 0, 0.71, 1.41 and 2.14 mM, the viscosity readings of the polymerized system were 82.25, 77.40, 78.30 and 79.00 seconds, respectively. If one assumes the polymerization, without the inhibitor present, to be 100%, then 2.14 mM NEM had only 4% inhibition (Figure 24 and Table XXIII). Drabikowski (1964) indicated that the reaction of the SH group with NEM did not lead to functional alterations, nor did this seem to be the case with other covalently reacting thiol reagents. This might explain the weak inhibitory effect of NEM on the polymerization of bovine G-actin.

It was found that a concentration of 71.42 mM and 142.85 mM iodoacetic acid (IAA) in the polymerization solution had a significant depressing effect on F-actin formation with respect to the control. However, the difference between the influence of these two concentrations was non-significant. When the concentration of the iodoacetic acid was increased from 0 to 214.28 mM, the viscosity reading was decreased from 82.25 seconds (100% polymerization) to 75.50 seconds (91.79% polymerization) which was highly significant (Tables XXIII and XXIV).

The effect of iodoacetamide (IAAM) on the polymerization was quite similar to iodoacetic acid (IAA). As the concentration of these two

Figure 24. Effect of N-Ethylmaleimide (NEM), Iodoacetic Acid (IAA), Iodoacetamide (IAAM) and p-Chloromercuribenzoate (p-CMB) on the Polymerization of G-Actin

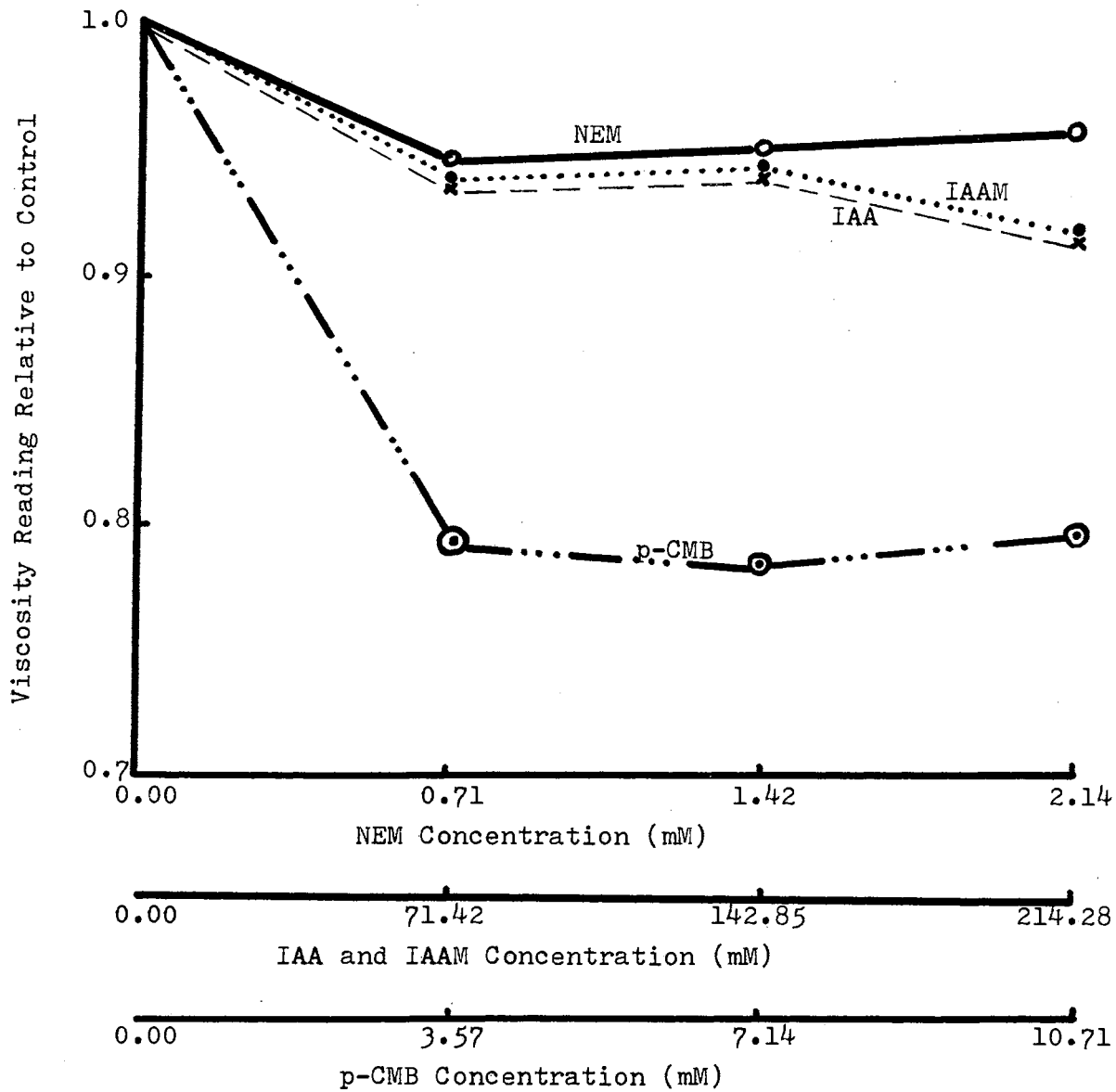

1.0
0.9
0.8
0.7
Viscosity Reading Relative to Control
NEM
IAAM
IAA
p-CMB
0.00
0.71
1.42
2.14
NEM Concentration (mM)
0.00
71.42
142.85
214.28
IAA and IAAM Concentration (mM)
0.00
3.57
7.14
10.71
p-CMB Concentration (mM)

TABLE XXIII

EFFECT OF N-ETHYLMALEIMIDE (NEM), IODOACETIC ACID (IAA), IODOACETAMIDE (IAAM) AND p-CHLOROMERCURIBENZOATE (p-CMB) ON THE POLYMERIZATION OF G-ACTIN

Factor	Concentration (mM)	VR[1,3]	RTC[2]
NEM	0.000	82.25	1.0000
	0.714	77.40	0.9410
	1.428	78.30	0.9519
	2.142	79.00	0.9604
IAA	0.000	82.25	1.0000
	71.420	76.90	0.9349
	142.850	77.25	0.9392
	214.280	75.50	0.9179
IAAM	0.000	82.25	1.0000
	71.420	77.20	0.9386
	142.850	77.35	0.9404
	214.280	76.15	0.9258
p-CMB	0.000	80.55	1.0000
	3.571	64.00	0.7945
	7.142	63.40	0.7870
	10.714	64.20	0.7970

[1]VR refers to the viscosity reading in seconds.

[2]RTC refers to viscosity readings relative to control.

[3]Each value is an average of two viscosity readings.

TABLE XXIV

ANALYSIS OF VARIANCE FOR THE EFFECT OF N-ETHYLMALEIMIDE (NEM), IDOACETIC ACID (IAA), IODOACETAMIDE (IAAM) AND p-CHLOROMERCURIBENZOATE (p-CMB) ON THE POLYMERIZATION OF G-ACTIN

Source	df	MS			
		NEM	IAA	IAAM	p-CMB
Total	7	-----	-----	------	-------
Concentration	4	8.9245***	17.3883***	14.8812***	139.3979***
Error (Readings)	3	0.0112	0.0375	0.0687	0.0112

***P < 0.005

sulfhydryl reagents increased, the viscosity relative to control was decreased slightly (Table XXIII and Figure 24). Poglazov (1966) indicated that cysteine in G-actin was the only constituent which reacted with iodoacetate or iodoacetamide.

The chemical, p-chloromercuribenzoate (p-CMB), was a very strong inhibitor (Figure 24). It took only 3.57 mM p-CMB to decrease the polymerization to 79.45% from the control. However, the effect of this inhibitor on the polymerization of G-actin was not significantly different among the concentrations of 3.57, 7.14 and 10.71 mM.

The amount of p-CMB-titratable sulfhydryl group of actin was in the range of 6.1 to 6.8 moles per 6.1×10^4 gm (Tonomura, et al., 1962). Two moles of them reacted instantaneously with p-CMB and were titratable with NEM. By titration of these two sulfhydryl groups with the reagent, the polymerization of G-actin was not inhibited and F-actin was not depolymerized. Remaining sulfhydryl groups reacted slowly with p-CMB and did not with NEM. The polymerization of G-actin was inhibited, and F-actin was depolymerized by the slow binding of p-CMB to the sulfhydryl groups of the latter class. The inhibition of polymerization by p-CMB was accompanied by removal of the ATP bound to G-actin. This might explain the inhibitory mechanisms of these organic sulfhydryl reagents on the polymerization of G-actin.

Effect of Thioglycolate, Cysteine and Ascorbic Acid on the Polymerization of G-Actin

The data in Table XXV show that when a 71.42 mM and 142.85 mM of thioglycolate were added to the reaction mixture, the polymerization was 95% and 96% respectively. Although they were not significantly different from each other, these values were significantly different from

TABLE XXV

EFFECT OF THIOGLYCOLATE, CYSTEINE AND ASCORBIC ACID ON THE POLYMERIZATION OF G-ACTIN

Factor	Concentration (mM)	VR[1,3]	RTC[2]
Thioglycolate	0.00	82.25	1.0000
	71.42	78.20	0.9507
	142.85	79.05	0.9610
	214.28	76.10	0.9252
Cysteine	0.00	79.85	1.0000
	71.42	63.50	0.7952
	142.85	65.05	0.8146
	214.28	65.75	0.8234
Ascorbic Acid	0.00	82.25	1.0000
	71.42	61.70	0.7501
	142.85	61.25	0.7446
	214.28	62.35	0.7580

[1]VR refers to the viscosity reading in seconds.

[2]RTC refers to viscosity readings relative to control.

[3]Each value is an average of two viscosity readings.

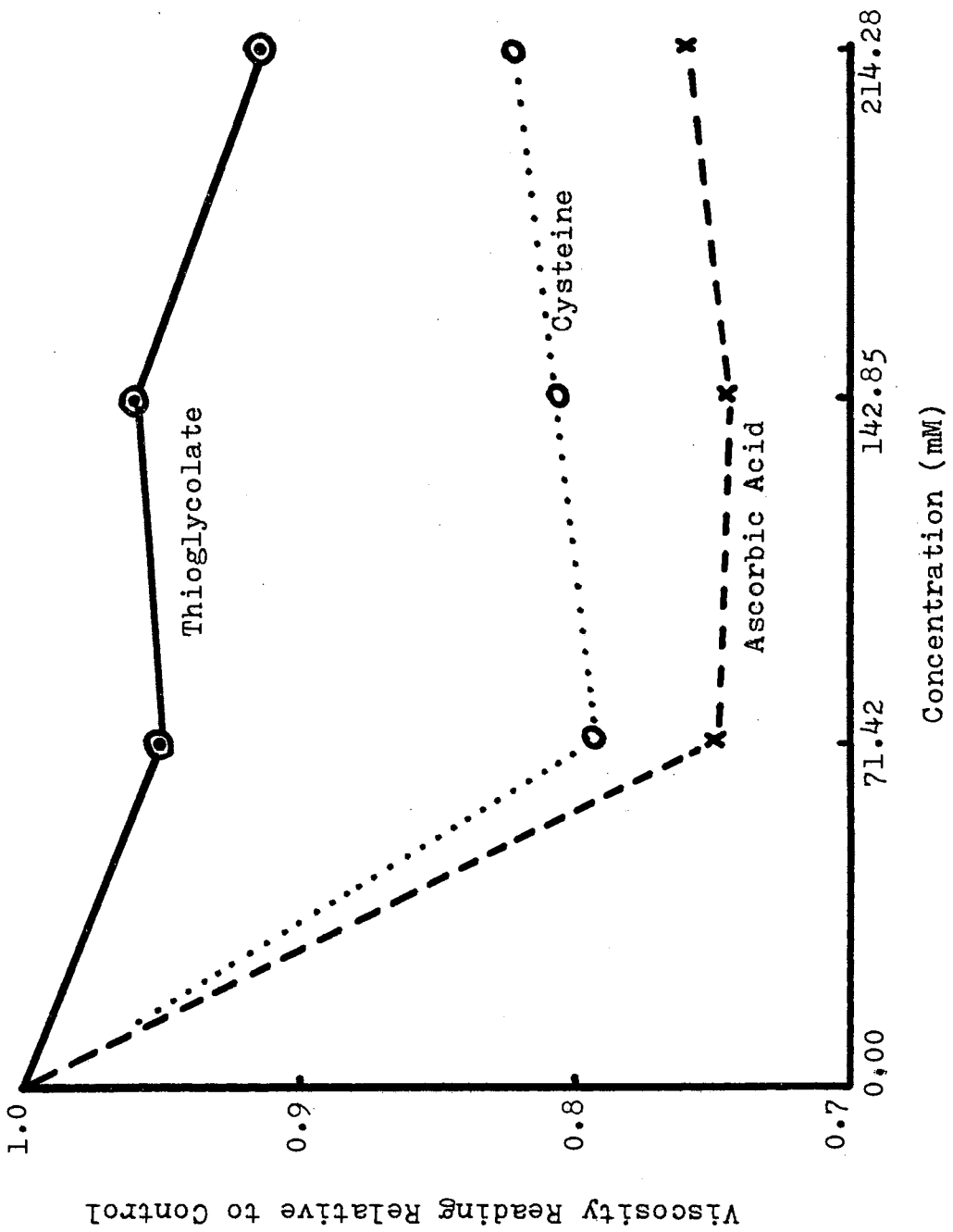
TABLE XXVI

ANALYSIS OF VARIANCE FOR THE EFFECT OF THIOGLYCOLATE, CYSTEINE AND ASCORBIC ACID ON THE POLYMERIZATION OF G-ACTIN

Source	df	MS		
		Thioglycolate	Cysteine	Ascorbic Acid
Total	7	-----	------	--------
Concentration	3	13.0500***	115.5212***	210.1912***
Error (Readings)	4	0.0875	0.0187	0.1037

***P < 0.005

Figure 25. Effect of Thioglycolate, Cysteine and Ascorbic Acid on the Polymerization of G-Actin


Viscosity Reading Relative to Control
1.0
0.9
0.8
0.7
0.00
71.42
142.85
214.28
Concentration (mM)
Thioglycolate
Cysteine
Ascorbic Acid

the control. When the thioglycolate concentration was increased to 214.28 mM, a 92% polymerization was observed.

The polymerization was decreased from 100% to 79% when cysteine concentration was increased from 0 to 71.42 mM. However, if the concentrations were further increased to 142.82 mM and 214.28 mM, their effect tended to lessen.

A significant 25% inhibition was observed when a 71.42 mM of ascorbic acid was added to the polymerization system. However, no significant differences were found among the concentrations of 71.42, 142,85 and 214.28 mM of ascorbic acid (Table XXV).

These reagents probably exert their influence by maintaining reduced conditions and preventing the formation of the disulfide bond, which is necessary to form the polymer, F-actin (Oosawa et al., 1961). Nevertheless, these organic reagents, particularly ascorbic acid and cysteine, had rather low pK values. Thus the pH of the polymerization system could be altered and the viscosity would be dropped. The order of the inhibitors' effect on the polymerization of bovine G-actin may be ranked as ascorbic acid > cysteine > thioglycolate.

Effect of EDTA and Urea on the Polymerization of G-Actin

When EDTA concentration was increased from 0 to 0.08 mM, the viscosity reading was dropped from 83.65 to 69.95 seconds. If the control (no EDTA added) was assumed as 100% polymerization, then there was only 83% (Table XXVII) polymerization observed when a 0.08 mM EDTA was added to the reaction mixture. The viscosity was decreased rapidly to 61 seconds when the EDTA concentration was increased to 0.17 mM. Although a highly significant inhibition ($P < 0.005$) was found to be due to the total

TABLE XXVII

EFFECT OF EDTA AND UREA ON THE POLYMERIZATION OF G-ACTIN

Factor	Concentration	VR[1,3]	RTC[2]
EDTA (mM)	0.0000	83.65	1.0000
	0.0857	69.95	0.8362
	0.1714	61.05	0.7298
	0.2571	59.75	0.7142
	0.3428	58.85	0.7035
	0.4285	58.45	0.6987
Urea (M)	0.0000	84.10	1.0000
	0.3420	82.55	0.9815
	0.6850	74.20	0.8822
	1.0280	67.45	0.8020
	1.3710	63.45	0.7548
	1.7140	61.05	0.7259

[1]VR refers to the viscosity reading in seconds.

[2]RTC refers to viscosity readings relative to control.

[3]Each value is an average of two viscosity readings.

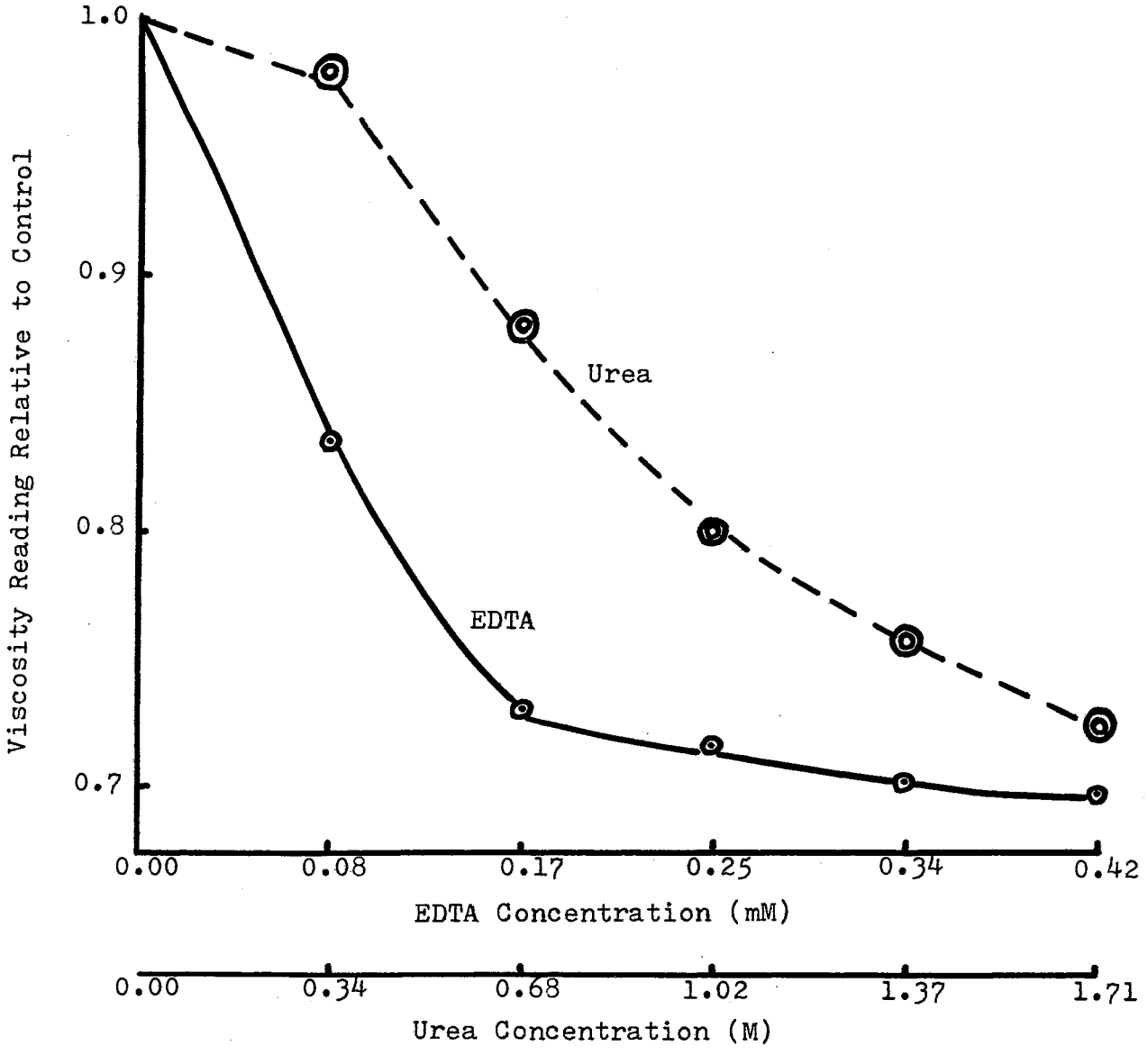
TABLE XXVIII

ANALYSIS OF VARIANCE FOR THE EFFECT OF EDTA AND UREA ON THE POLYMERIZATION OF G-ACTIN

Source	df	MS	
		EDTA	Urea
Total	11	------	------
Concentration	5	200.2433***	190.4613***
Error (Readings)	6	0.3516	0.0633

***P < 0.005

Figure 26. Effect of EDTA and Urea on the Polymerization of G-Actin


Viscosity Reading Relative to Control
1.0
0.9
0.8
0.7
Urea
EDTA
0.00
0.08
0.17
0.25
0.34
0.42
EDTA Concentration (mM)
0.00
0.34
0.68
1.02
1.37
1.71
Urea Concentration (M)

effect of EDTA concentrations (Table XXVIII) on the polymerization of bovine G-actin, there were no significant differences either among the concentrations of 0.17, 0.25 and 0.34 mM, or among the concentrations of 0.25, 0.34 and 0.42 mM (Table XXVII).

Martonosi, et al., (1961a) indicated that removal of the tightly bound Ca^{++} of G-actin in aqueous solution renders it unpolymerizable. Since EDTA is a very strong chelating compound for divalent cations, particularly for Ca^{++} and Mg^{++}, and since the structural integrity of G-actin prior to polymerization was destroyed by the formation of EDTA-Ca or EDTA-Mg complex, the inhibition on the polymerization of bovine G-actin was effected (Kasai, et al., 1965; Oosawa, et al., 1965).

The concentration of urea needed to inhibit the G-actin polymerization was considerably high as compared with EDTA. In order to obtain an 18% inhibition, only 0.17 mM EDTA was required; however, 1.71 M urea was needed to get the same degree of inhibition. A highly significant inhibition was found to be due to the urea concentrations (Table XXVIII).

Since urea is a protein denaturing reagent, the inhibition of polymerization could be attributed to the dissociation of H-bonds in either G-actin or F-actin. Thus helical content of actin was reduced, and conformational changes resulted (Oosawa, et al., 1961; Higashi, et al., 1965; Katz, 1965).

CHAPTER IX

SUMMARY AND CONCLUSIONS

Experimental material for this study was obtained from the longissimus dorsi (l.d.) muscles of hereford steer carcasses. The steers were of similar breeding, age and weight (approximately 860 kg).

The longissimus dorsi muscles were excised immediately post mortem, chilled in ice and were held in a $0^{o}C$ cooler of approximately 70% relative humidity. They were freed of surrounding fat and connective tissue, then minced twice through a grinder.

The starting material for the G-actin isolation was the minced bovine l.d. muscle from which the myofibrillar protein, myosin, had been previously extracted. After a series of washings of this minced muscle residue with $NaHCO_3$, distilled water and n-butanol, an acetone-dried muscle powder was obtained. Aliquots of the dried muscle powder were extracted with 2×10^{-4} M ATP solution. The extract which contained crude G-actin, was clarified via high speed centrifugation. The G-actin was then polymerized to F-actin by adding KCl, and by a series of washes with the ATP solution, each followed by ultracentrifugation, purification was effected. G-actin was recovered by depolymerizing the F-actin. This was accomplished by dialyzing the F-actin against aqueous ATP solution. Sedimentation patterns, chromatographic separation and polyacrylamide gel electrophoresis were used as criteria of purity.

The amino acid composition and electrophoretic characteristics of

bovine G-actin were determined. The effect of reaction conditions such as ionic strength, pH, time, protein concentration and temperature on the G- to F-actin transformation was investigated. The effect of certain chemicals including divalent cations, oxidizing, reducing and sulfhydryl reagents on the polymerization of G-actin was determined.

Sedimentation patterns obtained on the G-actin preparation showed a single sharp peak, indicating purity and molecular homogeneity. Chromatographic separation on Bio-Rad P-100 also suggested purity of the G-actin preparation. Electropherograms, obtained on polyacrylamide gels, showed the G- and F-actin preparations to consist of 9 and 8 electronegative components, respectively. Electrophoretically, G-actin differed from its polymer only in that it contained an additional highly mobile, anodic component. However, when the separating gel was reduced from 7% to 3.5%, only one band was observed in both G- and F-actin. The molecular weight of bovine G-actin calculated from the amino acid composition was 61,479.

The ionic strength for maximum and optimum polymerization was found at 0.228 and 0.085-0.257, respectively. The optimum pH for maximum polymerization was 5.8. Maximum polymerization at 24, 10 and 1°C occurred at 10, 80 and 120 minutes, respectively. A linear relationship was found between viscosity reading and concentration of G-actin in the polymerization solution. A complete loss in its polymerizability was observed when G-actin was heated at 55°C for 10 minutes.

When $MgCl_2$ and $CaCl_2$ were added to the reaction mixture to a concentration of 8 mM and 14 mM, respectively, polymerization was enhanced. The order of the inhibitors' effects on the polymerization of G-actin was as follows: $K_2CrO_4 > KNO_3 > KNO_2 > H_2O_2$ for oxidizing reagents;

ascorbic acid > cysteine > thioglycolate for reducing reagents; $HgCl_2$ > KI and NaI, p-chloromercuribenzoate > N-ethylmaleimide, Iodoacetic acid and Iodoacetamide for sulfhydryl reagents. Urea also inhibited the polymerization, but to a lesser degree than EDTA as far as the concentration was concerned.

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