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Studies on the extraction of myofibrillar proteins from rabbit striated muscle

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STUDIES ON THE EXTRACTION OF MYOFIBRILLAR PROTEINS FROM RABBIT STRIATED MUSCLE

by

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INTRODUCTION

Biochemists have long been interested in the myofibrillar proteins because this group of proteins contains the contractile elements of muscle. Moreover, considerable use has been made of myofibrillar protein solubility as a tool for characterization of muscle tissue growth and differentiation in several species. Protein solubility has also been useful for comparing properties of different muscles. Hence, methodology for extraction of protein from muscle immediately after death has attracted considerable attention and detailed procedures have been published for isolation and purification of actin, myosin, tropomyosin and the other more recently discovered myofibrillar proteins.

Interest and emphasis on studies of post-mortem muscle protein solubility have also increased because of the effect of rapid post-mortem glycolysis on muscle protein solubility, and the possibility that post-mortem tenderization is caused by a dissociation of actomyosin to actin and myosin. It is well-known that during post-mortem storage, excised muscles first become rigid and tough and then eventually become soft and tender. Accompanying these physical changes are biochemical changes which at present are still not completely characterized. It would be very beneficial if the physical methods of measuring rigor could be correlated to certain biochemical changes, particularly in the myofibrillar protein fraction. Protein solubility may be a very useful method for detecting biochemical changes in post-mortem muscle which can then be related to the known post-mortem changes. Although post-mortem changes in myofibrillar protein solubility would most likely be directly relatable

to post-mortem alterations in physical properties of muscle, post-mortem changes in sarcoplasmic protein solubility also remain important to the meat scientist because changes in this fraction may reflect autolytic breakdown of protein in the less soluble myofibrillar and stroma fractions.

Several studies of post-mortem changes in muscle protein solubility and their possible relation to meat quality have recently appeared in the literature. No clear relationship between protein solubility and tenderness has emerged from these studies. However, many of these early investigations have suffered from failure to characterize the extracted protein. Rather, it has usually been assumed that high ionic strength salt solutions extract "actomyosin" and that changes in the amount of protein extracted by high ionic salt solutions reflect changes in the solubility of "actomyosin". However, it is well-known that myosin alone may be specifically extracted by high ionic strength salt solutions and differences in the amount of protein solubilized by such solutions may reflect the differences in either actin or myosin solubility. Indeed, there have been several recent studies of actomyosin extraction from at-death rabbit muscle which indicate, in contrast to the current prevailing opinion, that many high ionic strength salt solutions will extract only myosin from minced muscle, even when extraction is prolonged for 24 hours or longer. Interpretation of those studies attempting to relate tenderness to post-mortem muscle protein solubility would be altered substantially if the extracts in such studies contained only myosin. Furthermore, the use of muscle protein solubility to help characterize the biochemical changes in muscle undergoing atrophy or hypertrophy, or

passing through other rapid physiological changes requires a thorough understanding of the process of myofibrillar protein extraction. For example, the identity of the rate-limiting factor in actomyosin extraction is not clear. Neither is it known if the in situ interaction between the thick and thin filaments actually lowers actomyosin extractability, although it is apparently assumed that it does. Therefore, the purpose of this investigation was to study the extraction of myofibrillar protein by different extracting solutions with particular emphasis on the relative rates of actin and myosin solubilization from whole minced muscle or myofibrils.

Limitations of the Study

Because of the infinite number of different extracting solutions and conditions, it was not possible to include all possible combinations of different pH values, ionic strengths and extracting salts in this study. Emphasis was placed on extraction pH values near neutrality, the ionic strength of all solutions was fixed at 0.65, and the extracting salts were selected to emphasize the possible, hitherto unsuspected role of Ca^{++} in actin solubilization. Neither was it possible to conduct a completely thorough study of the properties of extracted protein solutions, but rather tests were chosen that would be most sensitive to the relative amounts of actin and myosin in the extracts. Therefore this study does not attempt to assess the rate of solubilization of tropomyosin, troponin and the other minor myofibrillar proteins. The study was limited to rabbit muscle and it is recognized that different results may be obtained with other species.

Nomenclature and Abbreviations

The following abbreviations will be used throughout this thesis: ATP, adenosinetriphosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, 1,2-bis-(2-dicarboxymethylaminoethoxy)-ethane; Tris, Tris-(hydroxyethyl) aminomethane; $\Gamma/2$, an ionic strength calculated on a molarity basis; g, acceleration due to gravity = 980 cm/sec; hr, hour; u, micro or micron; min, minute; mM, millimolar; ml, milliliter; mg, milligram; η_{rel}, relative viscosity; cc, cubic centimeter; nm, nanometer; v/w, volume/weight.

REVIEW OF LITERATURE

Because of its long usage, there has been considerable confusion about the meaning of the term "myosin". The word, myosin, was first used by Kuhne (1859), to refer to the substance which formed a clot when the juice pressed out of frog muscle was allowed to stand at room temperature. It was realized soon afterward that Kuhne's "myosin" was a complex mixture, and the term, myosin, was reserved for that fraction of protein which appeared in strong salt ($\Gamma/2 > 0.5$) extractions of muscle as opposed to those more soluble proteins appearing in the press juice. Thus "myosin" solutions were prepared by extraction of minced muscle with strong salt solutions followed by precipitation of myosin by dilution (Danilewsky, 1881; Halliburton, 1887; Von Furth, 1895). Later "myosin" was used by Edsall (1930) to indicate the protein fraction of muscle which is extracted by 0.6 M KCl at pH 8.5 - 9.0, and precipitated when the solution is diluted to 0.1 M KCl at pH 7.0. However, in 1941 Banga and Szent Gyorgyi (1941) found that the earlier myosin preparations consisted of more than one protein. If minced muscle was extracted with a high ionic strength salt solution ($\Gamma/2 \geq 0.3$) for less than 1 hour, the extracted protein exhibited a relatively low viscosity, and the viscosity of the solution was not influenced by the addition of ATP. If, on the other hand, the muscle mince was extracted with the same salt solution for 20 - 24 hours or overnight, the extract had a high viscosity, and the viscosity was substantially lowered by addition of ATP. Banga and Szent Gyorgyi (1941) deduced that during prolonged extraction, a second protein

was extracted which then formed a complex with myosin. The term, "myosin B", was coined to refer to the complex solubilized by the 20 - 24 hour high ionic strength extraction of minced muscle. Other investigators have called this same complex, "natural actomyosin". On the other hand, myosin A was the term used by Banga and Szent Gyorgyi to refer to the single protein solubilized by short ($<$1 hour) extraction of minced muscle. In present nomenclature the term, myosin, is reserved for the single protein solubilized by the 1 - 2 hour extraction of minced muscle. Therefore myosin A is nearly equivalent to myosin, and furthermore, most of the so-called "myosin" preparations described before 1945 are in fact, not myosin at all but consist principally of myosin B or natural actomyosin.

About the same time that Banga and Szent Gyorgyi reported their findings, Schramm and Weber (1942) described some ultracentrifugal studies of "myosin" solutions prepared by overnight extraction with high ionic strength solutions, which demonstrated that such solutions contained both a slowly sedimenting component, $S_{20} = 6$, and a faster sedimenting component, $S_{20} = 20 - 36$. These two components could be separated by differential centrifugation, thereby providing further strong evidence that the protein then known as "myosin" was actually a complex of two proteins. Schramm and Weber (1942) referred to the more slowly sedimenting component as "L-myosin" (L = leicht) and to the faster component as "S-myosin" (S = Schnell). Thus L-myosin is nearly equivalent to myosin A or myosin itself and S-myosin is analogous to myosin B or natural actomyosin.

In 1942-43 Straub first isolated and characterized actin, a second major protein of the myofibril (Straub, 1942; 1943). It was immediately realized that actin was the second protein complexed with myosin in the 20 - 24 hour salt extracts of minced muscle, and it was shown that ATP possessed the ability to dissociate the actin-myosin complex (Mommaerts, 1942). This then explained the ability of ATP to markedly lower the viscosity of myosin B solutions. There has been much subsequent work corroborating this finding (Banga and Szent Gyorgyi, 1941; Dainty et al., 1944; Mommaerts, 1948; 1950; Tonomura and Yoshimura, 1960; Maruyama and Gergely, 1962). Moreover it has been shown that pyrophosphate acts in a manner similar to ATP (Gergely and Kohler, 1958; Acs et al., 1949; Tonomura and Morita, 1959; Granicher and Portzehl, 1964).

Banga and Szent Gyorgyi (1941) attributed the phenomenon of actomyosin extraction to the fact that once the endogenous ATP present in muscle at death had fallen to a level too low to effectively dissociate the actin-myosin interaction, the affinity between myosin in solution and actin in the residue breaks the bonds holding actin to the residue, pulling actin into solution where it complexes with myosin to form actomyosin.

The early work by Szent Gyorgyi's group at Szeged has remained unchallenged for many years and many of the preparative techniques devised by this group have continued in use virtually unchanged to the present time. The problem of myosin and actomyosin extraction from muscle was not re-examined until 1953, when Hanson and Huxley (1953) investigated different extraction techniques in an effort to localize actin and myosin

in the interdigitating thick and thin filament model of muscle structure that they had just discovered. These workers found that it was possible to extract almost pure myosin even from myofibrils or glycerinated muscle fibers, both of which do not contain any ATP, by use of either a 0.1 M sodium pyrophosphate, 0.001 M $MgCl_2$ solution at pH 7.0, or a 0.47 M KCl, 0.01 M pyrophosphate, 0.1 M phosphate, 0.001 M $MgCl_2$ solution (Hasselbach-Schneider solution) at pH 6.4. Prolonged extraction by either of these solutions caused almost complete disappearance of the A-band from myofibrils or glycerinated fibers without apparently affecting the I-band or the Z-disk. Furthermore it was possible to extract actin from the myosin-extracted residue. Since the A-band is the region containing the thick filaments and the I-band is the region containing the thin filaments, the results suggest that the thick filaments are composed of myosin whereas the thin filaments are composed principally of actin. Indeed, many subsequent studies using both selective extraction techniques (Hanson and Huxley, 1955; Corsi and Perry, 1958; Corsi et al., 1967) and fluorescent antibody labeling (Szent Gyorgyi and Holtzer, 1960) have now left little doubt that myosin is localized exclusively in the thick filaments and that actin is confined to the thin filaments. In fact, since Huxley (1963) and Kaminer and Bell (1966) have, by careful manipulation of the pH and ionic strength of purified myosin solutions, been able to reconstitute filaments closely resembling native thick filaments, it is now generally felt that the thick filaments are composed almost entirely of myosin. On the other hand, the thin filaments almost certainly contain other proteins in addition to actin (Endo et al., 1966; Ohtsuki

et al., 1967; Corsi et al., 1967).

Although the contractile machinery of muscle consists principally of actin and myosin, and the study of myofibrillar protein extraction must be concerned chiefly with the extraction of these two proteins, a number of other proteins have been found in the myofibril in lesser quantities. These proteins ostensibly regulate or affect the response of actin and myosin to Mg^{++} and ATP, and for this reason, they are often referred to collectively as "regulatory proteins". The first of these regulatory proteins to be discovered was tropomyosin whose isolation and properties were first described by Bailey (1946). Several studies (Perry and Corsi, 1958; Endo et al., 1966; Corsi et al., 1967) have shown that tropomyosin is located along the length of thin filament. Although there have been several suggestions that tropomyosin was also located in the Z-band (Corsi and Perry, 1958; Endo et al., 1966; Huxley, 1963), recent results (Stromer et al., 1969; and Caspar et al.[1]), indicate that the Z-line contains little if any tropomyosin. Troponin, a more recently discovered regulatory protein (Ebashi and Kodama, 1965), is apparently distributed in periodic fashion along the thin filament, but is not present in either the thick filaments or the Z-line (Ohtsuki et al., 1967). α-actinin, a third regulatory protein appears to be located either in the Z-disk or at the junction of the Z-disk with the thin filaments (Briskey et al., 1967b; Masaki et al., 1967; Goll et al., 1969 in press). All three of these regulatory proteins can be at least partially

[1]Caspar, D. L. D., C. Cohen and W. Longley, Children's Cancer Research Foundation, Boston, Massachusetts. X-ray diffraction of tropomyosin crystals and the Z-line. Private communication. 1969.

extracted from washed myofibrils in a form free of actin or myosin by swelling the myofibrils in low ionic strength solutions for a period of several days (Ebashi and Ebashi, 1965; Perry and Corsi, 1958; Perry et al., 1966; Schaub et al., 1967a; 1967b; Arakawa et al., 1969 in press). This extraction is enhanced if the pH is adjusted to 8.0 - 8.5. Because of their solubility in very low ionic strength solutions, it is difficult to ascertain the effects of the regulatory proteins on myosin and actomyosin extraction, although α-actinin, because of its supposed location at or near the Z-line, may well be important in rupture of the bonds between the thin or actin filaments and the Z-disk. Banga and Szent Gyorgyi (1941) suggested that such a rupture must precede actin solubilization. This problem will be discussed in more detail after first reviewing some more recent studies on actomyosin extraction.

According to Banga and Szent Gyorgyi's hypothesis on the mechanism of myosin and actomyosin extraction, it should be impossible by the use of high ionic strength salt solutions to extract anything other than actomyosin from washed myofibrils which do not contain any ATP. Conversely, if ATP or pyrophosphate is added to the extraction solution, it should be possible to extract myosin free from actin from washed myofibrils. Several early studies suggested that this was indeed the case (Hanson and Huxley, 1953; Hasselbach and Schneider, 1951). However, Perry and Corsi (1958) indicated that they found it impossible to extract actin-free myosin from either fresh or glycerinated myofibrils even though solutions of varying ATP or pyrophosphate concentration with and without added $MgCl_2$ were used. ATP was shown to be present throughout the ex-

traction period. Several years earlier, Perry (1955) had reported that whereas the Hasselbach-Schneider solution selectively extracted L-myosin from fresh whole minced muscle, this same solution extracted actomyosin from myofibrils prepared from fresh muscle. If, however, the muscle was allowed to pass into rigor before preparing the myofibrils, the Hasselbach-Schneider solution then selectively extracted myosin. Thus, the extraction of myosin and actomyosin from myofibrils did not appear to conform to the simple principles formulated earlier by Banga and Szent Gyorgyi (1941).

Between 1958 and 1966 most of the work on myosin and actomyosin extraction was directed toward investigation of the rate of actomyosin formation in high ionic strength suspensions of minced whole muscle.

A controversy had developed about 1956-58 between Morales's and Gergely's laboratories concerning the effect of ATP on myosin B or natural actomyosin solutions (Von Hippel et al., 1958; 1959; Gellert et al., 1959; Gergely, 1956; Gergely and Kohler, 1958). Morales interpreted his results as indicating that ATP caused myosin B particles to extend at constant molecular weight but Gergely maintained that ATP dissociated myosin B into two smaller particles, actin and myosin. Much of the disagreement was found to originate from the circumstance that Morales used a 5-hour extraction of minced whole muscle with Weber-Edsall solution to prepare myosin B, whereas Gergely used a 24-hour extraction with the same solution. Careful examination, using both analytical ultracentrifugation and light scattering on the myosin B preparations resulting from these two extraction methods (Von Hippel

et al., 1958; 1959), demonstrated that three classes of particles could be found in the 5-hour myosin B, but that the 24-hour myosin B appeared to contain only one or two classes of particles. About 65% of the protein in the 5-hour "myosin B" preparation was, in fact, free myosin and only 34% of the 5-hour "myosin B" consisted of large particles resembling actomyosin. These large particles could be further divided into a heavy group which appeared to elongate without dissociation upon the addition of ATP, and a light group which dissociated upon the addition of ATP. Gergely's 24-hour myosin B consisted principally of this light group of large particles with a trace of the heavy group, but did not contain any detectable free myosin. This difference in composition explained the observed differences in interaction with ATP.

Since the 5-hour myosin B ostensibly contained both myosin and actomyosin, actin extraction presumably commences sometime between 30 minutes and 5 hours after suspension of minced muscle in Weber-Edsall solution. Maruyama and his associates have carefully examined the time course of actomyosin extraction in an effort to ascertain when actin extraction begins and to relate the onset of actin extraction to the ATP content of the suspension (Noda and Maruyama, 1959; Ishiyama, 1960; Haga et al., 1965). Myosin A appeared to be the main component in both 1- and 5-hour extracts although minced muscle which had been allowed to sit at 2° for 1 hour after death of the rabbit and then extracted for 4 hours (to give a total post-mortem time of five hours) appeared to contain a relatively small amount of actomyosin as well as myosin. Extraction of minced muscle starting at 5 hours post-mortem and continuing to 24

hours post-mortem yielded some typical natural actomyosin which had a particle length somewhat larger than that of the normal 24-hour myosin B preparation. These observations were in close agreement with the results earlier reported by Morales (Von Hippel et al., 1958; 1959) except that Morales obtained 65% myosin A in their 5-hour extracts compared to the 90% myosin A yield found by Noda and Maruyama. A year later, Ishiyama (1960) followed actomyosin extraction while simultaneously measuring the ATP concentration of the muscle suspension. His results appeared to confirm the pioneering research of Banga and Szent Gyorgyi (1941) that the extraction of actomyosin starts only after the intrinsic ATP of the muscle mince is hydrolyzed by the ATPase activity of myosin.

A much more extensive investigation of the time course of actomyosin extraction was reported in 1965 by Haga et al. (1965). These workers used viscosity, turbidity, flow birefrigence, analytical ultracentrifugation, and ATPase measurements to study the nature of the protein solubilized from minced rabbit muscle by Weber-Edsall solution. They observed a remarkable increase in viscosimetric activity of extracts (indicating a higher actomyosin content) between 10-20 hours of extraction. The viscosimetric activity of the extracts reached a maximum value after 20 hours of extraction and did not change with longer extraction periods up to 30 hours. Also, an appreciable increase in turbidity occurred between 10 and 20 hours of extraction. This turbidity decreased greatly on addition of ATP and showed an almost constant value for myosin B which had been extracted for longer than 15 hours. These results indicate the formation of large macromolecules, which are sensitive to ATP, in the

minced muscle suspensions between 10 and 20 hours of extraction. Furthermore, Ca^{++}-modified ATPase activity of the extracted protein at 10 mM KCl was high after 1 or 5 hours of extraction and then decreased slowly with longer extraction times. On the other hand, the Mg^{++}-modified ATPase activity of the extracted protein was very low after 1 and 5 hours of extraction and then increased with increasing extraction time, with the largest increase occurring between 10 and 12 hours of extraction. Since myosin ATPase is inhibited by Mg^{++} but activated by Ca^{++} and actomyosin ATPase activity is activated by Mg^{++}, these results suggest that the extraction of actin occurs after 5 hours, with the greatest extraction occurring between 10-12 hours after initial suspension. Schlieren diagrams of analytical ultracentrifugal runs also indicated that after 5 hours of extraction, the area under the slow (about 6S) myosin peak decreased and the area under the fast (about 30-50S) actomyosin peak increased. This change was again most evident between 10 and 15 hours of extraction. The results of all their physico-chemical determinations being in good agreement, Haga et al. (1965) concluded that during high ionic strength extraction of minced muscle, myosin is first solubilized from muscle mince and then between 10 and 20 hours of extraction is transformed to actomyosin. Thus, Haga et al. placed the start of actin solubilization at approximately 10 hours after suspension of minced muscle in a high ionic strength solution at $2^{o}C$. This is about the same time that Ishiyama's earlier work (Ishiyama, 1960) showed that ATP disappeared in the muscle suspensions.

However, the manner in which the F-actin in the muscle residue was solubilized to combine with myosin and form actomyosin remained unclear. Recently, Haga et al. (1966a) have suggested that F-actin filaments, 1 u in length, are solubilized intact and form the core of the natural actomyosin particles. This may account for the uniformity of particle length usually seen in myosin B preparations. This length ranges between 1.0 to 2.4u (Haga et al., 1966a), close to the length of native thin (actin) filaments. Electron microscopic observations indicated that solubilization of actin was preceded by separation between the thin filaments and the Z lines (Haga et al., 1966b). Both 0.6 M KCl containing myosin or 0.6 M KCl containing 1 - 10 mM Ca^{++} solubilized actin filaments from a fibrous residue which had been prepared by selectively extracting myosin from minced muscle. A solution of 0.6 M KCl, however, did not liberate any protein from this fibrous residue, but vigorous mechanical disintegration did enhance F-actin extraction.

Although the studies between 1958 and 1966 appeared merely to confirm and extend Banga and Szent Gyorgyi's original ideas on myosin and actomyosin extraction, there have been two recent reports which indicate that ATP may not have the role of actin-myosin dissociation as is envisaged by the Banga-Szent Gyorgyi theory. Barber and Canning (1966) investigated the problem of myosin and actomyosin extraction from myofibrils, using myofibrils which had been purified free from contaminating ribonucleic acid and sarcoplasmic reticular membranes by means of density gradient centrifugation. Extraction of such purified myofibrils by a 0.5 M KCl, 0.1 M Tris, 2×10^{-4} M ATP solution at pH 8.0 yielded only myosin,

even when the extraction was prolonged overnight to insure complete degradation of the ATP. When elements of the sarcoplasmic reticulum were added to the purified myofibrils, this same salt solution specifically extracted actomyosin from the myofibrils, even with short extraction times. Although Barber and Canning could not offer an explicit explanation for their results, they did suggest that the Ca^{++}-binding ability of the sarcoplasmic reticulum vesicles may be involved.

The second report, which also appeared in 1966, was a paper by Mihalyi and Rowe (1966) which showed that actomyosin extraction, even from whole muscle minces, was prevented by the presence of 0.2 M phosphate in the extracting solution, even though the degradation rate of the intrinsic ATP of the muscle mince was not affected by the presence of phosphate. These authors proposed that inorganic phosphate as well as ATP and pyrophosphate act to preserve or strengthen the bonds between the thin filaments and the Z disk and that this preservation effect rather than the actomyosin dissociating effect of ATP or pyrophosphate is responsible for the prevention of actomyosin extraction by these two anions. The omission of phosphate from the extracting solutions led first to the appearance of myosin in the extracts after 30 - 60 minutes of extraction and then to the presence of actomyosin after 10 - 12 hours of extraction. This demonstrated that the inhibition of actomyosin extraction was, in fact, due to the presence of phosphate in the extracting solutions. Shortly after Mihalyi and Rowe's report, Chaudhry et al. (in press) also noted the failure of a high ionic strength phosphate containing solution to extract typical actomyosin from myofibrils, even in the absence of

pyrophosphate and ATP. However, the mechanism whereby phosphate, pyrophosphate, and ATP exert their effects remains unknown.

It occurred to us that there might be some connection between these last two reports on myosin and actomyosin extraction and the findings by Haga et al. (1966b) that Ca^{++} appeared to loosen the bonds between the I- and Z-filaments. The sarcoplasmic reticulum normally contains a substantial amount of bound Ca^{++} but loses the ability to hold this Ca^{++} in the absence of ATP; thus, the addition of the elements of the sarcoplasmic reticulum to the myofibrils in Barber and Canning's study (1966) would also mean the addition of substantial amounts of Ca^{++} to the extraction mixture. If Ca^{++} loosens the I-Z bonds, then added Ca^{++} might enhance actin solubilization and thereby explain the actomyosin extraction which Barber and Canning (1966) observed only in the presence of sarcoplasmic reticulum. Furthermore, since inorganic phosphate, pyrophosphate, and ATP are all good Ca^{++} chelators, the Ca^{++} chelating abilities of these anions may account for their ability to inhibit actomyosin extraction in Mihalyi and Rowe's (1966) studies. Therefore, we decided to re-examine the extraction of myosin and actomyosin from muscle, with particular emphasis on the possible role of Ca^{++} in actomyosin extraction.

MATERIALS AND METHODS

The report (Mihalyi and Rowe, 1966) that high ionic strength phosphate buffers would extract only myosin and not actomyosin from minced rabbit muscle formed the basis for this study. A number of preliminary experiments confirmed the lack of actomyosin extraction by phosphate-containing buffers if such extractions were done by using minced whole rabbit muscle and in the absence of stirring. On the other hand, a thorough homogenization of myofibrils preceding their extraction by high ionic strength phosphate buffers led to the appearance of a viscous actomyosin-like protein solution which appeared polydisperse in the analytical ultracentrifuge. The results of this investigation will be reported in the form of ten separate experiments, with each experiment consisting of two or more animals.

Preparative Procedures

The back and leg muscles of rabbits constituted the experimental material in this study. The rabbits were anesthetized by intravenous injection of 0.5 cc of d-tubocurarine chloride (3 mg/cc) mixed with 1.5 cc of 60 mg/ml nembutol and immediately exsanguinated and skinned. The back and leg muscles were excised, carefully cleaned of adhering fat and connective tissue and minced in a meat grinder. Samples of the minced muscle were then used either for direct extraction of myofibrillar proteins with different extraction media or for preparation of myofibrils which were then used for studies of myofibrillar protein extraction.

Extraction of minced whole muscle

Quadruplicate 5-gram samples of minced muscle were weighed directly into 250 ml centrifuge bottles, suspended by use of a stirring rod in five volumes (v/w) of a particular solution and kept without further stirring at 2°C. Salt solutions used for extraction of muscle mince in various experiments were as follow: (1) Potassium chloride-potassium phosphate, pH 6.5, $\Gamma/2$ = 0.65, composed of 0.2 M K-phosphate and 0.3 M KCl; (2) Potassium chloride-potassium phosphate, pH 7.6, $\Gamma/2$ = 0.65, composed of 0.2 M K-phosphate, 0.1 M KCl; (3) Potassium chloride - Tris-HCl, pH 7.6, $\Gamma/2$ = 0.65, composed of 0.2 M Tris, 0.5 M KCl; (4) Solution No. 3 plus 10 mM EGTA; (5) Solution No. 3 plus 10 mM Ca^{++}.

The preceding solutions were precooled to 2°C before use and all subsequent preparative procedures were conducted at 2°C using solutions precooled to 2°C. After a given period of extraction, one of the four centrifuge bottles containing minced muscle suspension was centrifuged in a Sorvall refrigerated centrifuge at 10,000 x g for 15 minutes. Extraction times of 3, 6, 12 and 24 hours were used in each experiment in this investigation. Following centrifugation, the supernatant was decanted into a graduated cylinder and diluted 15 times (v/v) with glass-distilled, deionized water to obtain an ionic strength of 0.04. The diluted extracts were centrifuged at 10,000 x g for 15 minutes, the precipitate dissolved in 0.5 M KCl, and the protein reprecipitated by 12-fold dilution to an ionic strength of 0.04. The precipitated protein was collected by centrifugation at 10,000 x g for 15 minutes and the dissolution-reprecipitation cycle repeated two more times. The precipitate

from the final reprecipitation was dissolved in 0.5 M KCl, kept over night in the refrigerator and then filtered through glass wool. The filtered solution was used for subsequent studies on the proportion of myosin and actomyosin extracted.

Preparation of myofibrils

Three different methods were used to prepare myofibrils in this study. The back and leg muscles of rabbits were used in all three preparations and all preparations were done at $2^{o}C$ using precooled solutions. In the most frequently used method (hereafter referred to as sucrose myofibrils), minced muscle was suspended in 10 volumes (v/w) of 0.25 sucrose, 0.05 Tris and 1 mM EDTA, pH 7.6, by using three 15-second homogenizations in a Waring blender, with an interval of 45 seconds between each homogenization. The myofibrils were sedimented at 1000 x g for 10 minutes in a MSE Model-6L centrifuge and then resuspended in 5 volumes (v/w) of the same sucrose solution by a 10-second homogenization. The myofibrils were again sedimented at 1000 x g for 10 minutes and resuspended in 5 volumes (v/w) of 0.05 M Tris, 1 mM EDTA, pH 7.6, by a 15-second homogenization. This suspension was passed through a polyethylene net to remove connective tissue, and the strained myofibrils were then sedimented at 1000 x g for 10 minutes. The sedimented myofibrils were washed once by suspension in 0.15 M KCl followed by centrifugation at 1000 x g for 10 minutes, and the sedimented myofibrils were then suspended in various solvents depending on the experiment.

In one experiment, myofibrils (phosphate myofibrils) were prepared

by a gentle suspension of minced muscle in a 0.03 M potassium phosphate buffer pH 7.4. A stirring rod was used to effect the suspension instead of the homogenization used for the sucrose myofibrils. After a 2-hour extraction, the suspension was centrifuged for 10 minutes at 10,000 x g, the supernatant discarded, and the myofibrillar residue resuspended by means of a stirring rod in 0.03 M potassium phosphate for a second 2-hour extraction. The myofibrils were again sedimented at 10,000 x g for 10 minutes and the sedimented myofibrils then used directly for extraction.

In a third kind of experiment, myofibrils were again prepared by gentle suspension of muscle mince with a stirring rod, but with a 0.03 M Tris buffer, pH 7.4, substituted for the 0.03 M potassium phosphate buffer (Tris myofibrils). The extraction times and sedimentation procedures were exactly as described for the phosphate myofibrils.

Extraction of myofibrils

Myofibrils obtained by the methods described in the preceding section were extracted by using one of the following salt solutions: (1) potassium chloride-potassium phosphate, pH 6.5, $\Gamma/2 = 0.65$, composed of 0.2 M K phosphate, 0.3 M KCl; (2) potassium chloride-potassium phosphate, pH 7.6, $\Gamma/2 = 0.65$, composed of 0.2 M K phosphate, 0.1 M KCl; (3) potassium chloride-Tris-HCl, pH 7.6, $\Gamma/2 = 0.65$, composed of 0.2 M Tris, 0.5 M KCl; (4) Solution No. 3 plus 10 mM EGTA; (5) Solution No. 3 plus 10 mM Ca^{++}.

In each experiment, 5-gram samples of myofibrils were placed in each of four 250 ml centrifuge bottles, and all four samples were then suspended

in one of the solutions just listed. After either 3, 6, 12, 24 hours of extraction, one of the myofibrillar suspensions was centrifuged for 15 minutes at 10,000 x g, and the supernatant filtered through glass wool. Aliquots of the filtered solution were dialyzed for 24 hours against four changes of 0.4 M KCl to remove the various anions used in different extracting solutions. The dialyzed solutions were used in the ATPase and turbidity studies described subsequently. Analytical ultracentrifuge and viscosity studies were done directly on the extracts without dialysis.

Enzymic treatment of myofibrils In one experiment, myofibrils were subjected to a four-minute digestion by trypsin or papain. Purified trypsin powder, twice recrystallized and free from salt, was purchased from Sigma Chemical Co. A crude papain powder was also purchased from Sigma Chemical Co. Both enzymes were dissolved in distilled water just prior to use. Digestion was done for four minutes at 25°C with enzyme to myofibrillar protein ratios of 1/300 (w/w). The digestion was done in 100 mM KCl, 100 mM Tris-HCl, pH 7.6. The trypsin reaction was stopped by addition of a fourfold excess of crystallized soybean trypsin inhibitor (Sigma Chemical Co.) followed by sedimentation of the undigested myofibrils at 1000 x g for 5 minutes at 2°C. The myofibril residue was washed three times with 150 mM KCl to remove any last traces of the trypsin-trypsin inhibitor complex and then subjected to extraction by high ionic strength solutions. No specific inhibitor of papain digestion which would not also affect the myofibrillar proteins was available. Therefore papain digestion was stopped by rapid sedimentation of the undigested myofibrils at 3000 x g for 1 minute at 2°C followed immediately by five washes with 150 mM KCl at 2°C. All five washes were completed within 15 minutes after the end of

the four-minute digestion period. The washed papain-treated myofibrils were then subjected directly to high ionic strength extraction. Prior to high ionic strength extraction, small aliquotes of both trypsin-treated and papain-treated myofibrils were observed under the phase microscope.

Protein determination

Protein analysis of both myofibrillar fractions from whole ground muscle and from prepared myofibrils was done by the biuret analysis (Gornall et al., 1949; Robson et al., 1968). Standards consisted of 2.5, 5.0, 7.5, and 10.0 mg of bovine serum albumin.

Analysis of Myosin and Actomyosin Content

After protein analysis, each extract of myofibrillar protein, whether from myofibrils or from minced whole muscle, was examined for actomyosin content by using three different tests. These tests were viscosity change upon addition of ATP, analytical ultracentrifugation, and comparison of the Ca^{++}- and Mg^{++}-modified ATPase activities. In a few experiments, the turbidity response to added ATP was also tested.

Viscosity measurements

The viscosity of the extracted myofibrillar proteins was measured at 25°C by using an Ostwald viscometer with an outflow time of 72.5 seconds for 5 ml of water. Five-ml aliquots of the extracted protein at a concentration of 2 mg/ml in 0.5 M KCl were placed in the viscometer and allowed to equilibrate for 3 minutes. Because of its thixotropic nature,

some difficulty is always encountered in attempting to accurately measure actomyosin's viscosity. However, since the viscosity of actomyosin in the absence of ATP is markedly higher than in the presence of ATP, this lack of precision was not a problem in our experiments. After equilibration, the viscosity of the myofibrillar protein extract was measured, and then 0.25 ml of 0.1 M ATP together with 0.25 ml of 0.1 M $MgCl_2$ were added directly to the viscometer and mixed with the myofibrillar protein extract to yield 5.5 ml of a 0.45 M KCl, 4.5 mM Mg-ATP solution containing 1.82 mg protein/ml. The viscosity of this resulting solution was measured as soon as possible after mixing. Relative viscosities were calculated from an average of three separate readings, both before and after ATP addition. The activity of the myofibrillar protein was then calculated from the formula first proposed by Portzehl et al. (1950). This formula is shown below:

$$\text{Activity} = \frac{\frac{\ln \eta_{rel}}{C} - \frac{\ln \eta_{rel\ ATP}}{C'}}{\frac{\ln \eta_{rel\ ATP}}{C'}} \times 100$$

where:

η_{rel} is the relative viscosity before ATP addition,

η_{relATP} is the relative viscosity after ATP addition, and

C and C' are the concentrations of the protein in solution without and with ATP.

Since the addition of 4 to 5 mM Mg-ATP at ionic strength above 0.4 causes dissociation and a marked viscosity drop in actomyosin but does not affect the viscosity of myosin, a higher activity number indicates the presence of a larger proportion of actomyosin in the myofibrillar protein extract.

Sedimentation studies

These were performed in the Spinco Model E analytical ultracentrifuge using the Schliern optical system at a temperature of 20°C. The solutions contained 4-10 mg protein/ml in the salt solutions used for extraction. Since the objective of this investigation was simply to ascertain whether myosin or actomyosin was present in the extracts and since actomyosin sediments much more rapidly than myosin (10 - 30s vs 5 - 6s) the sedimentation coefficients were not measured for each experiment, but instead the sedimentation diagrams were merely inspected for the presence of the typical actomyosin or myosin pattern.

ATPase determinations

Disodium ATP purcha .d from Sigma Chemical Company was converted to the Tris salt by treatment with Dowex-50 in the H^+ form followed by neutralization with Tris.

The enzymic assays were started by the addition of 0.25 ml of 0.01 M ATP. The conditions of the assays were as follows: 100 mM KCl, 50 mM Tris-acetate, pH 7.0, 1 mM $MgCl_2$ or $CaCl_2$, 0.2 mg protein/ml, 2.5 ml final volume.

After 15 minutes of incubation at 25°C, the reaction was stopped by addition of 0.5 ml of 15% trichloroacetic acid. The protein precipitate was removed by centrifugation at 1000 x g for 5 minutes and the inorganic phosphate content of the supernatant analyzed by the method of Taussky and Shorr (1953). Absorbancy was measured at wave-length of 385 mu exactly 900 seconds after addition of the molybdic acid reagent. A blank

tube containing ATP but no protein was run with each assay, and the inorganic phosphate concentration in this tube was subtracted from the phosphate concentration in the enzyme-containing tubes to correct for nonenzymatic breakdown of the ATP. Results were expressed as specific activity in u moles of inorganic phosphate released/minute/mg of protein. Since actomyosin is insoluble at the ionic strength of the assay, the assay mixture was stirred magnetically by a Mark I magnetic stirrer from Cole-Parmer.

Turbidity assay

These were done essentially as described by Seraydarian et al. (1967). The conditions of the assay were: 50 mM KCl, 20 mM Tris-acetate, pH 7.0, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM ATP, 0.5 mg protein/ml, 25-27°C, final volume of 3.0 ml. The reaction was initiated by the addition of 0.30 ml of 0.01 M ATP, and the turbidity at 660 nm followed immediately after mixing. A large increase in turbidity immediately after ATP addition is indicative of a substantial amount of actomyosin in the protein extract.

RESULTS

The extensive microscopic studies of H. E. Huxley (1963) point to the fact that actomyosin does not exist in muscle in the same form as encountered in solutions of natural, or synthetic actomyosin. Therefore, the extraction of actomyosin must proceed through disintegration of the myofibrillar structures, followed by the reassociation of the extracted proteins in a less structured form. Myosin is completely soluble in the high ionic-strength salt solutions normally used for actomyosin extraction. The actin filaments, on the other hand, appear to be firmly attached to the Z-disk. Consequently, the appearance of actomyosin in salt extracts is linked to the solubilization of actin from the thin filaments, and the study of actomyosin extraction becomes an investigation of how to break the bonds between the thin filaments and the Z-disk. The results of this study will be reported in the form of ten experiments, each designed to test the extraction properties of two different extracting solutions on the same muscle tissue sample. Each experiment involved at least two, and in many cases, more than two different animals.

Experiment I

This experiment was done to check Mihalyi and Rowe's (1966) observation that high ionic strength phosphate buffers will extract only myosin and not actomyosin from minced rabbit muscle. The two solutions used were a KCl-phosphate buffer at pH 7.6 ($\Gamma/2 = 0.65$) and a KCl-Tris-HCl buffer at pH 7.6 ($\Gamma/2 = 0.65$). The viscosimetric activity of the protein extracted by these two solutions is shown in Table 1. The KCl-phosphate

Table 1. Viscosimetric activity of protein extracted from minced rabbit muscle by KCl-phosphate or KCl-Tris-HCl at pH 7.6

Time of extraction (hr)	Extracting solution	
	KCl-phosphate	KCl-Tris-HCl
3	0.0	0.0
6	0.0	0.6
12	0.0	3.8
24	1.61	57.2

solution solubilized only myosin even with extraction times up to 24 hours, whereas the KCl-Tris-HCl buffer extracted actomyosin, with most of the actomyosin extraction occurring between 12 and 24 hours of extraction. The very small viscosimetric activity observed in the phosphate extract after 24 hours and in the KCl-Tris extract after 6 to 12 hours is not indicative of the presence of any significant amount of actomyosin because the presence of actomyosin will generally result in viscosimetric values above 50.

These viscosimetric results confirm Mihalyi and Rowe's findings and are substantiated by the ATPase activity measurements given in Table 2. There was almost no Mg^{++}-modified ATPase activity in the KCl-phosphate extracts, but the KCl-Tris extract possessed a significant Mg^{++}-modified ATPase activity, particularly after 24 hours of extraction. The analytical ultracentrifugal patterns shown in Figure 1 also point to the same conclusion. Only a single hyper-sharp peak with a sedimentation coefficient

Figure 1. Sedimentation patterns of protein extracted from minced whole rabbit muscle by KCl-Tris or KCl-phosphate solutions.

a) After extraction for 12 hr with 0.5 M KCl, 0.2 M Tris-HCl, pH 7.6, $\Gamma/2$ = 0.65, 4.0 mg/ml.

b) After extraction for 12 hr with 0.1 M KCl, 0.2 M K-phosphate, pH 7.6, $\Gamma/2$ = 0.65, 4.0 mg/ml.

c) After extraction for 24 hr with 0.5 M KCl, 0.2 M Tris-HCl, pH 7.6, $\Gamma/2$ = 0.65, 4.0 mg/ml.

d) After extraction for 24 hr with 0.1 M KCl, 0.2 M K-phosphate, pH 7.6, $\Gamma/2$ = 0.65, 4.0 mg/ml.

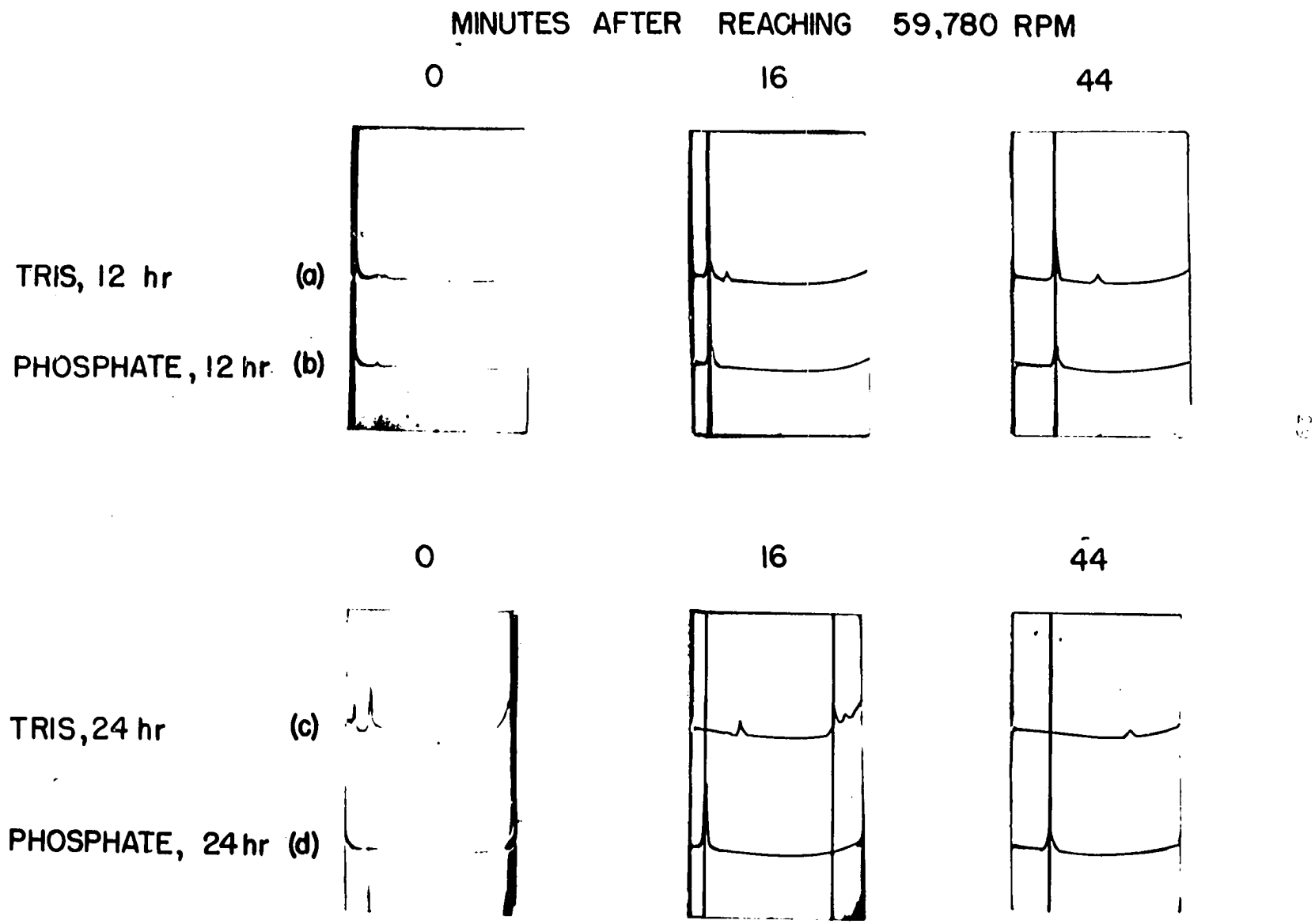

MINUTES AFTER REACHING 59,780 RPM
0
16
44
TRIS, 12 hr (a)
PHOSPHATE, 12 hr (b)
0
16
44
TRIS, 24 hr (c)
PHOSPHATE, 24 hr (d)

Table 2. ATPase activity of protein extracted from minced rabbit muscle by KCl-phosphate or KCl-Tris-HCl at pH 7.6

Time of extraction (hr)	KCl-phosphate			KCl-Tris-HCl		
	Mg^{++}	Ca^{++}	Mg^{++}/Ca^{++}	Mg^{++}	Ca^{++}	Mg^{++}/Ca^{++}
	(u mole/mg/min			(u mole/mg/min)		
3	.000	.255	.00	.010	.239	.04
6	.000	.270	.00	.013	.297	.04
12	.000	.265	.00	.017	.305	.06
24	.004	.248	.02	.044	.255	.17

of 5S is seen in the KCl-phosphate extract after either 12 or 24 hours of extraction, but a series of rapidly sedimenting peaks characteristic of actomyosin is seen in the 24-hour KCl-Tris extract.

Experiment II

Our first experiment suggested that high ionic strength phosphate-containing buffers would not extract actomyosin from minced muscle at pH 7.6. In order to extend this conclusion, this second experiment was designed to test the actomyosin extraction abilities of phosphate-containing buffers at several different pH values. KCl-phosphate solutions at pH 6.5 and 7.6 (both at an ionic strength of 0.65) were used. The viscosimetric results in Table 3 show that no actomyosin was extracted from minced rabbit muscle after 3, 6, 12, or 24 hours of extraction with KCl-phosphate solution at either pH 6.5 or 7.6. Although the viscosimetric activities appeared to increase slightly after 24 hours of extraction at

either pH 6.5 or 7.6, these activities are still very low compared to activities of over 50 which are typical of actomyosin, and it is possible that these low activities reflect the presence of aggregated myosin rather than actomyosin. The viscosimetric results are substantiated by the ATPase measurements showing almost no Mg^{++}-modified ATPase activity in any of the extracts (Table 4).

Table 3. Viscosimetric activity of protein extracted from minced rabbit muscle by KCl-phosphate at pH 6.5 or 7.6

Time of extraction (hr)	Extracting solution	
	KCl-phosphate pH 6.5	KCl-phosphate pH 7.6
3	1.35	0.0
6	1.35	0.0
12	1.34	0.0
24	5.45	1.61

Table 4. ATPase activity of protein extracted from minced rabbit muscle by KCl-phosphate at pH 6.5 or 7.6

Time of extraction (hr)	KCl-phosphate, pH 6.5			KCl-phosphate, pH 7.6		
	Mg^{++} (u mole/mg/min)	Ca^{++}	Mg^{++}/Ca^{++}	Mg^{++} (u mole/mg/min)	Ca^{++}	Mg^{++}/Ca^{++}
3	.014	.110	.13	.000	.255	.00
6	.012	.105	.11	.000	.270	.00
12	.018	.118	.15	.000	.265	.00
24	.027	.143	.19	.004	.248	.02

Sedimentation patterns of the KCl-phosphate extracts at either pH 6.5 or 7.6 (Figure 2), showed, in both cases, a single hyper-sharp peak sedimenting at the rate characteristic of myosin. No rapidly sedimenting actomyosin peaks were visible in any of the samples in this experiment, further supporting the conclusion based on the viscosimetric and ATPase activities.

Experiment III

The first two experiments established the fact that high ionic strength phosphate buffers would not extract actomyosin from minced rabbit muscle at pH values near neutrality, thereby confirming Mihalyi and Rowe's suggestion that phosphate appears to strengthen or stabilize the bonds between the I- and Z-filaments. Since Maruyama (1966) had sugtested that the I-Z bonds may be ruptured by mechanical agitation, the effect of gentle magnetic stirring on the extraction of minced muscle by phosphate buffers was investigated. The extracting solution used was KCl-phosphate, pH 7.6, $\Gamma/2 = 0.65$. The results in Table 5 indicate that constant stirring causes a significant extraction of actomyosin, even by phosphate-containing buffers. This extraction evidently commences between 6 and 12 hours of extraction and continues up to at least 24 hours of extraction. In the absence of any mechanical agitation, no actomyosin was extracted, in agreement with the result of the first two experiments in this study.

The results of the ATPase assays (Table 6) confirmed the viscosimetric data because a significant amount of Mg^{++}-modified ATPase activity

Figure 2. Sedimentation patterns of protein extracted from minced whole rabbit muscle by KCl-phosphate buffers at pH 6.5 or 7.6.

a) After extraction for 12 hr with 0.3 KCl, 0.2 M K-phosphate, pH 6.5, $\Gamma/2$ = 0.65, 4.8 mg/ml.

b) After extraction for 24 hr with 0.3 M KCl, 0.2 M K-phosphate, pH 6.5, $\Gamma/2$ = 0.65, 2.8 mg/ml.

c) After extraction for 12 hr with 0.1 M KCl, 0.2 M K-phosphate, pH 7.6, $\Gamma/2$ = 0.65, 4.0 mg/ml.

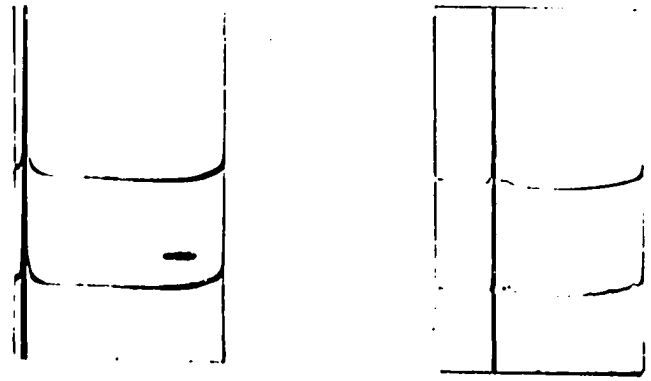
d) After extraction for 24 hr with 0.1 M KCl, 0.2 M K-phosphate, pH 7.6, $\Gamma/2$ = 0.65, 4.0 mg/ml.

MINUTES AFTER REACHING 59,780 RPM

8 56

pH 6.5 , 12 hr (a)

pH 6.5 , 24 hr (b)



8 56

pH 7.6 , 12 hr (c)

pH 7.6 , 24 hr (d)

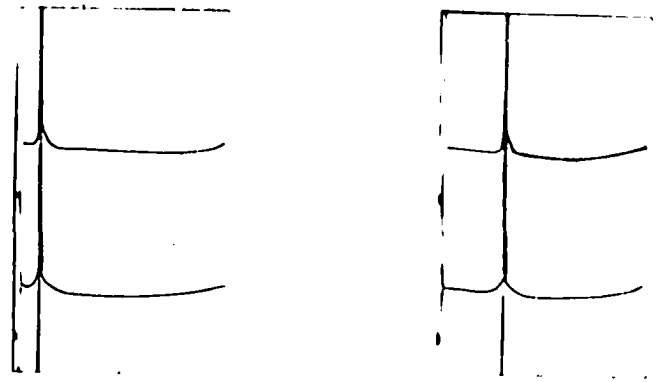


Table 5. Viscosimetric activity of protein extracted from minced rabbit muscle by KCl-phosphate at pH 7.6

Time of extraction (hr)	Extracting solution	
	KCl-phosphate (not stirred)	KCl-phosphate (stirred)
3	0.0	0.6
6	0.0	7.0
12	0.0	33.2
24	1.6	83.7

Table 6. ATPase activity of protein extracted from minced rabbit muscle by KCl-phosphate at pH 7.6

Time of extraction (hr)	KCl-phosphate					
	Not stirred			Stirred		
	Mg^{++}	Ca^{++}	Mg^{++}/Ca^{++}	Mg^{++}	Ca^{++}	Mg^{++}/Ca^{++}
	(u mole/mg/min)			(u mole/mg/min)		
3	.000	.255	.00	.015	.304	.05
6	.000	.270	.00	.027	.328	.08
12	.000	.265	.00	.057	.276	.21
24	.004	.248	.02	.085	.218	.39

existed in the stirred solutions, but no Mg^{++}-modified ATPase activity was detectable in the unstirred extracts. Moreover, ultracentrifuge patterns of the protein extracted from the stirred muscle mince (Figure 3) are characteristic of actomyosin, but the patterns of non-stirred muscle extracts exhibited only a single peak characteristic of myosin.

Figure 3. Sedimentation patterns of protein extracted from minced whole rabbit muscle by KCl-phosphate in presence or absence of stirring.

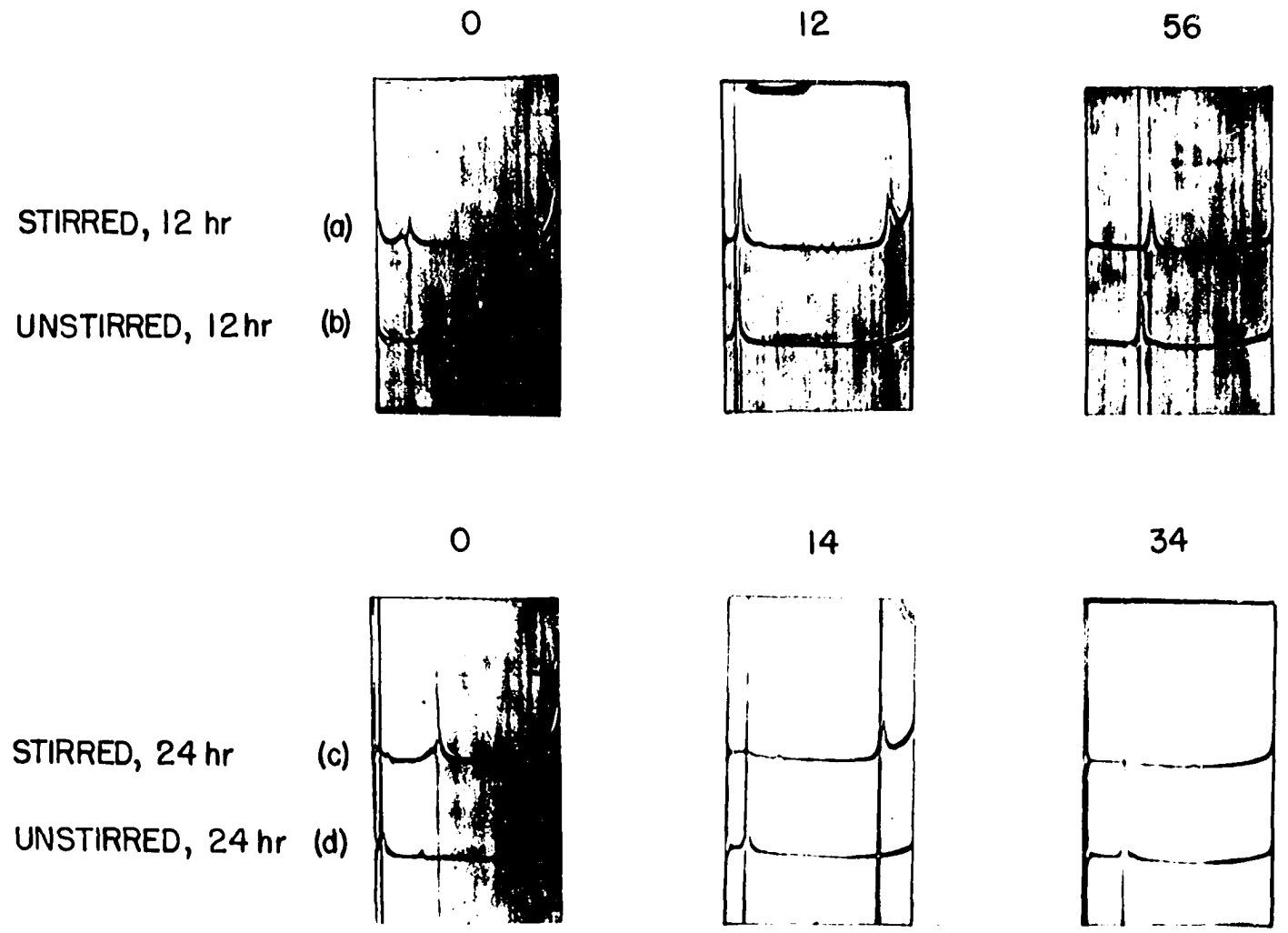
a) After extraction for 12 hr with 0.1 M KCl, 0.2 M K-phosphate, pH 7.6, $\Gamma/2$ = 0.65, stirred, 4.0 mg/ml.

b) After extraction for 12 hr with 0.1 M KCl, 0.2 M K-phosphate, pH 7.6, $\Gamma/2$ = 0.65, not stirred, 4.0 mg/ml.

c) After extraction for 24 hr with 0.1 M KCl, 0.2 M K-phosphate, pH 7.6, $\Gamma/2$ = 0.65, stirred, 4.0 mg/ml.

d) After extraction for 24 hr with 0.1 M KCl, 0.2 M K-phosphate, pH 7.6, $\Gamma/2$ = 0.65, not stirred, 4.0 mg/ml.

MINUTES AFTER REACHING 59,780 RPM



Experiment IV

Experiments I - III had investigated the effect of phosphate buffer on actomyosin extraction from minced whole muscle. Several early reports had indicated some unusual extraction patterns occurred when myofibrils were extracted instead of whole minced muscle. Since myofibrils contain no ATP, it would be expected that actomyosin extraction would commence immediately upon their suspension in a high ionic strength salt solution. Experiment IV was designed to test the effect of a high ionic strength phosphate buffer on actomyosin extraction from myofibrils. The Tris-myofibrils, prepared as described in the Material and Methods section, were handled as gently as possible during preparation to prevent any mechanical rupture of the I-Z bonds, which would lead to subsequent actomyosin extraction, regardless of the extraction conditions.

The viscosimetric results presented in Table 7 show that phosphate-containing solutions do not extract an appreciable amount of actomyosin from myofibrils during even a 24 hour extraction period. On the other hand, the substitution of Tris-buffer at the same pH and ionic strength leads to the extraction of a considerable amount of actomyosin. This extraction begins about 3 hours and continues out to 24 hours after the initial suspension. It was noticed that all our myofibril extracts contained an appreciable amount of material which was heterogeneous in the analytical ultracentrifuge and which exhibited a weak viscosimetric response to ATP. This material was present already after three hours of extraction and accounts for the relatively high viscosimetric results obtained for the KCl-phosphate extracts. It is unlikely that this material

represents actomyosin in the usual sense because it did not possess a Mg^{++}-modified ATPase activity (Table 8) and did not exhibit the typical actomyosin schlieren diagram in the analytical ultracentrifuge (cf. Figure 4). It may be a heterogeneous polymer of denatured myosin and actin.

Table 7. Viscosimetric activity of protein extracted from unhomogenized myofibrils by KCl-phosphate or KCl-Tris-HCl at pH 7.6

Time of extraction (hr)	Extracting solution	
	KCl-phosphate	KCl-Tris-HCl
3	13.3	24.2
6	24.5	48.3
12	24.5	60.1
24	22.8	74.5

Table 8. ATPase activity of protein extracted from unhomogenized myofibrils by KCl-phosphate or KCl-Tris-HCl at pH 7.6

Time of extraction (hr)	KCl-phosphate			KCl-Tris-HCl		
	Mg^{++}	Ca^{++}	Mg^{++}/Ca^{++}	Mg^{++}	Ca^{++}	Mg^{++}/Ca^{++}
	(u mole/mg/min)			(u mole/mg/min)		
3	.008	.037	.22	.006	.030	.20
5	.007	.030	.23	.017	.037	.46
12	.009	.043	.21	.036	.078	.46
24	.008	.035	.23	.051	.127	.40

Figure 4. Sedimentation patterns of protein extracted from unhomogenized or homogenized myofibrils by KCl-phosphate.

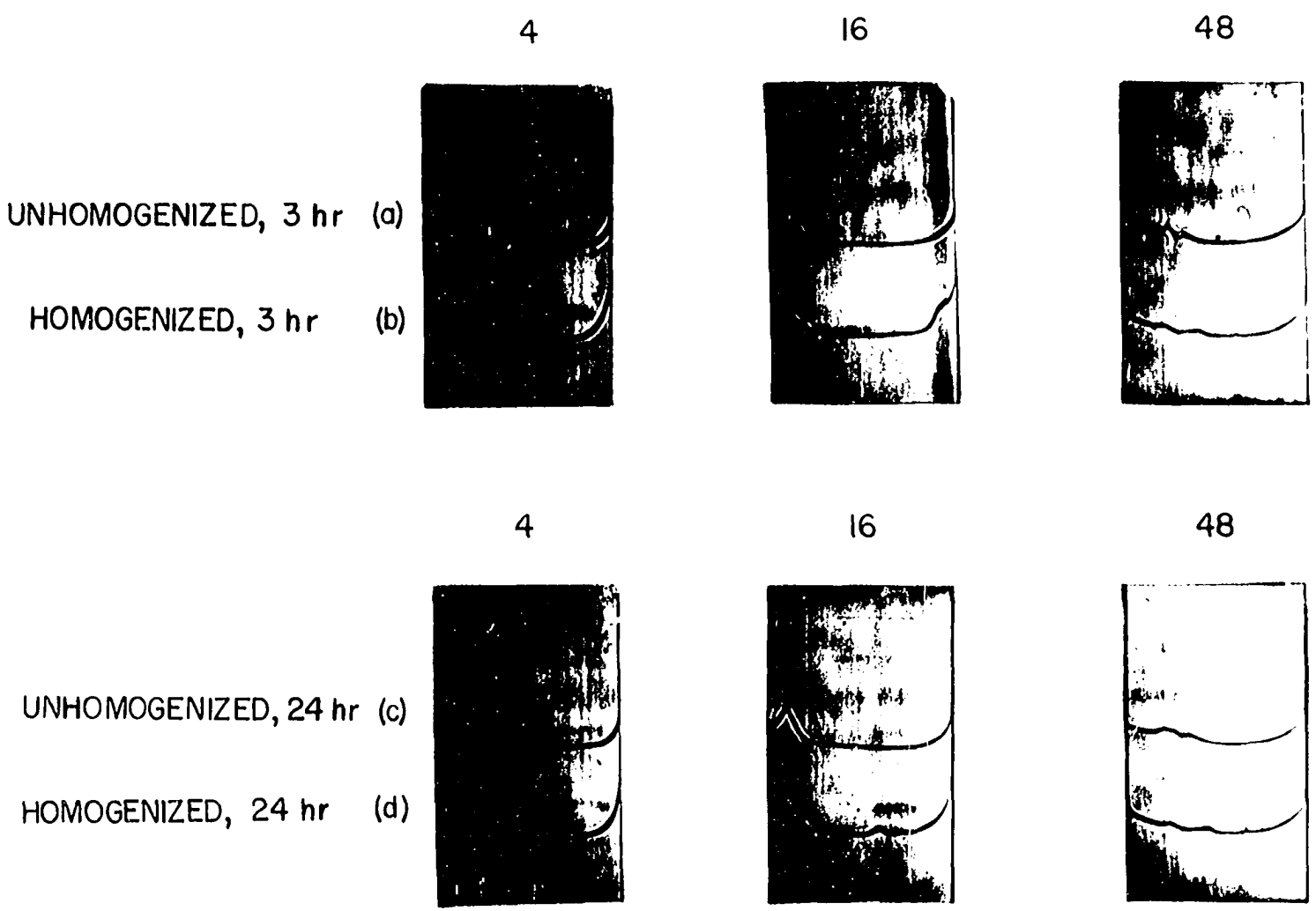
a) After extraction of unhomogenized myofibrils for 3 hr with 0.1 M KCl, 0.2 M K-phosphate, pH 7.6, $\Gamma/2$ = 0.65, 3.46 mg/ml.

b) After extraction of homogenized myofibrils for 3 hr with 0.1 M KCl, 0.2 M K-phosphate, pH 7.6, $\Gamma/2$ = 0.65, 3.86 mg/ml.

c) After extraction of unhomogenized myofibrils for 24 hr with 0.1 M KCl, 0.2 M K-phosphate, pH 7.6, $\Gamma/2$ = 0.65, 4.56 mg/ml.

d) After extraction of homogenized myofibrils for 24 hr with 0.1 M KCl, 0.2 M K-phosphate, pH 7.6, $\Gamma/2$ = 0.65, 3.93 mg/ml.

MINUTES AFTER REACHING 59,780 RPM



This suggestion is supported by the low Ca^{++}-modified ATPase activities observed for all the myofibrillar extracts.

Results of the ATPase test confirmed the viscosimetric data since the KCl-Tris extracts possessed a significant Mg^{++}-modified ATPase activity whereas the KCl-phosphate extracts exhibited no such activity (Table 8).

Experiment V

Experiment IV demonstrated that high ionic strength phosphate buffers would not extract actomyosin from myofibrils which had been prepared in a manner that would minimize mechanical rupture of I-Z bonds. Experiment V was designed to test whether the same phosphate buffers would extract actomyosin from myofibrils which had been subjected to homogenization during their preparation and therefore probably had some I-Z bonds mechanically broken. The viscosimetric results presented in Table 9 show that both KCl-phosphate and KCl-Tris extract considerable amounts of actomyosin from homogenized myofibrils. In fact, the viscosimetric data suggest that actomyosin extraction begins very quickly from homogenized myofibrils and a considerable amount of actomyosin is extracted within the first three hours after initial suspension. This occurs before even the KCl-Tris solution could extract actomyosin from the unhomogenized myofibrils (cf. Experiment IV). Thus, homogenization during myofibril preparation must lead to considerable rupture or weakening of the bonds between the I- and Z-filaments.

Table 9. Viscosimetric activity of protein extracted from homogenized myofibrils by KCl-phosphate or KCl-Tris-HCl at pH 7.6

Time of extraction (hr)	Extracting solution	
	KCl-phosphate	KCl-Tris-HCl
3	77.0	74.1
6	99.6	83.7
12	99.9	72.9
24	102.6	72.5

The experiment on ATPase activity of the KCl-phosphate and KCl Tris-HCl extracts (Table 10) confirmed the viscosimetric results. Both the ATPase data and viscosimetric results suggest that when homogenized myofibrils are extracted, KCl-phosphate solutions extract more actomyosin than a KCl-Tris solution. There is no evident explanation for this.

Table 10. ATPase activity of protein extracted from homogenized myofibrils by KCl-phosphate or KCl-Tris-HCl at pH 7.6

Time of extraction (hr)	KCl-phosphate			KCl-Tris-HCl		
	Mg^{++} (u mole/mg/min)	Ca^{++}	Mg^{++}/Ca^{++}	Mg^{++} (u mole/mg/min)	Ca^{++}	Mg^{++}/Ca^{++}
3	.061	.134	.46	.018	.102	.18
6	.067	.120	.56	.017	.105	.16
12	.095	.137	.69	.028	.151	.19
24	.096	.137	.70	.046	.161	.29

Experiment VI

Experiments IV and V showed that high ionic strength KCl-phosphate solutions would not extract actomyosin from myofibrils unless the myofibrils were mechanically disrupted during their preparation. Experiment VI was conducted to extend and confirm this conclusion by using a direct comparison of KCl-phosphate extraction of two kinds of myofibrils, one homogenized and one not homogenized during preparation. The unhomogenized myofibrils in this experiment were prepared by gentle suspension in a 0.03 M K-phosphate solution at pH 7.4 while the homogenized myofibrils were again prepared by homogenization in sucrose. Both kinds of myofibril preparations were extracted with KCl-phosphate, pH 7.6, $\Gamma/2 = 0.65$.

The viscosimetric activity (Table 11) of the extracts confirmed the previous conclusions since a considerable amount of actomyosin was extracted from the homogenized myofibrils, but the extracts from the unhomogenized myofibrils exhibited only a very low viscosimetric activity, reminiscent of other KCl-phosphate extracts of myofibrils. This low viscosimetric activity was probably due to heterogeneous polymers of denatured myosin and actin. The ATPase results (Table 12) were again in good agreement with viscosimetric data and indicated that actomyosin was extracted only from the homogenized myofibrils. The very low Ca^{++}-modified ATPase activity of extracts from the unhomogenized myofibrils lends support to the suggestion that the small amount of viscosimetric activity observed in these extracts originated from polymers of denatured myosin and actin. The conclusions of this experiment are further substantiated by the sedimentation patterns of the protein extracts (Figure 4).

Table 11. Viscosimetric activity of protein extracted from rabbit myofibrils by KCl-phosphate at pH 7.6

Time of extraction (hr)	Unhomogenized myofibrils	Homogenized myofibrils
3	19.4	77.0
6	26.8	99.6
12	31.2	99.9
24	35.2	102.6

Table 12. ATPase activity of protein extracted from rabbit myofibrils by KCl-phosphate at pH 7.6

Time of extraction (hr)	Unhomogenized myofibrils			Homogenized myofibrils		
	Mg^{++}	Ca^{++}	Mg^{++}/Ca^{++}	Mg^{++}	Ca^{++}	Mg^{++}/Ca^{++}
	(u mole/mg/min)			(u mole/mg/min)		
3	.017	.053	.32	.061	.134	.46
6	.013	.055	.24	.067	.120	.56
12	.011	.055	.20	.095	.137	.69
24	.015	.040	.38	.096	.137	.70

As pointed out before the sedimentation patterns of protein extracted from myofibrils is very heterogeneous but it is possible to detect some rapidly sedimenting material characteristic of actomyosin in the protein extracted from homogenized myofibrils. This material is evident after only 3 hours of extraction. However, no actomyosin is visible in sedimentation patterns of the protein extracted from the unhomogenized myofibrils, even after 24 hours of extraction.

Experiment VII

The previous experiments demonstrated that KCl-phosphate at pH 7.6 would not extract actomyosin from myofibrils unless they had been homogenized during their preparation. Experiment VII was done to determine whether a KCl-phosphate solution at pH 6.5, closer to the isoelectric points of myofibrillar proteins, would also extract actomyosin from homogenized myofibrils. The viscosimetric activities presented in Table 13 clearly show that KCl-phosphate solutions at pH 6.5 will extract actomyosin from myofibrils as rapidly and as efficiently as KCl-phosphate at pH 7.6. Evidently, once the I-Z bonds have been weakened or broken, lowering the pH below 7.6 does not substantially lessen actomyosin extraction. Helander (1957) had shown that actomyosin extraction from whole minced rabbit muscle is suppressed at pH values below 7.6. It is possible, therefore, that the primary effect of high pH is to aid in weakening or rupturing the I-Z bonds.

Table 13. Viscosimetric activity of protein extracted from homogenized myofibrils by KCl-phosphate at pH 6.5 or 7.6

Time of extraction (hr)	KCl-phosphate (pH 6.5)	KCl-phosphate (pH 7.6)
3	75.2	77.0
6	131.2	99.6
12	108.4	99.9
24	99.9	102.6

Again, the ATPase data (Table 14) are in good agreement with the viscosimetric results and confirm the conclusion that actomyosin extraction by KCl-phosphate solutions from homogenized myofibrils proceeds equally well at either pH 6.5 or 7.6

Table 14. ATPase activity of protein extracted from homogenized myofibrils by KCl-phosphate at pH 6.5 or 7.6

Time of extraction (hr)	KCl-phosphate pH 6.5			KCl-phosphate pH 7.6		
	Mg^{++}	Ca^{++}	Mg^{++}/Ca^{++}	Mg^{++}	Ca^{++}	Mg^{++}/Ca^{++}
	(u mole/mg/min)			(u mole/mg/min)		
3	.046	.126	.37	.061	.134	.46
6	.034	.116	.29	.067	.120	.56
12	.049	.143	.34	.095	.137	.69
24	.090	.168	.54	.096	.137	.70

Experiment VIII

The first seven experiments of this study demonstrated that high ionic strength phosphate-containing buffers at pH values near neutrality are unable to extract actomyosin from either minced whole rabbit muscle or rabbit myofibrils unless the muscle is treated mechanically to weaken or rupture the bonds between the I- and the Z-filaments. In an effort to understand how phosphate may be acting to strengthen or stabilize the I - Z-bonds, the report of Haga et al. (1966b) came to mind. These investigators had shown that Ca^{++} appeared to enhance actin solubilization from fibrous myofibrillar residues from which all myosin had been extracted. Since phosphate is a good Ca^{++} chelator, it was hypothesized that Ca^{++} was necessary to loosen actin filaments from their attachment to the Z-line, and that the effect of phosphate was to chelate this Ca^{++}, preventing it from loosening the I- Z-bonds and thereby preventing actomyosin extraction. To test this hypothesis, minced rabbit muscle was

extracted by a KCl-Tris solution at pH 7.6 containing either 10 mM EGTA or 10 mM Ca^{++}. EGTA is a strong Ca^{++} chelator but has a low (approximately 10^4) binding constant for Mg^{++}.

The viscosimetric activities of the protein extracts solubilized by these two solutions are shown in Table 15. The extract from the KCl-Tris solution containing 10 mM Ca^{++} exhibited a considerably higher viscosimetric activity than the extract from the solution containing EGTA. However, even in the presence of 10 mM EGTA, a small but significant amount of activity appeared in the supernatant. Although these viscosity results suggest that Ca^{++} promotes and EGTA inhibits the extraction of actomyosin from minced rabbit muscle, the interpretation is complicated by the results of the ATPase tests on these extracts (Table 16). For reasons which are not clear, neither the EGTA- nor the Ca^{++}-extract exhibited any significant amount of Mg^{++}-modified ATPase activity. It is unlikely that the myosin in the Ca^{++}-extract was denatured, since the Ca^{++}-modified ATPase activity of this extract is high. Ultracentrifuge studies of these extracts (Figure 5) clearly show that KCl-Tris-EGTA extract contains principally myosin with perhaps a very small amount of rapidly sedimenting material resembling actomyosin also present. This result is in agreement with the viscosity and ATPase assays on this extract. The KCl-Tris-Ca^{++} extract, on the other hand, exhibits a very unusual sedimentation diagram (Figure 5) consisting of several rapidly sedimenting peaks, possibly indicative of actomyosin, together with a more slowly sedimenting peak possessing a sedimentation coefficient characteristic of myosin.

Figure 5. Sedimentation patterns of protein extracted from minced whole rabbit muscle by KCl-Tris-HCl in the presence of 10 mM EGTA or 10 mM Ca^{++}.

a) After extraction for 12 hr with 0.5 M KCl, 0.2 M Tris-HCl, 10 mM EGTA, pH 7.6, $\Gamma/2$ = 0.65, 4.0 mg/ml.

b) After extraction for 24 hr with 0.2 M KCl, 0.2 M Tris-HCl, 10 mM EGTA, pH 7.6, $\Gamma/2$ = 0.65, 4.0 mg/ml.

c) After extraction for 12 hr with 0.5 M KCl, 0.2 M Tris-HCl, 10 mM $CaCl_2$, pH 7.6, $\Gamma/2$ = 0.65, 7.6 mg/ml.

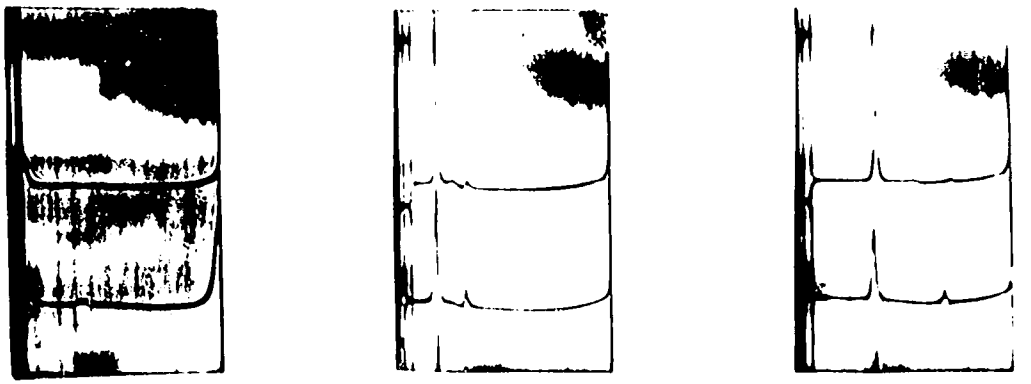
d) After extraction for 24 hr with 0.5 M KCl, 0.2 M Tris-HCl, 10 mM $CaCl_2$, pH 7.6, $\Gamma/2$ = 0.65, 8.1 mg/ml.

MINUTES AFTER REACHING 59,780 RPM

4 24 56

EGTA, 12 hr (a)

EGTA, 24 hr (b)



4 24 56

Ca^{++}, 12 hr (c)

Ca^{++}, 24 hr (d)

In an attempt to obtain further information on the nature of the KCl-Tris-EGTA and KCl-Tris-Ca^{++} extracts, the turbidity response of these extracts to added ATP was measured at low ionic strength. Suspensions of pure myosin do not give a turbidity response upon the addition of ATP, but the presence of a very small amount of actin will cause a measurable increase in turbidity upon ATP addition (Briskey et al. 1967a). The results of this test (Figure 6) indicate that both the KCl-Tris-EGTA and the KCl-Tris-Ca^{++} extracts contain actomyosin. However the turbidity response to the KCl-Tris-Ca^{++} extract is two to three times higher than the turbidity response of the KCl-Tris-EGTA extract. The amount of turbidity response is not linearly proportional to actomyosin content, and the interpretation of these results is further complicated by the ostensibly low Mg^{++}-modified ATPase activity of the KCl-Tris-Ca^{++} extracts. The Mg^{++}-modified ATPase activity of actomyosin suspension is presumably coupled to the turbidity response, and therefore the turbidity response of the KCl-Tris-Ca^{++} extract may be anomalously low. However it is obvious that the KCl-Tris-Ca^{++} extract must contain much more actomyosin than the KCl-Tris-EGTA extract.

Considered together, these results plainly show that the presence of EGTA in a KCl-Tris solution hinders the extraction of

Figure 6. Turbidity response for protein solubilized after 3 and 12 hr of extraction of minced whole rabbit muscle by either 0.5 M KCl, 0.2 M Tris-HCl, 10 mM EGTA or 0.5 M KCl, 0.2 M Tris-HCl, 10 mM $CaCl_2$.

Conditions of turbidity assay: 0.5 mg protein/ml, 50 mM KCl, 20 mM Tris-acetate, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM ATP, pH 7.0, 26°C. Reaction initiated by the addition of ATP at 0 minute.

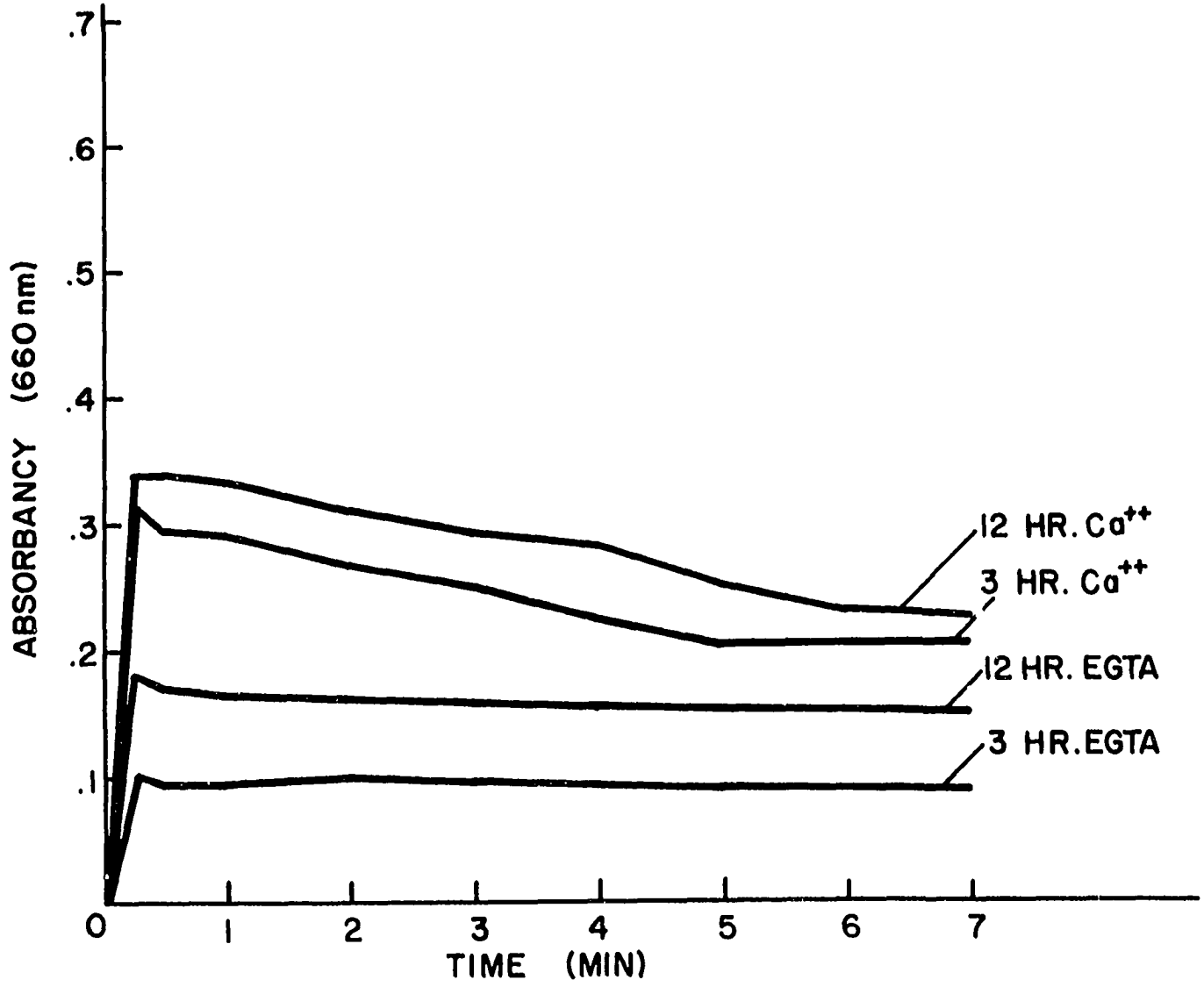

ABSORBANCY (660 nm)
.7
.6
.5
.4
.3
.2
.1
0
1
2
3
4
5
6
7
TIME (MIN)
12 HR. Ca++
3 HR. Ca++
12 HR. EGTA
3 HR. EGTA

Table 15. Viscosimetric activity of protein extracted from minced rabbit muscle by KCl-Tris-HCl + 10 mM EGTA or KCl-Tris-HCl + 10 mM Ca^{++} at pH 7.6

Time of extraction (hr)	Extracting solution	
	KCl-Tris-HCl + 10 mM EGTA	KCl-Tris-HCl + 10 mM Ca^{++}
3	3.8	17.0
6	16.3	28.9
12	18.0	44.0
24	28.5	87.2

Table 16. ATPase activity of protein extracted from minced rabbit muscle by KCl-Tris-HCl + 10 mM EGTA or KCl-Tris-HCl + 10 mM Ca^{++} at pH 7.6

Time of extraction (hr)	Extracting solution					
	KCl-Tris-HCl + 10 mM EGTA			KCl-Tris-HCl + 10 mM Ca^{++}		
	Mg^{++}	Ca^{++}	Mg^{++}/Ca^{++}	Mg	Ca^{++}	Mg^{++}/Ca^{++}
	(u mole/mg/min)			(u mole/mg/min)		
3	.010	.267	.04	.012	.288	.04
6	.010	.308	.03	.012	.304	.04
12	.013	.273	.05	.018	.293	.06
24	.030	.267	.11	.018	.250	.07

actomyosin from minced whole rabbit muscle, a result which confirms our original hypothesis. The presence of Ca^{++} in the KCl-Tris solution gives anomalous results, apparently causing solubilization of an abnormal actomyosin which does not exhibit a high Mg^{++}-modified ATPase activity.

Experiment IX

Experiment VIII had confirmed our expectation that the presence of Ca^{++} was a requirement for extraction of a significant amount of actomyosin from muscle. However, addition of Ca^{++} to the extracting solution did not cause a marked increase in the rate of actomyosin solubilization as expected (although viscosity results suggested a small increase in this rate, (cf. Tables 1 and 15), but led instead to extraction of an anomalous actomyosin. Therefore, an experiment was done to test the effects of Ca^{++} and EGTA on actomyosin solubilization from myofibrils. Homogenized myofibrils were used so that comparisons could be made between actomyosin extracted by an EGTA-containing solution and actomyosin extracted by a Ca^{++}-containing solution. The extracting solutions used were again KCl-Tris-HCl at pH 7.6 containing either 10 mM EGTA or 10 mM Ca^{++}.

The viscosimetric results are shown in Table 17. The presence of EGTA appeared to hinder actomyosin solubilization even from homogenized myofibrils (cf. Tables 9 and 17) although some actomyosin was clearly extracted even in the presence of EGTA. On the other hand, the presence of Ca^{++} caused the almost immediate appearance of actomyosin in the extract (cf. Tables 9 and 17). The ATPase assays of these extracts are shown in Table 18. Again, the presence of Ca^{++} in the extracting solution appeared to decrease the Mg^{++}-modified ATPase activity of the extract, since the Mg^{++}-modified ATPase activity of the KCl-Tris-Ca^{++} extract is considerably lower than the Mg^{++}-modified ATPase activity of the KCl-Tris-EGTA extract.

However, the Ca^{++}-modified ATPase activity of the KCl-Tris-Ca^{++} extract is slightly higher than the Ca^{++}-modified ATPase activity of the KCl-Tris-EGTA extract.

Table 17. Viscosimetric activity of protein extracted from homogenized myofibrils by KCl-Tris-HCl + 10 mM EGTA or KCl-Tris-HCl + 10 mM Ca^{++} at pH 7.6

Time of extraction (hr)	Extracting solution	
	KCl-Tris-HCl + 10 mM EGTA	KCl-Tris-HCl + 10 mM Ca^{++}
3	33.8	93.8
6	37.9	100.0
12	43.9	100.0
24	46.6	100.0

Table 18. ATPase activity of protein extracted from homogenized myofibrils by KCl-Tris-HCl + 10 mM EGTA or KCl-Tris-HCl + 10 mM Ca^{++} at pH 7.6

Time of extraction (hr)	Extracting solution					
	KCl-Tris-HCl + 10 mM EGTA			KCl-Tris-HCl + 10 mM Ca^{++}		
	Mg^{++} (u mole/mg/min)	Ca^{++}	Mg^{++}/Ca^{++}	Mg^{++} (u mole/mg/min)	Ca^{++}	Mg^{++}/Ca^{++}
3	.197	.196	1.00	.094	.219	.43
6	.145	.181	.80	.088	.221	.40
12	.162	.206	.79	.094	.224	.42
24	.109	.210	.52	.094	.217	.43

Experiment X

The previous experiments in this study have all been based on the assumption that rupture of bonds between the I- and Z-filaments was the rate limiting step in actin solubilization. To test this assumption more directly, myofibrils were treated very briefly with trypsin, and then extracted with phosphate-containing buffers. Very brief digestion of myofibrils with trypsin has been shown to almost completely remove the Z-disk (Stromer et al., 1967). To extend this experiment, a second myofibril sample was treated with papain, a proteolytic enzyme whose effects on myofibrils are not completely understood. Both the trypsin-treated and papain-treated myofibrils were extracted with KCl-phosphate, pH 7.6, $\Gamma/2 = 0.65$.

Phase micrographs of control (untreated), trypsin-treated (4 min at 25°C) and papain-treated (4 min at 25°C) are shown in Figure 7. The most obvious structural consequence of either trypsin or papain treatment of myofibrils was the almost total removal of the Z-line. This being so, it would be expected that these myofibrils would very quickly liberate actomyosin upon extraction.

The viscosimetric results shown in Table 19 confirm this expectation. Actomyosin was very quickly extracted from both trypsin-treated and papain-treated myofibrils, and such extraction appeared virtually complete as early as three hours after initial suspension. Furthermore, the viscosimetric activities observed in this experiment were the highest encountered in the entire study. The ATPase results are entirely in agreement with this conclusion (Table 20) since Mg^{++}-modified ATPase activities of the

Figure 7. Phase micrographs of control (untreated), trypsin-treated and papain-treated myofibrils.

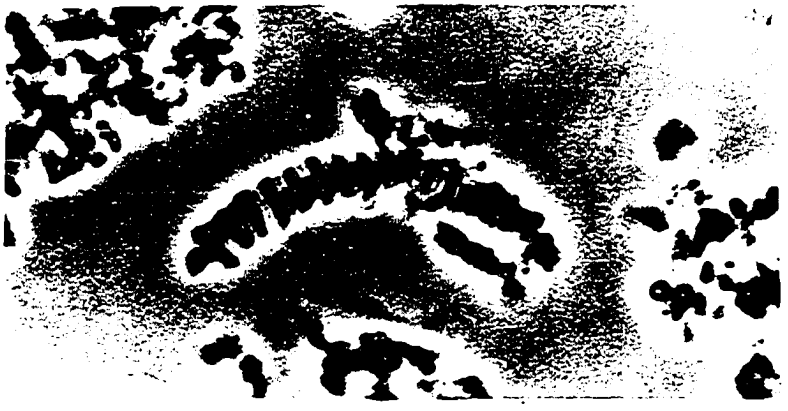
a) Phase micrograph of myofibrils without any enzymatic digestion but treated at 25°C. 4 min.

b) Phase micrograph of myofibrils after trypsin digestion.

c) Phase micrograph of myofibrils after papain treatment.

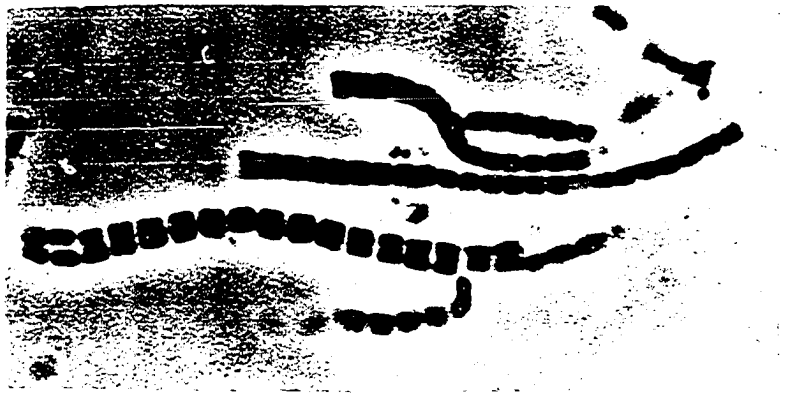
Conditions of papain or trypsin digestion: 1 mg papain or trypsin/300 mg myofibrillar protein, 100 mM KCl, 100 mM Tris acetate, pH 7.6, 25°C, 4 min.

(a)



CONTROL MYOFIBRILS

(b)



TRYPSINESED MYOFIBRILS

(c)

PAPAIN TREATED MOYFIBRILS

extracts were high already after only three hours of extraction. The ultracentrifugal patterns of the extracts also showed that the 3-hour extracts consisted primarily of actomyosin with only a very small amount of free myosin (Figure 8, a and b). The sedimentation patterns of these extracts did not change significantly, even when the extraction was prolonged for 24 hours (Figure 8, c and d).

Table 19. Viscosimetric activity of protein extracted from rabbit myofibrils treated with trypsin and papain by KCl-phosphate at pH 7.6

Time of extraction (hr)	Extracting solution	
	KCl-phosphate (trypsin-treated)	KCl-phosphate (papain-treated)
3	140.4	129.8
6	133.9	131.8
12	117.9	133.4
24	120.4	134.2

Table 20. ATPase activity of protein extracted from rabbit myofibrils treated with trypsin and papain, by KCl-phosphate at pH 7.6

Time of extraction (hr)	KCl-phosphate					
	Trypsin-treated			Papain-treated		
	Mg^{++} (u mole/mg/min)	Ca^{++}	Mg^{++}/Ca^{++}	Mg^{++} (u mole/mg/min)	Ca^{++}	Mg^{++}/Ca^{++}
3	.051	.223	.23	.042	.235	.18
6	.056	.221	.25	.039	.223	.18
12	.067	.204	.33	.045	.215	.21
24	.095	.251	.38	.060	.242	.25

Figure 8. Sedimentation patterns of protein extracted from trypsin-treated or papain-treated myofibrils by KCl-phosphate.

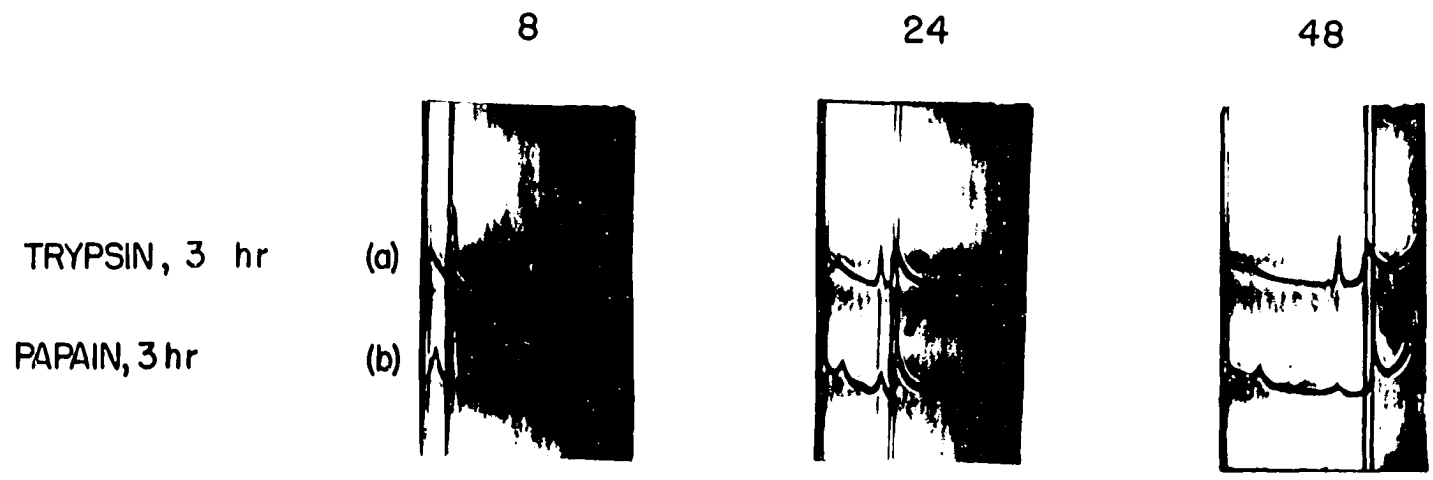
a) After 3 hr of extraction with 0.1 M KCl, 0.2 M K-phosphate, pH 7.6, $\Gamma/2 = 0.65$, myofibrils treated for 4 min with trypsin first, prior to extraction, 5.9 mg/ml.

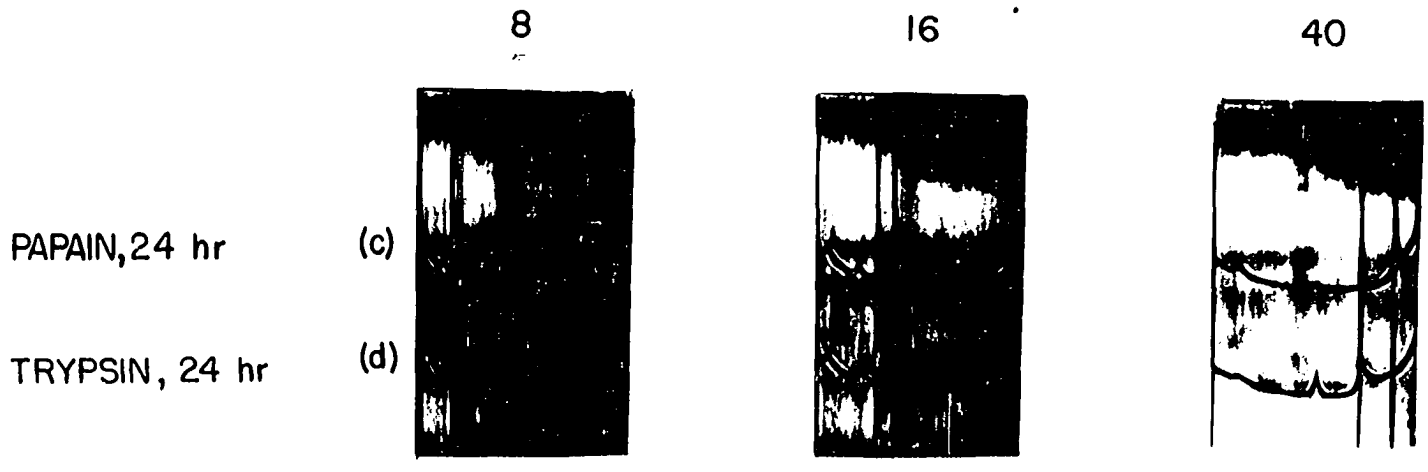
b) After 3 hr of extraction with 0.1 M KCl, 0.2 M K-phosphate, pH 7.6, $\Gamma/2 = 0.65$, myofibrils treated for 4 min with papain first, prior to extraction, 6.1 mg/ml.

c) After 24 hr of extraction with 0.1 M KCl, 0.2 M K-phosphate, pH 7.6, $\Gamma/2 = 0.65$, myofibrils treated for 4 min with papain first, prior to extraction, 5.1 mg/ml.

d) After 24 hr of extraction with 0.1 M KCl, 0.2 M K-phosphate, pH 7.6, $\Gamma/2 = 0.65$, myofibrils treated for 4 min with trypsin first, prior to extraction, 4.68 mg/ml.

MINUTES AFTER REACHING 59,780 RPM





The turbidity response of the KCl-phosphate extracts of trypsin and papain-treated myofibrils is shown in Figure 9. Both extracts exhibited a sizeable turbidity response after only 3 hours of extraction, again indicating that the 3-hour extracts consisted primarily of actomyosin. It was noticed that the extracts from trypsin-treated myofibrils exhibited a lower and oftentime slower rate of turbidity development than the extracts from papain-treated myofibrils. This is probably a reflection of the weakening effect which trypsin has on the actin-myosin interaction (Goll et al.).[1]

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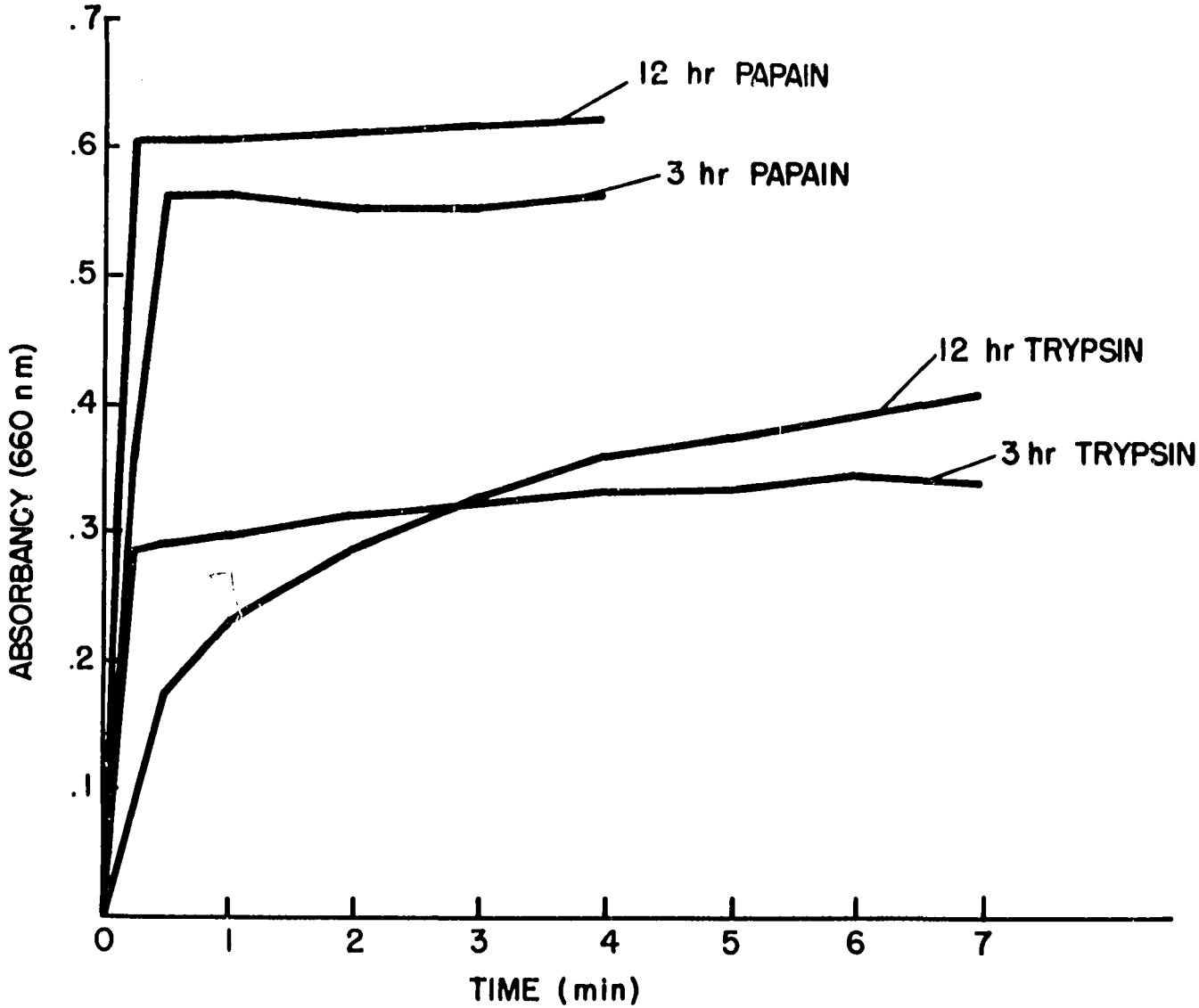
[1]Goll, Darrel E., R.M. Robson, and D. W. Henderson, Iowa State University, Ames, Iowa. An effect of trypsin on the actin-myosin interaction. Private communication. 1969.

Figure 9. Turbidity response of protein solubilized by 3 and 12 hr extraction of trypsin or papain-treated myofibrils with ~0.1 M KCl, 0.2 M K-phosphate, pH 7.6, $\Gamma/2 = 0.65$.

Conditions of turbidity assay: 0.5 mg myofibrillar/protein/ml, 50 mM KCl, 20 mM Tris-acetate, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM ATP, pH 7.0, 26°C. Reaction initiated by the addition of ATP at 0 min.


.7
.6
.5
.4
.3
.2
.1
ABSORBANCY (660 nm)
12 hr PAPAIN
3 hr PAPAIN
12 hr TRYPSIN
3 hr TRYPSIN
0
1
2
3
4
5
6
7
TIME (min)

DISCUSSION

The results of the ten experiments described in this investigation clearly demonstrate that high ionic strength ($\Gamma/2 = 0.65$) phosphate-containing buffers, at either pH 7.6 or 6.5, extract only myosin and not actomyosin from minced rabbit muscle or rabbit myofibrils. Even though the extraction times were extended to 24 hr, almost no actomyosin extraction occurred in phosphate-containing buffers. The use of KCl-Tris-HCl buffer at identical pH values and ionic strengths and under identical extraction conditions on the same muscle preparations resulted in actomyosin extraction, with most of the actomyosin solubilization occurring between 12 and 24 hour of extraction. Therefore, the lack of actomyosin extraction is clearly a property of the presence of phosphate, and is not due to the particular muscle residue or to an inappropriate choice of pH, ionic strength, or other extraction conditions. These results confirm and extend the findings of Mihalyi and Rowe (1966) who also found that the presence of phosphate prevented actomyosin solubilization from minced whole rabbit muscle.

This finding is quite surprising in view of the presently accepted concepts of actomyosin extraction (Banga and Szent Gyorgyi, 1941; Haga et al., 1966b) which ascribe a central role in prevention of actomyosin extraction to the presence of ATP. Thus, it is currently thought that when minced muscle is suspended in a high ionic-strength salt solution, myosin is first extracted into solution. After the intrinsic ATP present in the muscle at death has fallen to a level so low that it cannot effectively dissociate the actin-myosin complex, the affinity between

myosin in solution and actin in the thin filaments breaks the bond between the Z-disk and the thin filament, releasing actin into solution to form actomyosin. In terms of this concept, actomyosin extraction would start immediately upon suspension of myofibrils, which do not contain any ATP, in the KCl-phosphate or KCl-Tris-solution. Mihalyi and Rowe (1966), found that the presence of phosphate in the extracting buffer did not affect the rate of breakdown of the intrinsic ATP in muscle. These investigators suggested that the effect of phosphate (or of pyrophosphate or ATP) was to stabilize bonds between the thin filaments and the Z-line, thereby preventing the release of actin from the residues into solution to form actomyosin. However no evidence was given in Mihalyi and Rowe's (1966) paper to support this hypothesis. Thus, it is possible that phosphate, pyrophosphate, or ATP may be affecting several properties of a muscle suspension in a high ionic-strength solution, and that any one of these effects may prevent actin solubilization.

The results of several experiments in the present study plainly showed that mechanical shear forces, either as a result of homogenization during myofibril preparation or due to stirring during extraction of minced whole muscle, caused actomyosin extraction even in the presence of phosphate buffer. In fact, under conditions of such mechanical shear, Tris- and phosphate-containing buffers appeared equal in their ability to extract actomyosin. However, the demonstration that mechanical shear increases the rate of actin solubilization in phosphate-containing buffers does not prove that such shear forces are rupturing or breaking bonds between the I- and the Z-filaments. Such shear forces could be fracturing

the actin filaments themselves, causing the release of F-actin fragments into solution.

The experiments involving extraction of trypsin or papain-treated myofibrils with phosphate-containing buffers do provide evidence that the rate of actin solubilization depends on the rate of rupture of the I-Z bonds. As judged by examination in the phase microscope, the only visible effect of 4-minute treatment of myofibrils with papain or trypsin was a removal of the Z-line. That this is the only structural effect of trypsin has been further documented by electron microscopic examination (Stromer et al., 1967). When trypsin- or papain-treated myofibrils were suspended in a high ionic-strength phosphate-containing solution, actomyosin solubilization commenced at once and was virtually complete within 3 hours. Under identical conditions, only a very small amount of actomyosin was solubilized until after 12 hours of extraction from homogenized myofibrils that had not been treated with trypsin or papain. In fact, viscometric activities of the 3-hour extract from the trypsin- or papain-treated myofibrils were higher than for the 24-hour extracts from untreated myofibrils. This indicates that not only a very rapid, but also a very complete solubilization of actin occurs after Z-lines have been removed by proteolytic enzymes.

Thus, the results of this study are in agreement with the findings of Haga et al. (1966b) who presented electron microscopic evidence that the extraction of actomyosin from minced whole rabbit muscle was preceded by the appearance of a break between the Z-line and the I-band. Furthermore, Maruyama (1966) found that the average particle length in actomyosin

solutions was 1 u, the same as the length of their filaments *in vivo*. This suggests that thin or actin filaments are solubilized intact rather than as small pieces that have been broken off the end of thin filament in succession until it has been solubilized. Together, the evidence indicates that F-actin is solubilized as intact filaments, and that the rate-limiting step in actin solubilization is the rate of rupture of the I-Z bonds.

Therefore the results of this study clearly show that unless the muscle is first treated to weaken or rupture the I-Z-bonds, phosphate-containing buffers will not solubilize actin from either minced whole muscle or myofibrils. However, it was not clear how phosphate (or presumably pyrophosphate and ATP as well) was acting to cause this effect.

Haga *et al.* (1966b) have reported that the presence of Ca^{++} appears to enhance the solubilization of "natural" F-actin filaments from fibrous muscle residues which have had all their myosin extracted. Since phosphate, pyrophosphate, and ATP are all strong Ca^{++} chelators, it seemed possible that Ca^{++} weakens the I-Z bonds, and that the effect of phosphate (or pyrophosphate or ATP) was to chelate Ca^{++}, thereby preventing its effect at the I-Z junction. This possibility has been strongly supported in this study by experiments showing that the addition of low amounts of EGTA, a strong Ca^{++} chelator, to a high ionic-strength KCl-Tris-HCl buffer prevented actomyosin extraction by Tris solution from minced whole rabbit muscle. Moreover, EGTA appeared to significantly slow the rate of actomyosin extraction even from homogenized myofibrils.

On the other hand, the addition of Ca^{++} to the high ionic-strength KCl-Tris-HCl extracting solution appeared to enhance the rate of actomyosin solubilization. The effect of Ca^{++} was somewhat difficult to ascertain exactly, since the presence of 10 mM Ca^{++} appeared to cause solubilization of an anomalous actomyosin which exhibited three to four peaks in the analytical ultracentrifuge and had an abnormally low Mg^{++}-activated ATPase activity. The viscosity measurements, however, clearly indicated that the presence of Ca^{++} increased the rate of appearance of viscosimetric activity in extracts of either minced whole muscle or homogenized myofibrils.

The mechanism of calcium's effect on the I-Z bonds is entirely unknown. It has recently been shown[1] that incubation of muscle fiber strips in physiological saline solutions containing 1 mM Ca^{++} leads to disappearance of Z-lines in myofibrils prepared from these strips. Substitution of 5 mM EGTA for Ca^{++} results in stabilization of Z-line structure in the fibers. Robson[2] has shown that Ca^{++} causes some precipitation of α-actinin, a protein supposedly located in the Z-line. Whether this effect of Ca^{++} on α-actinin presages an effect of Ca^{++} on the interaction between α-actinin in the Z-line and F-actin in the thin filaments must remain speculative for the present.

The findings presented in this study have fundamental implications for studies of post-mortem changes in myofibrillar protein solubility.

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[1]Busch, W. A., Iowa State University, Ames, Iowa. Effect of Ca^{++} on rabbit muscle fiber. Private communication, 1969.

[2]Robson, R. M., Iowa State University, Ames, Iowa. Effect of Ca^{++} on α-actinin. Private communication. 1969.

Many of these studies have ostensibly not examined the nature of their protein extracts in any detail, but rather have assumed that high ionic strength salt solutions will always extract actomyosin. Even for many of those studies where the solubilized protein has been characterized, this characterization consisted only of measurement of the amount of protein precipitated at various ionic strength. Thus, the protein precipitated at ionic strengths between 0.2 and 0.3 is termed "actomyosin", and the additional protein precipitated by further dilution to ionic strength of 0.05 to 0.1 is called "myosin". Yet, it is known that significant amounts of aggregated myosin are precipitated at an ionic strength of 0.2 to 0.25. Many studies on post-mortem changes in myofibrillar protein solubility have been interpreted in terms of an interaction between actin and myosin filaments soon after death. This interaction presumably leads to a decrease in protein solubility. Post-mortem storage times of longer than 24 hours are accompanied by an increase in myofibrillar protein solubility, which has been interpreted as a dissociation of the actin-myosin complex. It has recently been shown (Henderson, 1968) that post-mortem storage causes progressive loss of Z-line structure. Results of the present study show that loss of the Z-line or rupture of I-Z bonds causes a considerable enhancement in rate of actomyosin formation. Therefore it now appears that post-mortem Z-line degradation accounts for the increase in myofibrillar protein solubility with post-mortem storage times of 24 hour or greater, and that this increased solubility should not be interpreted in terms of an actin-myosin dissociation. In fact, it is not clear why the actin-myosin interaction should have any great effect

on myofibrillar protein solubility at all, and the earlier interpretation concerning interaction and dissociation of the thick and thin filaments should be completely re-examined.

The results of this study also indicate that turbidity is the most sensitive assay for the presence of actin in myofibrillar protein extracts. In fact, it appears possible to obtain a sizeable turbidity response to ATP addition even in extracts whose viscosity did not change upon the addition of ATP, that did not exhibit any detectable Mg^{++}-modified ATPase activity characteristic of actomyosin, and that did not possess any visible actomyosin boundary in the analytical ultracentrifuge. The relation of the turbidity assay to the contraction process is not clear, and so the significance of the apparent sensitivity of the turbidity assay to the presence of actin is also unknown. Therefore, turbidity was not used extensively for following the extraction of actomyosin in this study.

SUMMARY

This investigation has studied the extraction of myofibrillar protein by different salt solutions, with particular emphasis on the relative rates of actin and myosin solubilization from whole minced muscle or myofibrils. Rabbit muscle was used throughout. The ionic strength of all extracting solutions was adjusted to exactly 0.65 by varying the KCl concentration. The presence of myosin and actomyosin in the extracts was determined by using three independent methods: 1) analytical ultracentrifugation, 2) ratio of Mg^{++}-to Ca^{++}-modified ATPase activity, and 3) the effect of 5 mM Mg^{++}-ATP on viscosity of the extracts in 0.5 M KCl. The results of the third test were expressed in terms of viscosimetric activity.

The findings in this study showed that, in the presence of phosphate buffer at either pH 6.5 or 7.6, actomyosin is not extracted from minced whole muscle or myofibrils, unless extraction is accompanied by some treatment severe enough to loosen the actin filament from the Z-line. This treatment may consist of stirring the muscle suspension during extraction or of homogenization of the myofibrils during their preparation. If the extraction is done under identical conditions of pH, ionic strength, and temperature, but Tris-HCl buffer is substituted for the phosphate buffer, actomyosin is extracted even in the absence of stirring or homogenization. Thus, the prevention of actomyosin extraction is caused by the presence of the phosphate anion. This finding raises considerable doubt concerning Banga and Szent Gyorgyi (1941) theory that actomyosin

extraction commences as soon as the intrinsic ATP in muscle is depleted. In the presence of Tris-HCl buffer, actomyosin extraction begins after 10-12 hours of extraction of minced whole muscle and after 3-6 hours of extraction of unhomogenized myofibrils.

It was observed that protein extracted from myofibrils by either Tris-containing or phosphate-containing buffers appeared very heterogeneous in the analytical ultracentrifuge. This protein also exhibited an abnormally low Ca^{++}-modified ATPase activity and possessed some viscosimetric activity, even though it had no Mg^{++}-modified ATPase activity and did not exhibit an actomyosin boundary in the analytical ultracentrifuge. It was concluded that high ionic-strength extraction of myofibrils results in the solubilization of some heterogeneous polymers of denatured myosin and actin.

Evidence was presented which suggests that rupture of the bonds between the I- and Z-filaments is the rate-limiting step in actomyosin solubilization. When examined in the phase microscope, the only effect of a 4-min trypsin or papain treatment of myofibrils was removal of the Z-line. Extraction of these treated myofibrils with phosphate-containing buffers resulted in almost complete extraction of actomyosin within three hours. This is much faster than actomyosin extraction from myofibrils even in the presence of Tris-HCl buffer.

This study also showed that the presence of 10 mM EGTA, a strong Ca^{++}chelator, in high ionic strength, Tris-containing buffers prevented actomyosin extraction in a manner similar to that observed for phosphate-

containing buffers. Therefore, it appears that the presence of Ca^{++} weakens or ruptures the I-Z bonds and thereby facilitates actin solubilization. Since phosphate is a strong Ca^{++} chelator, this would explain the lack of actomyosin extraction in phosphate-containing buffers. Some direct evidence supporting this conclusion was obtained when it was shown that addition of 10 mM Ca^{++} to high ionic-strength Tris-containing buffers increased the rate of actomyosin extraction from either minced whole muscle or myofibrils. However, actomyosin extracted in the presence of Ca^{++} appears to have an abnormally low Mg^{++}-modified ATPase activity and also exhibited an unusual appearance in the analytical ultracentrifuge. It is possible that even very low (10^{-5} to 10^{-4} M) amounts of Ca^{++} are sufficient to weaken or rupture the I-Z bonds and that higher concentrations of Ca^{++} cause some fragmentation of the actin filament itself. This fragmentation may cause formation of several classes of actomyosin based on length of actin filaments associated with myosin and may thus explain the appearance of three to four boundaries in the sedimentation patterns of actomyosin extracted in the presence of 10 mM Ca^{++}.

CONCLUSIONS

1. KCl-phosphate solutions at pH 7.6 and an ionic strength of 0.65 extract only myosin and not actomyosin from either minced whole rabbit muscle or rabbit myofibrils, even after extraction times up to 24 hours. Extraction of the same muscle preparations with KCl-Tris-HCl solutions of identical pH and ionic strength causes solubilization of actomyosin, with this solubilization beginning after 10-12 hours of extraction. Therefore, the presence of phosphate in high ionic-strength solutions at pH 7.6 suppresses the extraction of actomyosin from muscle residues. This suppression is not related to ATP content of the residue.

2. Changing pH of the KCl-phosphate extracting solution from 7.6 to 6.5 does not cause any difference in ability of phosphate-containing solutions to extract actomyosin from minced whole rabbit muscle or rabbit myofibrils.

3. Actomyosin may be solubilized from either rabbit myofibrils or minced whole muscle by high ionic-strength phosphate solutions at pH 7.6, if the myofibrils are homogenized during their preparation, or if the whole muscle mince is continually stirred during extraction. Evidently, such mechanical shear forces cause rupture or weakening of the bonds holding actin into the fibrous myosin-extracted residue. In the presence of high ionic-strength phosphate-containing solutions, actin solubilization from mechanically treated residues commences within 3 hours of extraction of myofibrils or within 12 hours of extraction of minced whole muscle.

4. Extraction of myofibrils by high ionic-strength salt solutions result in a protein solution that appears very heterogeneous in the analytical ultracentrifuge, and that possesses a very low Ca^{++}-modified ATPase activity. This occurs both in the presence and absence of actomyosin extraction. These protein solutions also exhibit a small viscosity decrease upon the addition of ATP even in the absence of any actomyosin. Direct extraction of myofibrils apparently causes solution of polymers of denatured myosin and actin.

5. The rate-limiting step in actin 'solubilization' from muscle by high ionic-strength salt solutions is the rate of rupture of bonds holding thin or actin filaments to the Z-line. Removal of the Z-line by brief enzymic digestion with either papain or trypsin results in immediate actomyosin solubilization when the enzyme-treated myofibrils are subsequently suspended in high ionic-strength salt solutions. Actomyosin solubilization from such myofibrils is almost complete within 3 hours of extraction and occurs even in the presence of phosphate. Therefore, phosphate must act to stabilize or strengthen the bonds between I- and Z-filaments, thereby preventing actin solubilization.

6. The presence of EGTA, a strong Ca^{++} chelator, in high ionic-strength KCl-Tris solutions prevents actomyosin extraction from minced whole muscle and suppresses the rate of actin solubilization from even homogenized myofibrils. Therefore, the absence of Ca^{++} must strengthen or stabilize the I-Z bonds, and the inhibition of actin solubilization by high ionic-strength phosphate-containing solutions is probably due to

the Ca^{++}-chelating abilities of phosphate. Addition of Ca^{++} to high ionic-strength KCl-Tris solutions increases the rate of actin solubilization by such solutions, but the actomyosin formed has an anomalously low Mg^{++}-modified ATPase activity, and an unusual "multi-peaked" appearance in the analytical ultracentrifuge.

7. Turbidity response to ATP addition is more sensitive test for the presence of actin in myosin-containing solutions than analytical ultracentrifugation, the appearance of a Mg^{++}-modified ATPase activity, or the ability of ATP to cause a viscosity decrease.

BIBLIOGRAPHY

Acs, G., K. Biro, and F. B. Straub. 1949. The interaction between actomyosin and polyphosphates. Hung. Acta Physiol. 2: 84-92.

Arakawa, N., R. M. Robson, and D. E. Goll. ca. 1969. Preparation of an α-actinin active at ionic strengths near the physiological range. (To be published.)

Bailey, K. 1946. Tropomyosin - a new asymmetric component of muscle. Nature 157: 367-368.

Banga, I. and A. Szent Gyorgyi. 1941. Preparation and properties of myosin A and B. Univ. Szeged Inst. Med. Chem. Stud. 1: 5-15.

Barber, M. L. S. and R. E. Canning. 1966. Extraction of contractile proteins from myofibrils prepared by rate-zonal centrifugation. Nat. Cancer Inst. Monogr. 21: 345-359.

Briskey, E. J., K. Seraydarian, and W. F. H. M. Mommaerts. 1967a. The modification of actomyosin by α-actinin II. The effect of α-actinin upon contractility. Biochim. Biophys. Acta 133: 412-423.

Briskey, E. J., K. Seraydarian, and W. F. H. M. Mommaerts. 1967b. The modification of actomyosin by α-actinin. III. The interaction between α-actinin and actin. Biochim. Biophys. Acta 133: 424-434.

Chaudhry, H. M., F. C. Parrish, and D. E. Goll. ca. 1969. Molecular properties of post-mortem muscle. 6. Effect of temperature on protein solubility of rabbit and bovine muscle. (To be published in J. Food Science.)

Corsi, A., U. Muscatello, and I. Ronshetti. 1967. Electron microscope observations of the location of actin and tropomyosin in the rabbit myofibril. J. Ultrastructure Research 19: 260-272.

Corsi, A. and S. V. Perry. 1958. Some observations on the localization of myosin, actin and tropomyosin in the rabbit myofibril. Biochem. J. 68: 12-17.

Dainty, M., A. Kleinzeller, A. S. C. Lawrence, M. Miall, D. M. Needham, and S. C. Shen. 1944. Studies on the anomalous viscosity and flow birefringence of protein solutions. III. Changes in these properties of myosin solutions in relation to adenosine-triphosphate and muscular contraction. J. Gen. Physiol. 27: 355-398.

Danilewsky, A. 1881. Myosin and siene. Darstellung, Eigenschaften, etc. Z. Physiol. Chem. 5: 158-165.

Ebashi, S. and F. Ebashi. 1965. α-actinin: a new structural protein from striated muscle. J. Biochem. 58: 7-12.

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

Edsall, J. T. 1930. Studies in the physical chemistry of muscle globulin. II. On some physico-chemical properties of muscle globulin (myosin). J. Biol. Chem. 89: 289-313.

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

Gellert, M. F., P. H. von Hippel, H. K. Schachman, and M. F. Morales. 1959. Studies on the contractile proteins of muscle. 1. The ATP-myosin B interaction. Journal American Chemical Society 81: 1384-1392.

Gergely, J. 1956. The interaction between actomyosin and adenosinetriphosphate. Light scattering studies. J. Biol. Chem. 220: 917-926.

Gergely, J. and H. Kohler. 1958. Light scattering studies on the stepwise formation and dissociation of actomyosin. In The Committee of Muscle Chemistry of Japan, eds. Conference on the chemistry of muscular contraction. Pp. 14-21. Tokoyo Igaku Shoin Ltd., Osaka.

Goll, D. E., W. F. H. M. Mommaerts, M. Reedy, and K. Saraydarian. ca. 1969. Studies on α-actinin-like proteins liberated during trypsin digestion of α-actinin of myofibrils. (To be published in Biochim. Biophys. Acta).

Gornall, A. G., C. T. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177: 751-766.

Granicher, D. and H. Portzehl. 1964. The influence of magnesium and calcium pyrophosphate chelates, of free magnesium ions, free calcium ions, and free pyrophosphate ions of the dissociation of actomyosin in solution. Biochim. et Biophys. Acta 86: 567-578.

Haga, T., K. Maruyama, and H. Noda. 1965. Formation of actomyosin during the extraction of muscle mince with weber-edsall solution. Biochim. et Biophys. Acta 94: 226-237.

Haga, T., K. Maruyama, and H. Noda. 1966a. The particle length of myosin B in solution. Biochim. Biophys. Acta 120: 459-461.

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

Halliburton, W. D. 1887. On muscle-plasma. J. Physiol. 8: 133-202.

Hanson, J. and H. E. Huxley. 1953. Structural basis of the cross-striations in muscle. Nature 172: 530-532.

Hanson, J. and H. E. Huxley. 1955. The structural basis of contraction in striated muscle. Symp. Soc. Exptl. Biol. 9: 228-264.

Hasselbach, W. and G. Schneider. 1951. Der L-myosin-und actingehalt des kaninchenmuskels. Biochem. Z. 321: 462-475.

Helander, E. 1957. On quantitative muscle protein determination. Acta Physiologica Scandinavica 41: 1-99.

Henderson, D. W. 1968. Effect of temperature on post-mortem structural changes in rabbit, bovine and porcine skeletal muscle. Unpublished Ph.D. thesis. Library, Iowa State University of Science and Technology, Ames, Iowa.

Huxley, H. E. 1963. Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle. J. Molecular Biol. 7: 281-308.

Ishiyama, I. 1960. Effects of various inorganic cations on the catalytic action of molybdate on ATP hydrolysis. Deut. Z. Ges. Gerichtl. Med. 50: 583-585.

Kaminer, B., and A. L. Bell. 1966. Myosin filamentogenesis: Effects of pH and ionic concentration. J. Mol. Biol. 20: 391-401.

Kuhne, W. 1859. Untersuchungen uber Bewegungen und veranderungen der contractilen Substanzen. Arch. Anat. Physiol.: 748-759.

Maruyama, K. 1966. Some physico-chemical properties of potassium-chloride-extracted F-actin from myofibrils. Biochem. Z. 345: 108-121.

Maruyama, K. and J. Gergely. 1962. Interaction of actomyosin with adenosinetriphosphate at low ionic strength. I. Dissociation of actomyosin during the clear phase. J. Biol. Chem. 237: 1095-1099.

Masaki, T., M. Endo, and S. Ebashi. 1967. Localization of 65 components of α-actinin at Z-band. J. Biochem. 62: 630-632.

Mihalyi, E. and A. J. Rowe. 1966. Studies on the extraction of actomyosin from rabbit muscle. Biochem. Z. 345: 267-285.

Mommaerts, W. F. H. M. 1942. Quantitative studies on some effects of adenyltriphosphate on myosin B. Studies Inst. Med. Chem. Univ. Szeged 1: 37-42.

Mommaerts, W. F. H. M. 1948. The reaction between actomyosin and adenosin-triphosphate. J. Gen. Physiol. 31: 361-375.

Mommaerts, W. F. H. M. 1950. A consideration of experimental facts pertaining to primary reaction in muscular activity. Biochim. et Biophys. Acta 4: 50-57.

Noda, H. and K. Maruyama. 1959. Flow birefringence properties of various myosin B preparations. Biochim. et Biophys. Acta 32: 263-264.

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

Perry, S. V. 1955. Myosin adenosinetriphosphase. In E. P. Colowick and N. O. Kaplan, eds. Methods in enzymology. Vol. 2. Pp. 582-588. Academic Press, New York, New York.

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

Perry, S. V., V. Davies, and D. Hayter. 1966. Natural tropomyosin and the factor sensitizing actomyosin adenosine-triphosphatase to ethylenedioxybis (ethyleneamine) tetra acetic acid. Biochem. J. 99: 1c-2c.

Portzehl, H., G. Schramm, and H. H. Weber. 1950. Aktomyosin und seine komponenten, I. Mitt. Z. Naturforsch. 5b: 61-74.

Robson, R. M., D. E. Goll, and M. J. Temple. 1968. Determination of protein in "Tris" buffer by the biuret reaction. Analytical Biochemistry 24: 339-341.

Schaub, M. C., D. J. Hartshorne, and S.V. Perry. 1967a. The adenosinetriphosphatase activity of desensitized actomyosin. Biochem. J. 104: 263-269.

Schaub, M. C., S. V. Perry, and D. J. Hartshorne. 1967b. The effect of tropomyosin on the adenosinetriphosphatase activity of desensitized actomyosin. Biochem. J. 105: 1235-1243.

Schramm, G. and H. H. Weber. 1942. Über monodisperse myosinlösungen. Kolloid. Z. 100: 242-247.

Seraydarian, A., E. J. Briskey, and W. F. H. M. Mommaerts. 1967. The modification of actomyosin by α-actinin. 1. A survey of experimental conditions. Biochim. Biophys. Acta 133: 399-411.

Straub, F. B. 1942. Actin. Studies Inst. Med. Chem. Univ. Szeged 2: 3-15.

Straub, F. B. 1943. Actin II. Studies Inst. Med. Chem. Univ. Szeged 3: 23-37.

Stromer, M. H., D. E. Goll, and L. E. Roth. 1967. Morphology of rigor-shortened bovine muscle and the effect of trypsin on pre- and post-rigor myofibrils. J. Cell Biol. 34: 431-445.

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

Szent Gyorgyi, A. G. and H. Holtzer. 1960. Fixation of muscle proteins with antibodies. Biochim. Biophys. Acta 41: 14-19.

Taussky, H. H. and E. Shorr. 1953. A microcolorimetric method for the determination of inorganic phosphorus. J. Biol. Chem. 202: 675-685.

Tonomura, Y. and F. Morita. 1959. The binding of pyrophosphate to myosin A and myosin B. J. Biochem. 46: 1367-1378.

Tonomura, Y. and J. Yoshimura. 1960. Inhibition of myosin B-adenosinetriphosphatase by excess substrate. Arch. Biochem. Biophys. 90: 73-81.

Von Furth, O. 1895. Ueber du eiweisskorper des muskelplasmas. Arch. Exptl. Path. Pharmakol. 36: 231-237.

Von Hippel, P. H., M. F. Gellert, and M. F. Morales. 1958. Some physical enzymatic phenomena in solutions of myosin B. In The Committee of Muscle Chemistry of Japan, eds. Conference on the chemistry of muscle contraction. Pp. 1-13. Tokyo Igaku Shoin Ltd., Osaka.

Von Hippel, P. H., M. F. Gellert, and M. F. Morales. 1959. Studies on the contractile proteins of muscle. II. Polymerization reactions in the myosin B system. Journal American Chemical Society 81: 1393-1400.

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