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THE ISOLATION AND PHYSICOCHEMICAL CHARACTERIZATION OF COMPONENTS OF THE TROPONIN SYSTEM WITH SPECIAL REFERENCE TO TROPONIN A

by

AUSTIN CARLOS MURRAY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "THE ISOLATION AND PHYSICOCHEMICAL CHARACTERIZATION OF COMPONENTS OF THE TROPONIN SYSTEM WITH SPECIAL REFERENCE TO TROPONIN A" submitted by AUSTIN CARLOS MURRAY in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The troponin system, which binds Ca^{2+} and in the presence of tropomyosin acts to make the ATPase activity of actomyosin dependent upon the level of Ca^{2+}, has recently been shown to consist of 3 or 4 different proteins. One of these proteins, inhibitory factor, inhibits the ATPase activity of actomyosin in the presence or in the absence of Ca^{2+}. Another component, troponin A, overcomes the effect of inhibitory factor upon the binding of Ca^{2+}, and a third factor appears to be involved in the binding of troponin to tropomyosin.

This investigation has shown that troponin isolated by the recognized procedures consists of three major protein species of molecular weights 40,000, 25,000 and 22,000. Attempts to separate these proteins by gel filtration and by ion exchange chromatography in benign media, although not entirely successful, gave information as to the preferred interactions among these proteins. Two complexes were isolated, the first of which contained species of molecular weights 40,000, 25,000 and 22,000, and the second of which contained species of molecular weights 25,000 and 22,000. These complexes were biologically active by the superprecipitation technique. Both were characterized with respect to their sedimentation equilibrium molecular weights, and their absorption and circular dichroism.

By measuring the biological activity of various isolated protein fractions and correlating this with the per cent compositions of the protein species of molecular weights 40,000, 25,000, and 22,000, the essential inhibitory factor of the troponin system was pinpointed as the component of molecular weight 25,000.

Troponin A, the component of molecular weight 22,000, was prepared from troponin by two different approaches, the first involving a high ionic strength, low pH precipitation, and the second involving DEAE-Sephadex column chromatography in 6 M urea. Pure troponin A was obtained by the latter method and was characterized with respect to sedimentation properties, sedimentation equilibrium molecular weight, viscosity, amino acid content, UV absorption, and circular dichroism. Since this protein was thought to bind Ca^{2+}, the physicochemical characterization was carried out in the presence and absence of Ca^{2+}. The occurrence of a conformational change in troponin A upon interaction with Ca^{2+} was reflected by changes in sedimentation rate, intrinsic viscosity and circular dichroism. Upon increasing the free Ca^{2+} level of troponin A solutions from $\sim 10^{-8}$ M to $\sim 10^{-5}$ M, troponin A assumed a more compact shape and underwent a large increase in apparent α-helical content with very little change in molecular weight. The possibility that this conformational change is important in the regulation of the contractile process has been suggested.

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GLOSSARY

myofibril	-	a fibrillar substructure of a muscle fiber, containing the contractile proteins
I band	-	the portion of the myofibrillar actin filaments not overlapping the myosin filaments (see figure 1)
I filament	-	actin filament (see figure 1)
Z line	-	a myofibrillar structure to which the I filaments are attached (see figure 1)
actomyosin	-	a complex of actin and myosin
natural actomyosin	-	a native complex of actin and myosin containing also tropomyosin and troponin
synthetic actomyosin	-	actomyosin prepared by the combination of actin and myosin
desensitized actomyosin	-	actomyosin resulting after treatment of natural actomyosin to remove or destroy troponin and tropomyosin
heavy meromyosin	-	the ATPase -active fragment produced by trypsin treatment of myosin

GLOSSARY (cont'd.)

relaxing protein native tropomyosin EGTA-sensitizing factor metin	-	a complex of tropomyosin and troponin
troponin	-	a system of proteins which in combination with tropomyosin causes the ATPase activity of actomyosin to become sensitive to the presence of Ca^{2+}
troponin B	-	a mixture of proteins which inhibit the ATPase activity of synthetic or desensitized actomyosin
inhibitory factor	-	the protein of the troponin system which inhibits the ATPase activity of synthetic or desensitized actomyosin
troponin A Ca^{2+}-sensitizing protein	-	the protein of the troponin system which binds Ca^{2+} strongly and makes the influence of inhibitory factor sensitive to the presence of Ca^{2+}
troponin A (H&M)	-	troponin A prepared by three times repetition of the Hartshorne-Mueller high ionic strength, low pH precipitation

GLOSSARY (cont'd.)

troponin A (urea)	-	troponin A prepared by DEAE-Sephadex column chromatography in 6 M urea as described in the "Results" section of this thesis

ABBREVIATIONS

ATP	adenosine triphosphate
CD	circular dichroism
CM	carboxymethyl
DEAE	diethylaminoethyl
dn/dc	specific refractive increment
DTT	dithiothreitol
$E^{1\%}_{1\ cm}$	absorbance of a 1% solution in a 1 cm light path
EDTA	ethylenediaminetetraacetate
EGTA	ethyleneglycol bis (β-aminoethylether)-N,N'-tetraacetate
ln y	natural logarithm of protein concentration in fringe displacement units
MW	molecular weight
nm	nanometer
OD	optical density
p[Ca]	negative logarithm of the calcium concentration
QAE	quaternary aminoethyl
$S_{20,w}$	sedimentation coefficient corrected to water at 20°C
$S^{o}_{20,w}$	intrinsic sedimentation coefficient
SDS	sodium dodecyl sulfate
SE	sulfoethyl
tris	tris-(hydroxymethyl)aminomethane
UV	ultraviolet
$\bar{v}$	partial specific volume
η_o	solvent viscosity
$[\eta]$	intrinsic viscosity

ABBREVIATIONS (Cont'd.)

η_{sp}	specific viscosity
$[\theta]_{\lambda}$	mean residue ellipticity at a wavelength λ
λ	wavelength in nanometers
μl	microliter
ρ	solvent density
β	Scheraga-Mandelkern function

I. INTRODUCTION

A. Historical Background

In view of the fact that troponin was discovered only seven years ago, an enormous flurry of activity still exists in this particular field. To the best of my knowledge no extensive review on the subject has appeared. For this reason a historical background will be included in this thesis. The review of Huxley (1969) may prove useful in illustrating the structural relationship between actin and myosin. Additionally, figure 1 is presented to familiarize the reader with muscle structure.

As recently as twenty years ago, the major contractile proteins of muscle were considered to be myosin and actin. Tropomyosin was known to be present in smaller amounts. Muscular activity was believed to be facilitated through the breakdown of ATP by actomyosin. However, very little information existed as to what factors initiated contraction and/or relaxation. Heilbrun and Wiercinski (1947) had shown that Ca^{2+} was the only one of several physiological substances, which when injected into muscle, caused localized contraction. It thus appeared that Ca^{2+} was in some way involved.

Marsh (1951, 1952) was among the first to recognize the existence of a physiological relaxing factor in muscle. This labile factor affected the volume response of muscle fibres to ATP. It caused lengthening of the muscle fibre in the presence of ATP and at the same time suppressed ATPase activity. However Ca^{2+} overcame the effects of this relaxing factor which was later shown by Bendall (1954) to be myokinase. Goodall and Szent-Györgyi (1953) found relaxation at

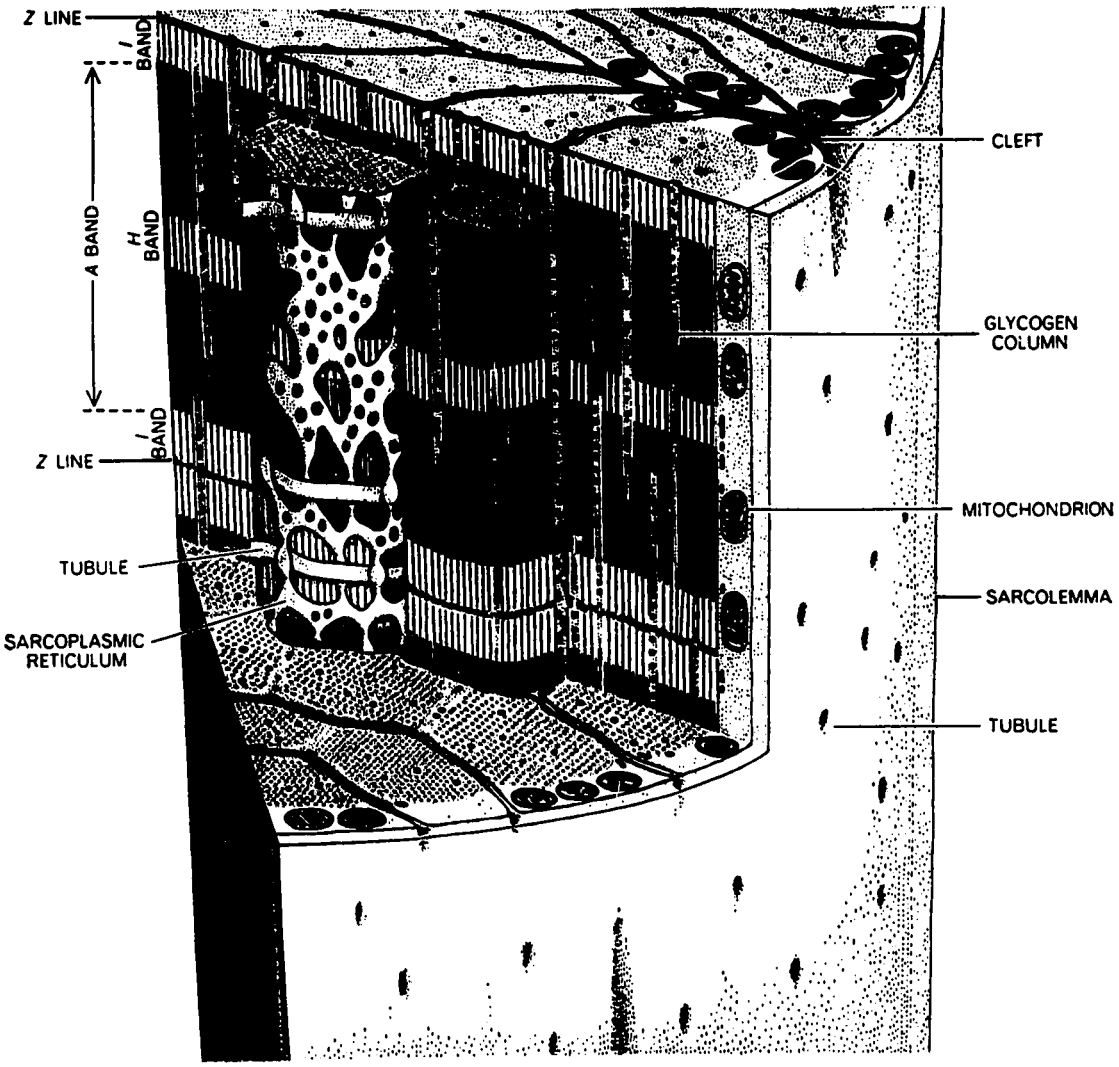


Figure 1. Structure of a typical voluntary muscle fibre. [From Hoyle (1970)]

high ATP levels which they attributed to a water-soluble protein which was in some way associated with creatine phosphate. This relaxation, which was inhibited by Ca^{2+}, was found by Lorand (1953) to be caused by ATP-creatine transphosphorylase. Ebashi (1958, 1961) isolated a protein relaxing factor from sarcoplasmic reticulum which bound Ca^{2+} and the binding depended upon the presence of ATP.

Factors other than protein, when added to muscle also caused relaxation. For example, high concentrations of ATP effected relaxation (Szent-Györgyi, 1951). Bolzer (1954), Watanabe (1955), and Perry and Grey (1956) showed that the addition of EDTA to muscle caused relaxation. Perry and Grey (1956) found that the relaxing effect of EDTA was due to inhibition of ATPase activity. Weber (1959) suggested that by binding Ca^{2+}, ATP and EDTA reduce the Ca^{2+} concentration to a level which is too low for the occurrence of the ATPase activity of actomyosin. Ebashi (1960) observed a correlation between the extent of relaxation by EDTA and several of its analogues and the amount of Ca^{2+} bound by these compounds. EGTA caused the most relaxation and also bound the most Ca^{2+}.

Contraction was known to be closely connected with the ATPase activity of actomyosin (Szent-Györgyi, 1947). Ebashi (1961) was able to simulate muscle contraction by the superprecipitation technique. Superprecipitation had earlier been defined by Szent-Györgyi (1947) as a change of actomyosin from a loose flocculent suspension into a granular precipitate of small volume, suggestive of extreme shrinkage and dehydration of the particles. Ebashi visualized superprecipitation as an increase in turbidity (optical density at 660 nm) of actomyosin

suspensions upon the addition of proper concentrations of ATP. EGTA caused relaxation of such systems, that is, it delayed the onset of turbidity. The superprecipitation technique has been described in detail (Maruyama and Gergely, 1962, 1962a, Levy and Fleisher, 1965, 1965a).

Perry and Grey (1956) had shown that the ATPase activity of natural actomyosin was affected by EDTA while that of synthetic actomyosin was not. Protein ('extra protein') other than myosin and actin had been extracted from myofibrils (Perry, 1953, Perry and Corsi, 1958, Perry and Zydowo, 1959) and was found to contain several different proteins, the significance of which was not obvious at the time.

In 1963 Ebashi isolated the protein component which made synthetic actomyosin sensitive to the presence of EGTA by low ionic strength extraction of myofibrils. This protein fraction was similar to the tropomyosin isolated by Bailey (1948), except for its effects on the superprecipitation of actomyosin (Ebashi, 1963, Ebashi and Ebashi, 1964), and it was termed native tropomyosin. The superprecipitation of synthetic actomyosin proceeded the same in the presence and absence of Ca^{2+} (Ebashi and Ebashi, 1964). However, when native tropomyosin was added to the system, contraction of the gel occurred in the presence of Ca^{2+}, while a delay in superprecipitation (relaxation) was observed in the absence of Ca^{2+}. Ebashi and Kodama (1965) found that their native tropomyosin (relaxing protein) could be separated into tropomyosin and a protein factor which was very different from tropomyosin. This protein factor was called troponin. Viscosity studies indicated that troponin interacted with and promoted the aggregation of tropomyosin.

At about the time of Ebashi's discovery, Szent-Györgyi and Kaminer (1963) reasoned that if Ca^{2+} had an affinity for a protein of the actomyosin system, negatively charged groups (sulfate, phosphate, carboxyl) were probably involved. Macromolecules exhibiting such a charge might display metachromasia, the ability to cause spectral shifts in certain dyes. A protein substance extracted from actomyosin showed this property and was termed 'metin'. No biological function was assigned to metin. Later, Azuma and Watanabe (1965, 1965a) separated metin into two components. The major component was tropomyosin, and the minor one acted like Ebashi's troponin.

Since 1965 a number of methods have been suggested for the preparation of native tropomyosin (relaxing protein, tropomyosin-troponin complex, EGTA-sensitizing factor) and troponin. Watanabe and Staprans (1966) further purified the relaxing protein of Ebashi and Ebashi (1964) by Sephadex G-200 column chromatography. The pure relaxing protein eluted in the void volume. Katz (1966) isolated relaxing protein from actin which had been prepared from an acetone-dried muscle powder. The 'extra protein' of Perry and Corsi (1958), prepared by low ionic strength extraction of myofibrils, sensitized the ATPase activity of actomyosin to Ca^{2+} (Perry et al., 1966). Hartshorne and Mueller (1967) extracted EGTA-sensitizing factor from natural actomyosin by low ionic strength extraction. They found that a troponin-rich fraction precipitated at 40-53% saturation with ammonium sulfate and a tropomyosin-rich fraction resulted at 53-60% saturation. Troponin could be further purified by precipitation of

contaminating tropomyosin at pH 4.6. Maximum EGTA-sensitizing activity occurred at a ratio of tropomyosin to troponin of 1:1.3 w/w. At this same ratio a single peak was observed in the ultracentrifuge. Yasui et al. (1968) prepared troponin and tropomyosin by high ionic strength extraction of an ethanol-ether muscle powder. Troponin was specifically precipitated at pH 4 at low ionic strength. Optimal sensitization of actomyosin was obtained at ratios of troponin to tropomyosin of greater than unity. Parker and Kilbert (1970) obtained troponin by extraction of myofibrils with mersalyl. This implied that sulfhydryl groups were involved in the binding of troponin to the myofibril. Fukazawa et al. (1970) isolated relaxing protein by a short term, low temperature, mild extraction of I-Z-I brushes (structure consisting of intact Z lines to which actin filaments are still attached). Because the procedure was more gentle than any previously applied one, perhaps it resulted in a complex more truly native. This complex was shown to be an extremely tight one and was capable of delaying the onset of superprecipitation of actomyosin for very long periods of time.

Soon after the discovery of troponin, Ebashi's group demonstrated the myofibrillar location of troponin and tropomyosin. Mueller (1965) had shown that no EGTA-sensitizing activity existed in muscle except in the myofibrils. Other workers have indicated that tropomyosin binds preferentially to F-actin (Laki et al., 1962, Martonosi, 1962) as does native tropomyosin (Maruyama et al., 1964). Endo et al. (1966) employed both a fluorescent protein technique and a fluorescent antibody technique in an attempt to locate native tropomyosin. After trypsin treatment of myofibrils, they were mixed

with native tropomyosin which had been labelled with a fluorescent dye, fluoresceinisothiocyanate (FITC). Strong fluoresence of the I band resulted. After a brief trypsin treatment of natural actomyosin, FITC-troponin, but not FITC-tropomyosin labelled the I band. After a longer trypsin treatment FITC-tropomyosin but not FITC-troponin labelled the I band. These results suggested that native tropomyosin is located along the I band. Ohtsuki et al. (1967), using immuno-electron microscopy, noted a periodic staining along the whole I filament with ferritin-labelled anti-troponin. Twenty-four periods of 400 Å each were seen on each side of the Z line. Troponin, and most likely tropomyosin, are located along the I filament at 400 Å intervals.

Ebashi et al. (1967) found that troponin bound 5 moles Ca^{2+} per 100,000 g protein with a binding constant of 6×10^5 M^{-1}. They suggested that the binding and detachment of Ca^{2+} to and from troponin might be important in the regulation of contraction. Fuchs and Briggs (1968) and Yasui et al. (1968) noted that 2.4 moles Ca^{2+} was bound per 100,000 g protein with a binding constant of 2.4×10^6 M^{-1}. The number of binding sites almost doubled by reaction of troponin with sulfhydryl reagents. On the other hand, sulfhydryl blocking reagents and DTT did not interfere with Ca^{2+} binding (Ebashi et al., 1968).

Another major breakthrough occurred when Hartshorne and Mueller (1968) showed that troponin can be separated into two different proteins, troponin A and troponin B. This was accomplished by dissolving troponin in 1.2 M KCl and making the solution 0.1 M HCl. The precipitate was troponin A. The supernatant was neutralized and

dialyzed at low ionic strength to precipitate out troponin B. Troponin B inhibited the ATPase activity of synthetic actomyosin. Tropomyosin enhanced this inhibition. Troponin A relieved the inhibition due to troponin B in the presence, but not in the absence of Ca^{2+}. Troponin, then, can be divided into two components with respect to function: one activity is inhibitory (troponin B), and a second one is Ca^{2+}-sensitizing (troponin A). Schaub and Perry (1969) obtained a similar separation by a method involving SE-Sephadex column chromatography in 6 M urea.

Hartshorne et al. (1969) and Hartshorne and Pyun (1971) characterized troponin A and troponin B with respect to sedimentation properties and optical properties. From SDS polyacrylamide gels, troponin A was shown to have a molecular weight of 18,500, and troponin B was made up of two major bands corresponding to molecular weights of 39,000 and 26,000. Troponin A bound 8 moles Ca^{2+} per 10^5 g protein with a binding constant of 5×10^6 M^{-1}. Troponin B bound little Ca^{2+} but when mixed with tropomyosin a complex sedimenting faster than either of the constituents resulted, indicating that troponin B strongly interacts with tropomyosin. Troponin A was also isolated by Greaser and Gergely (1970) by DEAE-Sephadex column chromatography of troponin in 6 M urea. From high speed sedimentation equilibrium studies the molecular weight was found to be 22,000. Maximally 4.5 moles Ca^{2+} was bound per 10^5 g protein with a binding constant of $7\text{-}12 \times 10^5$ M^{-1}. The Ca^{2+} binding of troponin A most likely involves carboxyl groups. It is noteworthy that Nockolds et al. (1972), upon determining the structure of a Ca^{2+}-binding carp myogen by X-ray crystallography, found that the bound Ca^{2+} was coordinated with one

glutamic and three aspartic acid carboxyl groups in a tetrahedral arrangement.

Drabikowski et al. (1971) separated troponin into 4 different components by chromatography on DEAE-Sephadex A-50. Peak I protein strongly inhibited the ATPase activity of actomyosin whether or not EGTA and tropomyosin were present. Peak IV protein had no activity by itself but it abolished the inhibitory effect of peak I protein in the absence of EGTA and tropomyosin. In this way peak IV protein behaved like troponin A. The precise role of the other two components was not made obvious.

Ebashi et al. (1971) improved his previous method for extracting troponin (Ebashi and Ebashi, 1964; Ebashi and Kodama, 1965) by making the procedure much simpler and more rapid. Troponin gave 3 major bands on SDS-polyacrylamide gels. These bands corresponded to molecular weights of 40,000, 22,000, and 17,000. The first two components were isolated by SE-Sephadex column chromatography in 6 M urea. These two components together were shown to have the characteristic function of troponin. Ebashi designated these components troponin I (MW = 40,000) and troponin II (MW = 22,000). Troponin II bound 8-9 moles Ca^{2+} per 10^5 g protein while troponin I bound essentially none. The third fraction from the SE-Sephadex chromatography had a molecular weight of 17,000 and appeared to correspond to troponin A (Hartshorne and Mueller, 1968).

Greaser and Gergely (1971) separated purified troponin into 4 major protein fractions by DEAE-Sephadex A-50 column chromatography in 6 M urea. The molecular weights of these fractions, as determined from SDS-polyacrylamide gels, were 14,000, 24,000, 35,000, and 21,000, in order of elution from the column. Reconstitution experiments indicated that the last three fractions were required to make the ATPase activity of actomyosin sensitive to Ca^{2+}. Activity could only be regained when these fractions were mixed prior to the removal of urea. This suggests that the correct refolding of one or more protein(s) may require the presence of the other components.

Schaub and Perry (1971) isolated the major component of inhibitory factor preparations by SE-Sephadex chromatography in urea. By gel filtration in guanidine-HCl (Fish et al., 1969), the molecular weight of this component was found to be 23,000. This protein inhibited the ATPase activity of myofibrils, natural actomyosin, and desensitized actomyosin. By increasing the amounts of actin, but not myosin or tropomyosin, the inhibitory activity was neutralized. Wilkinson et al. (1972) showed that inhibitory factor preparations contained proteins of molecular weights 37,000 and 23,000 and often one of molecular weight 14,000, depending on the method of preparation. The proteins of the inhibitory factor preparation were separated by elution from a CM-cellulose column with a gradient of decreasing ethanol concentration. The protein of molecular weight 23,000 was the major active fraction and its inhibitory activity was enhanced by tropomyosin. The component of 14,000 molecular weight possessed somewhat less inhibitory activity which was often not

enhanced by tropomyosin. This component could not be detected in myofibrils and Wilkinson et al. (1972) suggested that it might be formed during the preparation of troponin. The 37,000 molecular weight component appeared to serve no apparent function, although Ebashi et al. (1971) indicated that its role had to do with binding to tropomyosin.

Schaub et al. (1972) purified Ca^{2+}-sensitizing factor (troponin A) by successive chromatography on SE-Sephadex and on QAE-Sephadex in 6 M urea. This protein was demonstrated to be the only myofibrillar protein to retain Ca^{2+} even after gel filtration in the presence of 2-6 M urea, and it relieved the effect of inhibitory factor in the presence of Ca^{2+} and tropomyosin. Both tropomyosin and Ca^{2+}-sensitizing factor could be replaced by their carboxymethylated derivatives and the relaxing system was still effective.

In summary, on the basis of the above work, troponin appears to be composed of either 2 or 3 functional proteins. Two distinct functions have been associated with two of these proteins, that of inhibition of actomyosin ATPase activity, and that of making this inhibition sensitive to the presence of Ca^{2+}. Troponin exerts its effect in the presence of tropomyosin. A third component of troponin is believed involved in the binding of troponin to tropomyosin.

In view of the fact that limited physico-chemical characterization of the protein components has occurred, such information will be discussed only in light of the results presented later in this report.

B. Troponin as a Myofibrillar Protein

The model of Stewart and Levy (1970) has been chosen as a basis for the explanation of the contribution of troponin to the operation of the contractile apparatus. Troponin, which is an integral part of native tropomyosin or relaxing protein (Ebashi and Kodama, 1965), is bound to the F-actin filament at 400 Å intervals (Ohtsuki et al., 1967) probably through the tropomyosin moiety (Laki et al., 1962, Martonosi, 1962). Contraction depends upon the association of actin and myosin, and relaxation is caused by their dissociation (Szent-Györgyi, 1947). Ca^{2+} is implicated as the substance triggering contraction and its removal causes relaxation. Troponin binds Ca^{2+} more strongly than any other myofibrillar protein (Ebashi et al., 1968). Removal of troponin destroys any control Ca^{2+} has over the contractile system. In some way, the binding of Ca^{2+} to troponin leads to contraction.

Schliselfeld and Bárány (1968) showed that myosin and heavy meromyosin can bind two moles of ATP per protein molecule. Levy and Ryan (1965) suggested the existence of two ATP binding sites per myosin molecule - one hydrolytic site (responsible for breaking down ATP) and one inhibitory site (responsible for interactions of myosin and actin).

Stewart and Levy (1970) postulate that Ca^{2+} exerts its effect by changing the nature of the interaction between troponin and the inhibitory site of myosin. This is exemplified diagramatically in Figure 2. In the absence of Ca^{2+}, the binding of ATP to the inhibitory site of myosin probably causes repulsion of the negatively charged troponin of the actin complex. Stewart and Levy (1970) suggest

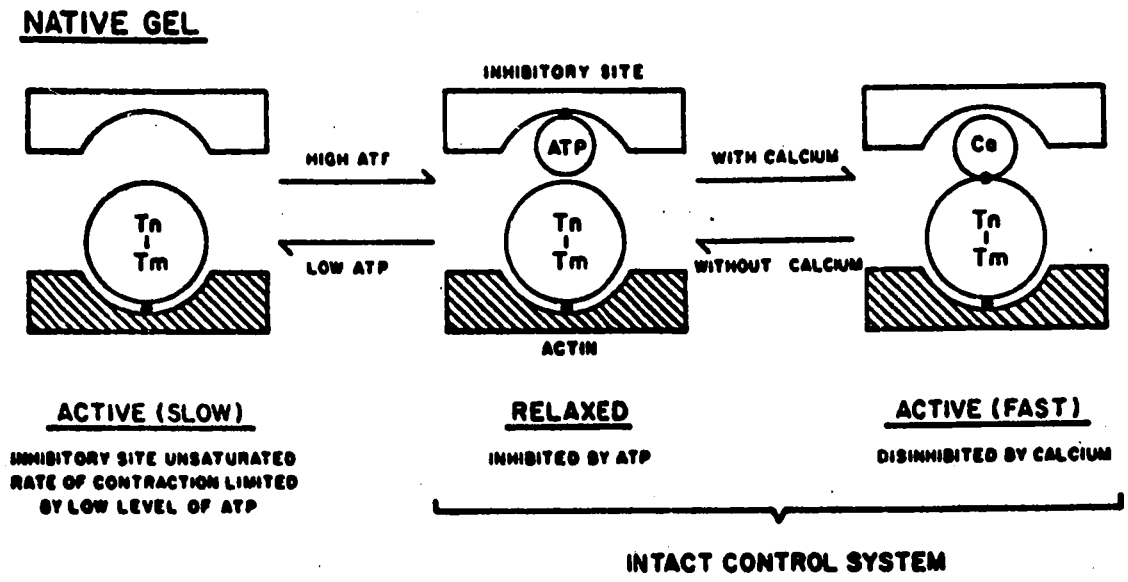


Figure 2. Model for the role of Ca^{2+} in the control of muscle contraction. Tn-Tm represents the troponin-tropomyosin complex. [From Stewart and Levy (1970)]

that upon binding of Ca^{2+} by troponin, the ATP at the inhibitory site of myosin is displaced. The hydrolytic site of myosin can interact with actin and ATPase activity and contraction can then proceed. The movement of Ca^{2+} to and from the myofibril has been described by Ebashi et al. (1969) and Hoyle (1970).

The role of troponin is grossly oversimplified here. In fact, the binding of Ca^{2+} appears to cause conformational changes in the troponin moiety (Han and Benson, 1970) or in the troponin-tropomyosin system (Tonomura et al., 1969). Ahmed et al. (1970) have shown that the addition of small amounts of Ca^{2+} to tropocalcin, a protein obtained from troponin by Ca^{2+} precipitation, causes considerable increases in apparent α-helical content. This aspect will be mentioned in greater detail later in this report.

It is hoped that the explanation of this model will function as a starting point for subsequent considerations of the role of troponin in the control of the contractile process.

C. Purpose of this Study

The present study was undertaken in 1969 with the intention of isolating troponin from rabbit skeletal muscle, physico-chemically characterizing it as well as its complex with tropomyosin, and studying details of the biological role of troponin and native tropomyosin with respect to interaction with actomyosin under near physiological conditions. However, as this rapidly developing field progressed, the objectives changed. After troponin itself was shown to be not a single protein, but rather a complex of several proteins, the separation and characterization of these components

were thought to be of first priority. Attempts at separation of the troponin led to isolation of various complexes which were characterized in part. Because the protein components of troponin were so tightly bound, harsher isolation procedures were obviously needed. Under denaturing conditions interactions between components were limited and separation was possible. Troponin A was the easiest component to isolate by this method so it became the principal subject of the investigation. The goal then has been to characterize troponin A physicochemically as well as biologically, in terms of its effect on superprecipitation in the presence and absence of Ca^{2+} and the other proteins of the relaxing system.

II. MATERIALS AND METHODS

A. Isolation of Troponin

The troponin used in this study was prepared from rabbit skeletal muscle by several different procedures.

The method of Yasui et al. (1968) is outlined as follows: Four hundred grams of minced rabbit skeletal muscle was mixed at 4°C with an equal volume of water and allowed to stand for 30 min. The juice was squeezed out through cheese cloth and an equal volume of ethanol was added to the residue. The liquor was again squeezed out and the residue was treated with 4 volumes ethanol-water (1:1). The residue was washed twice with 95% ethanol and twice with ether, and was then allowed to dry. This preparation of a muscle powder was in accordance with the methodology of Bailey (1948). The dried muscle powder was extracted with 7 volumes 1 M KCl, 0.5 mM DTT, pH 7 for 18 hr at room temperature. The muscle residue was removed by centrifugation and reextracted in the same manner for 4 hr. The pH of the combined supernatants at 4°C was lowered to 4.6 and the resulting precipitate was removed by centrifugation. The supernatant was taken to pH 7.0 and dialyzed overnight against two changes of 10 volumes of distilled water. The pH was lowered to 4.0 and the precipitate was saved and dissolved in water at pH 7.0. Ammonium sulfate was added to 40% saturation and any precipitate was discarded through centrifugation. Ammonium sulfate was added to 60% saturation. The resulting precipitate was centrifuged out and redissolved in water at pH 7.0. KCl was then added to a concentration of 1 M. The pH was lowered to 4.6 and any resulting precipitate was discarded. The supernatant (troponin) was taken to pH 7.0, dialyzed extensively versus

distilled water, and lyophilized. DTT (0.5 mM) was present in all solutions. Yields of troponin ranged from 50 to 100 mg per 100 g wet muscle.

The method of Ebashi et al. (1968) is described below. One hundred grams of minced rabbit skeletal muscle was extracted at 0°C with 300 ml 0.15 M potassium phosphate, pH 6.5, 0.3 M KCl (Guba-Straub solution) for 10 min. The residue remaining after squeezing the liquid off through cheese cloth was washed twice for 10 min each time with 300 ml 0.2 mM $NaHCO_3$, 0.02 M KCl and twice for 5 min each time with 300 ml water. The remaining residue was suspended in 100 ml water and left for 4 hr at room temperature. The supernatant was collected by centrifugation. For each 100 ml, 22.5 g solid ammonium sulfate was added at 0°C. The resulting precipitate was discarded and the supernatant was dialyzed against several changes of 0.2 mM $NaHCO_3$ overnight. The solution was centrifuged at 12,000 x g for 30 min and then dialyzed extensively against distilled water until no further precipitation occurred. The precipitate (troponin) was collected, dissolved in water at pH 7.0, dialyzed extensively versus water at pH 7.0 and then lyophilized.

Troponin was also prepared by the procedure of Hartshorne and Mueller (1969). Myofibrillar suspensions were initially prepared from minced muscle by the method of Perry and Zydowo (1959). Natural actomyosin, which is the actin-myosin complex to which is still attached tropomyosin and troponin, was extracted from the myofibrillar suspension with 0.01 M $NaCO_3$, 0.04 M $NaHCO_3$, 0.6 M KCl (Weber's solution) as was done by Perry and Corsi (1958). The actomyosin was precipitated by addition of 14 volumes of water at pH 7.0 and then

treated with the Bailey (1948) sequence of organic solvents. The dry natural actomyosin powder was extracted with 1 M KCl, 2 mM DTT, pH 7.0, for 18 hr and centrifuged at 20,000 x g for 3 hr. The supernatant was saved and dialyzed overnight against 4 volumes water at pH 7.0. Ammonium sulfate was added to 40% saturation and the precipitate was discarded by centrifugation. The supernatant was made 60% saturated in ammonium sulfate. The precipitate was saved through centrifugation and dissolved in 1 M KCl, 2 mM DTT. The pH was lowered to 3.5. Any precipitate was discarded. The pH was raised to 4.6 and any precipitate was discarded. The resulting supernatant (troponin) was dialyzed extensively versus distilled water and lyophilized. The yield of troponin was about 1.5 g per 1000 g wet muscle.

An additional preparative approach, based on the procedure of Yasui et al. (1968), combined features of the above methods. This variation included an 18 hr, room temperature extraction of an ethanol-ether muscle powder prepared as described above, with 7 volumes 50 mM tris, pH 7.6, 1 M KCl, 1 mM DTT. The muscle residue was centrifuged out at 2900 rpm, reextracted with 7 volumes of the same buffer for 4 hours, and recentrifuged. The supernatants were combined and cooled to $4^{o}C$, and the muscle residue was discarded. The pH of the combined supernatants was lowered to 4.6 and the resulting precipitate was discarded. The pH was then raised to 7 and the solution was dialyzed overnight versus 4 volumes distilled water. Any precipitate was discarded. Ammonium sulfate was added to the solution to 40% saturation and any resulting precipitate was removed by centrifugation. The ammonium sulfate concentration was subsequently increased to 70% saturation and the precipitate which resulted was saved by centrifugation

at 5700 rpm and dissolved in water. The resulting solution was made 1 M with respect to KCl and the pH was lowered to 4.6. Any precipitate was discarded. The pH was finally readjusted to 7 and the solution was dialyzed extensively against distilled water and lyophilized. Two to three grams of crude troponin resulted per 100 g muscle powder.

Because of an ether fire in the department, the decision was made to no longer prepare muscle powder with the Bailey (1948) sequence of organic solvents. Instead, the first part of the procedure was the similar to that of Ebashi et al. (1971). Wet minced muscle was extracted for 10 min with Guba-Straub solution, then washed in rapid succession three times with 3 volumes of 0.3 mM $NaHCO_3$, 0.05 M KCl and two times with 3 volumes of 0.3 mM $NaHCO_3$. Muscle residue and wash solution were separated by centrifugation. Two volumes of a solution of 75 mM tris, pH 7.6, 1.5 M KCl, 1 mM DTT was then added to the residue. After 12 hr extraction, a solid gel resulted which would not centrifuge out very easily. To overcome this problem, the pH was lowered to 4.8 and the resulting precipitate was centrifuged out at 2900 rpm. The supernatant was neutralized and treated as in the previous procedure beginning with the overnight dialysis against water. Resulting troponin yields were considerably lower than when a muscle powder was used.

All protein preparations were stored as lyophilized powder under vacuum at $4^{o}C$.

B. Biological Activity by the Superprecipitation Technique

The biological activity of troponin and its components was measured in terms of their effect upon the superprecipitation of actomyosin. A modification of the technique of Ebashi (1961) was used. Superprecipitation of actomyosin was manifested as an increase in turbidity as actomyosin changed from a loose flocculent precipitate into a granular precipitate of small volume. Under appropriate ionic conditions, addition of proper amounts of ATP to desensitized actomyosin (actomyosin from which all extra proteins had been removed) resulted in superprecipitation. The rate at which turbidity developed was independent of the amount of Ca^{2+} present. When troponin was added to the system, addition of the same amount of ATP caused a delay in the onset of turbidity in the absence of Ca^{2+} but had little effect on superprecipitation in the presence of Ca^{2+}.

Natural actomyosin was prepared following the procedure of Weber and Edsall (1954). The components of natural actomyosin most sensitive to trypsin attack are troponin and tropomyosin (Ebashi and Kodama, 1966), troponin more so than tropomyosin. Limited trypsin treatment selectively destroyed these components. Desensitized actomyosin was prepared by trypsin treatment (Ebashi and Ebashi, 1964) as follows. Five ml natural actomyosin (8-10 mg/ml) in 0.5 M KCl was added to 20 ml distilled water. One hundred µl of trypsin (1 mg/ml) was added with constant stirring, and the pH was maintained at 7.0. After 20 minutes the reaction was stopped by addition of 100 µl soya bean trypsin inhibitor (2 mg/ml).

The reaction mixture for the superprecipitation assay contained: 1 ml 60 mM imidazole pH 7.0, 4 mM $MgCl_2$, 1 ml desensitized actomyosin (no precaution was taken to remove trypsin and trypsin inhibitor), and 1 ml water. The $OD_{660\ nm}$ of the actomyosin was recorded with time on a Beckman DBG recording spectrophotometer for about 10 min. Then 20 µl 50 mM ATP (adjusted to pH 7.0 with NaOH) was added, and after rapid mixing, the optical density was recorded versus time. The amount of ATP added varied somewhat depending upon the actomyosin. The ATP level was usually adjusted to obtain maximum superprecipitation. The reaction was then repeated with the inclusion of 0.1 mM EGTA. If superprecipitation did not proceed similarly in the presence and absence of EGTA, this was taken as an indication that the actomyosin was not properly desensitized. An example of a typical superprecipitation experiment is shown (figure 3). Curves A and B represent reactions in the absence and presence of EGTA, respectively. The assay was then repeated in the presence of EGTA with the addition of 50 µg troponin per ml (curve C, figure 3). Active troponin clearly delayed the onset of turbidity.

C. Dye Binding studies

Spectra of toluidine blue in the presence or in the absence of troponin A were obtained using a Beckman DBG spectrophotometer with a recorder attachment. The solutions to be measured contained 200 µl of 2.4×10^{-4} M toluidine blue (Toluidine Blue O, Fisher Scientific) in 5 mM tris, pH 7.6 as well as varying amounts of a solution containing 1 mg troponin A per ml 5 mM tris, pH 7.6. The total solution volume was then made to 2.2 ml with 5 mM tris, pH 7.6. The change in the

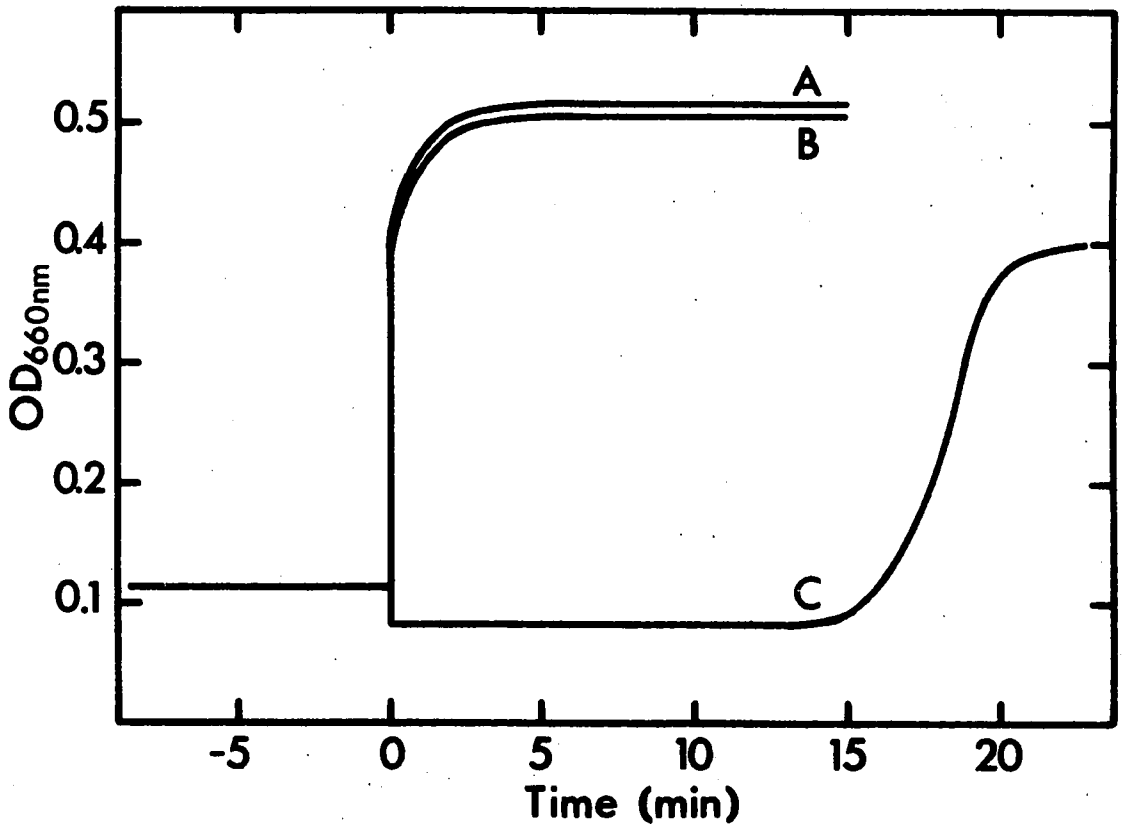


Figure 3. Illustration of the superprecipitation technique. Curves A and B represent the superprecipitation of desensitized actomyosin in the absence and in the presence of 0.1 mM EGTA, respectively. Curve C resulted when 50 µg troponin/ml was present in addition to 0.1 mM EGTA. ATP was added at time 0.

spectrum of toluidine blue with addition of troponin A was plotted as decrease in $OD_{634\ nm}$ versus troponin A concentration and the titrimetric endpoint was obtained by extending the linear limbs of the titration curve in a fashion similar to that of Stone and Bradley (1967).

D. Chromatographic Techniques

Gel filtration on Sephadex G-200 and ion exchange chromatography on QAE-Sephadex A-50 and on DEAE-Sephadex A-25 were conducted as recommended in Sephadex literature, with one exception. Chromatography in urea consumed large quantities of urea at considerable expense. This problem was overcome by equilibrating the DEAE-Sephadex A-25 and pouring the column in the buffer with urea omitted. Then about 5 column volumes of buffer with urea was passed through the column before the protein solution was applied.

All column chromatography was performed at 4°C. Constant flow rates were maintained by L.K.B. peristaltic pumps. Effluent was collected by a Gilson LB-1 fraction collector.

Solution conductivities were measured on a Radiometer type CDM 2d conductivity meter.

More specific details of chromatographic technique are presented in the 'Results' section.

E. SDS-polyacrylamide Gel Electrophoresis

For SDS-polyacrylamide gels, methodology similar to that of Shapiro *et al.* (1967) was employed. Protein samples were heated at 80°C for 10 min in 1% SDS, 1 mM DTT, electrophoresed on 5 or 10%

polyacrylamide gels, stained with Coomassie Blue for 3-5 hours, and destained with a Canalco horizontal destainer. Five percent gels were electrophoresed for 2-1/4 hrs at 5 mA/tube, 10% gels for 3-1/4 hrs at 6-1/2 mA/tube. Gels were scanned in a Gilford 240 spectrophotometer at 550 nm. Relative quantities of each component present were determined by measuring planimetrically the area under each peak after scanning, or by cutting out and weighing the peaks.

Protein molecular weights were determined from SDS-polyacrylamide gels by comparing their migration distances with those of proteins of known molecular weights.

F. Determination of Protein Concentrations and Extinction Coefficients

The concentrations of protein solutions were measured by Beckman Model E ultracentrifuge in double sector cells by synthetic boundary runs with interference optics. The concentration in each case was determined by measuring the number of fringes crossed upon moving the cross hairs of a Nikon microcomparitor exactly horizontally across an interference pattern on a photographic plate, and applying the following expression (Richards et al., 1968):

$$J = \frac{a\, c\, \frac{dn}{dc}}{\lambda} \qquad (1)$$

where J is the number of fringes;

a is the cell thickness in the direction of the optical path (12 mm);

c is the solute concentration in mg/ml;

λ is the wavelength of the light used (5461 Å);

$\frac{dn}{dc}$ is the specific refractive increment of the solute (a value of 0.185 ml/g was used).

The method of Lowry et al. (1951) was also used occasionally to determine protein concentrations.

Absorption spectra were measured on a Cary 15 spectrophotometer or on a Beckman DBG recording spectrophotometer. Routine optical density readings were obtained from a Gilford 240 spectrophotometer. Extinction coefficients were calculated by combination of absorption and concentration data.

G. Sedimentation Velocity

Sedimentation studies were performed on a Beckman Model E ultracentrifuge in either 12 mm 2^{o} Kel F cells or 12 mm 4^{o} synthetic boundary cells at 60,000 rpm. Temperatures were maintained near 20^{o}C. The Schlieren optical tract was used except in the case of low concentrations of troponin A (H&M) at which time the UV optics were employed. Photographs were taken at 8, 16, or 32 min intervals (depending upon rates of sedimentation and diffusion) on Kodak metallographic plates. Distances from the maximum ordinate to the reference hole were measured from a photographic plate with a Nikon model 6C microcomparitor and the true distance from the center of rotation to the center of the protein boundary was calculated.

The sedimentation coefficient, S (in sec), can be related to the distance, x (in cm), from the center of rotation to the maximum ordinate at time t (in sec), and the angular velocity, ω (in radians/sec), by the following expression:

$$S = \frac{1}{\omega^2} \times \frac{d \ln x}{dt} \qquad (2)$$

S values were evaluated by plotting log x versus time (in min), and multiplying the slope by:

$$\frac{2.303}{60\ \omega^2}$$

To express the sedimentation coefficient in terms of water at 20°C, the following equation (Schachman, 1959) was used:

$$S_{20,w} = S \times \left(\frac{\eta_T}{\eta_{20}}\right)_w \times \left(\frac{\eta}{\eta_o}\right)_T \times \frac{1 - \bar{v}\rho_{20,w}}{1 - \bar{v}\rho_T} \qquad (3)$$

where $\left(\frac{\eta_T}{\eta_{20}}\right)_w$ is the ratio of the viscosity of water at the experimental temperature, T, to that at 20°C;

$\left(\frac{\eta}{\eta_o}\right)_T$ is the viscosity of the solvent relative to that of water at temperature, T;

$\rho_{20,w}$ is the density of water at 20°C;

ρ_T is the density of solvent at T°;

$\bar{v}$ is the partial specific volume of the solute.

The value of $\bar{v}$ for troponin A calculated from amino acid analysis (this study) was 0.73 ml/g. This value was used in all calculations. Density and viscosity values were taken from International Critical Tables or from the Handbook of Chemistry and Physics (1970-71).

H. Sedimentation Equilibrium Molecular Weight Determinations

Sedimentation equilibrium runs were carried out on a Beckman Model E ultracentrifuge equipped with Rayleigh interference optics. A 12 mm double sector cell was used with charcoal-filled Epon centerpiece and with sapphire windows. Interference patterns were recorded on Kodak type IIG spectroscopic plates. Solution column lengths were approximately 3 mm in all cases. All experiments were performed near 20°C. Runs were assumed to be at equilibrium when no further changes in fringe positions occurred.

Minimum molecular weights were determined by the high speed, meniscus depletion approach of Yphantis (1964). This technique makes use of the fact that if the protein concentration at the meniscus approaches zero (as it does if the speed is high enough), then the vertical displacement of an interference fringe is proportional to the concentration of protein present. The following relationship allows calculation of the molecular weight of the lowest molecular weight species present:

$$M_w = \frac{2\ RT}{(1 - \bar{v}\rho)\omega^2} \times \frac{d \ln c}{dr^2} \qquad (4)$$

where M_w is the weight average molecular weight;

R is the gas constant;

T is the experimental temperature;

c is the protein concentration at a distance, r, from the axis of rotation;

$\bar{v}$ is the partial specific volume of the protein;

ρ is the solvent density.

Vertical fringe displacement (y) was measured at a number of r values. The minimum slope of a plot of ln y versus r^2 permitted direct calculation of the molecular weight of the lowest molecular weight species present.

For low speed sedimentation equilibrium experiments the approach of Richards et al. (1968) was used. Molecular weights were calculated from equation 4. Since these experiments were carried out at low speeds, the concentration of protein at the meniscus was significant. The protein concentration at the meniscus was found from the following expression:

$$c_m = c_o - \frac{r_b^2 (c_b - c_m) - \int_{cm}^{cb} r^2 \, dc}{r_b^2 - r_m^2} \qquad (5)$$

where c_m is the protein concentration at the meniscus;

c_o is the initial concentration of protein;

r_m is the distance of the meniscus from the center of rotation;

r_b is the distance of the cell bottom from the center of rotation;

c_b is the protein concentration at the cell bottom;

c is the protein concentration at any distance r from the center of rotation.

The protein concentration at any distance from the center of rotation was determined by adding the fringe displacement at any point r to the concentration at the meniscus (in fringes). A plot of ln c (or ln y) versus r^2 yielded a slope which could be used to determine the weight average molecular weight at any position across the cell.

The technical aspects involved in carrying out sedimentation equilibrium runs or measuring interference patterns were as described by Van Holde (1967) and by Chervenka (1969).

I. Spectrophotometeric Titration

As is well known, ionization of tyrosine to form the phenolate ion causes a shift in the UV spectrum to longer wavelengths. If spectral changes are determined as difference spectra, ionized tyrosine exhibits a difference peak having its maximum at 295 nm and has a molar difference extinction coefficient of about 2300 (Kronman and Robbins, 1970). At pH 7.0 tyrosine hydroxyl groups are unionized. Therefore, from increase in $OD_{295\ nm}$ at any pH value, the number of tyrosines ionized can be estimated. The pK for ionization of tyrosines quite accessible to the solvent is usually near 10. However, if tyrosines are completely or partially buried within a protein and perhaps inaccessible to solvent, the pK value is higher and the tyrosine ionization may exhibit time dependence. Determination of pK values and time dependence of tyrosine ionization may yield qualitative information concerning the accessibility of tyrosines to the external solvent.

The ionization of tyrosine hydroxyl groups was followed by means of a Cary 15 spectrophotometer. One cm quartz cells of 1 ml volume were employed. A protein solution at a given alkaline pH and one at pH 7.0 were compared for spectral changes at 295 nm. At time 0, 0.2 ml protein solution was mixed with 0.7 ml buffer and the $OD_{295\ nm}$ of this solution was compared directly with that of an equal protein concentration at pH 7.0. Because of the time required for mixing of alkaline samples, the recording usually did not begin until a time of about 40 sec. Buffered solutions consisted of 0.1 M tris, 1 M KCl for pH 7.0 to 9.0, 0.1 M carbonate-bicarbonate, 1 M KCl for pH 9.0 to 11.0, and mixtures of 1 M KCl and 1 M KOH for pH 11.0 to 13.5. Solutions contained also 10^{-5} M $CaCl_2$. pH values were measured both before and after addition of protein solution on a Radiometer type TTT1C pH meter with scale expander. Final protein concentrations were about 10^{-4} M.

J. Circular Dichroism

The circular dichroism measurements were made on a Cary model 6001 circular dichroism attachment to a Cary 60 recording spectropolarimeter equipped with a water cooled lamp housing maintained at 27°C. The instrument was calibrated with an aqueous solution of d-10-camphor sulfonic acid with a difference in molecular extinction (E_L-E_R) of 2.16. Constant nitrogen flushing was employed. Cells of 0.5 mm pathlength were used over the range of 185-250 nm with protein concentrations of approximately 0.1%. For the near UV region (250-320 nm), 1 cm cells were used with protein concentrations of about 0.3%. The mean residue ellipticity is given by:

$$[\theta] = \frac{\theta M}{100\ c\ell}$$

where M is the mean residue molecular weight (taken as 115 throughout this thesis);

θ is the observed ellipticity in degrees;

ℓ is cell path length in dm;

c is the protein concentration in gm/cm^3.

The units of $[\theta]$ are $degree \cdot cm^2$ per decimole.

Apparent % α-helix was estimated using polyglutamic acid at pH 3.8 as a prototype for 100% α-helix and the same polymer at pH 9.3 as a model for a random coil (Cassim and Yang, 1967).

K. Amino Acid Analysis

Amino acid analyses were performed on a Beckman 120B amino acid analyzer after hydrolysis of samples in duplicate for 24, 48, 72, and 96 hr at 110°C. Troponin A was not very soluble in constant boiling HCl. This problem was overcome by initially measuring the concentration of troponin A in water and then taking aliquots of aqueous troponin A to dryness. Constant boiling HCl was added, and the tubes were evacuated and sealed for hydrolysis. Oxidized samples were prepared according to Moore (1963). For the analyzer, 0.1-0.25 mg hydrolyzed protein or 0.1 μmole of each standard amino acid was suitable. Yields of serine, threonine, and methionine were extrapolated to zero time of hydrolysis. Otherwise the yields were taken as an average of the 72 and 96 hr hydrolyses.

Tryptophan was determined by the procedure of Oprenska-Blauth et al. (1963).

For estimation of sulfhydryl groups, the method of Sedlak and Lindsay (1968) was employed. Newly purchased DTT, which had been stored at -10°C and opened just previous to use, was used as a standard. A representative standard curve is shown (figure 4).

L. Viscometry

Measurements of viscosity were made in Ostwald-type viscometers of 1 ml capacity. Viscometers were cleaned with chromic acid. Just prior to use, they were rinsed, first with soap solution (Ninol, Beckman Instruments), then several times with distilled water and finally several times with acetone. All solvents and protein solutions were passed through 5μ Millipore filters. Viscometer temperatures were maintained at 20° ± 0.01°C by a thermostatically controlled water bath with a refrigeration unit. Viscometer flow times were 4-5 min with water. Flow times obtained from protein concentrations of 0.2 - 0.8% after 5-6 repetitions showed deviations of 0.2 sec or less. From the flow times of solvent and protein solutions, the specific viscosity was calculated. The reduced viscosity was obtained by dividing the specific viscosity by the protein concentration. By plotting the reduced viscosity versus protein concentration, the intrinsic viscosity was evaluated. In all this work, the treatise of Yang (1961) was followed.

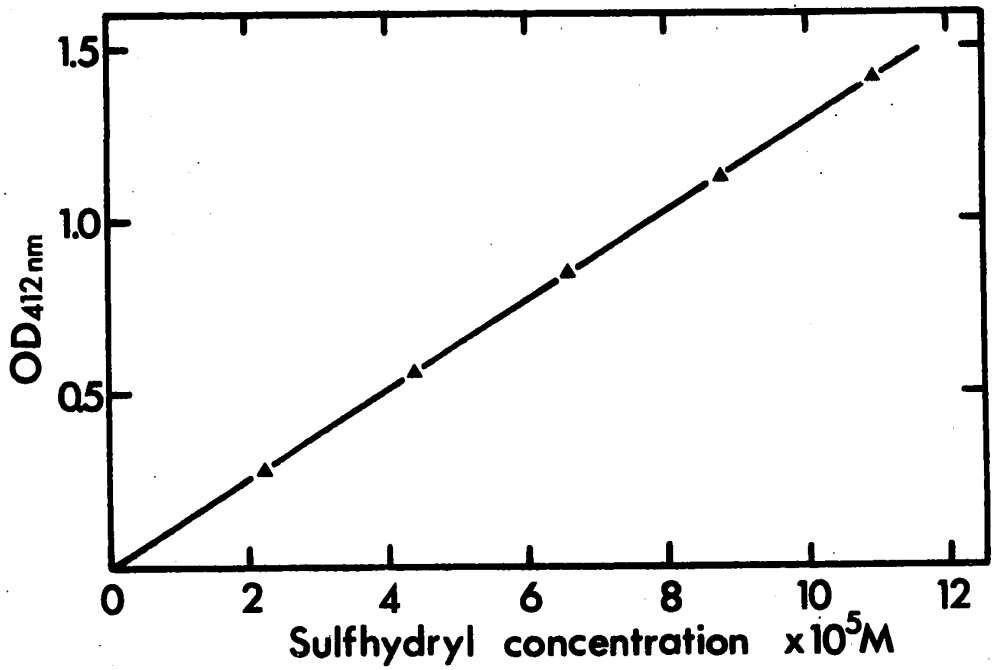


Figure 4. Standard curve for the determination of free sulfhydryl groups.

III. RESULTS

A. Troponin and its Constituents

1. Troponin

Troponin from rabbit skeletal muscle was isolated by a number of different procedures described in the 'Materials and Methods' Chapter. The terms "troponin" and "crude troponin" will be used interchangeably. Troponin can be considered crude in that it contains several distinct proteins, the molar ratio of which varies somewhat from preparation to preparation. All preparations were biologically active by the superprecipitation technique (see page 57).

The extent of homogeneity of these troponin preparations was determined by sedimentation velocity measurements. A typical sedimentation experiment on troponin isolated by the method of Yasui *et al.* (1968) is shown in figure 5. The solvent system included 0.5 M KCl, 0.067 M phosphate, pH 7.0, 1 mM DTT. The protein sedimented essentially as a single boundary. The $S_{20,w}$ values were determined at a number of concentrations (figure 6), from which it was seen that little concentration dependence of $S_{20,w}$ value existed. The $S^{\circ}_{20,w}$ value was 3.7S, in good agreement with the value of 3.8S obtained by Hartshorne *et al.* (1969).

In order to get an idea of the number of protein components in these troponin preparations, SDS-polyacrylamide gels were run (figure 7) and molecular weights were estimated from gels calibrated with standard proteins. Three major components of molecular weights 40,000 25,000 and 22,000 were always present. Additionally one of molecular

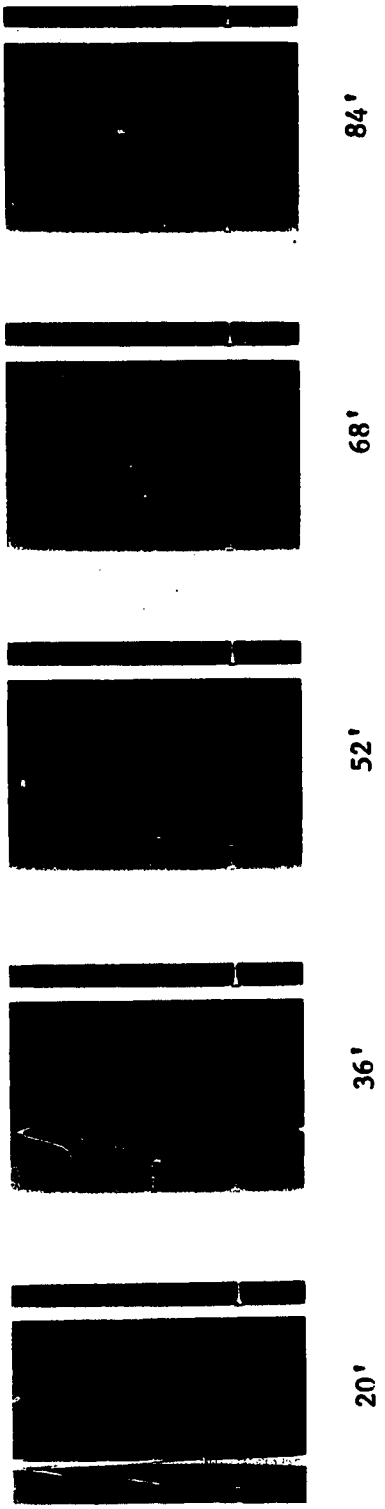


Figure 5. Sedimentation profiles of troponin. Two Kel F cells were used, one with a wedge window. Protein concentrations were 4.0 mg./ml. (upper) and 6.0 mg./ml. (lower). Time of photograph after reaching speed is indicated. Solvent - 0.05 M phosphate pH 7.0, 0.5 M KCl, 0.5 mM DTT. Bar angle - 55°.

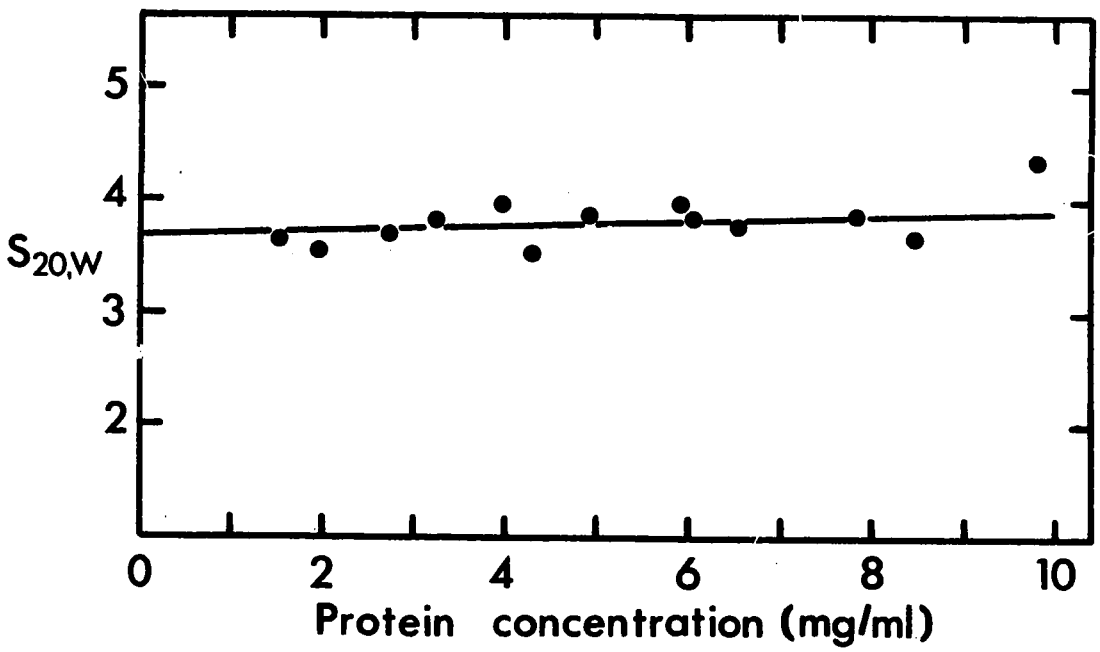


Figure 6. Concentration dependence of the $S_{20,w}$ value of troponin. Solvent - 0.05 M phosphate pH 7, 0.5 M KCl, 0.5 mM DTT.

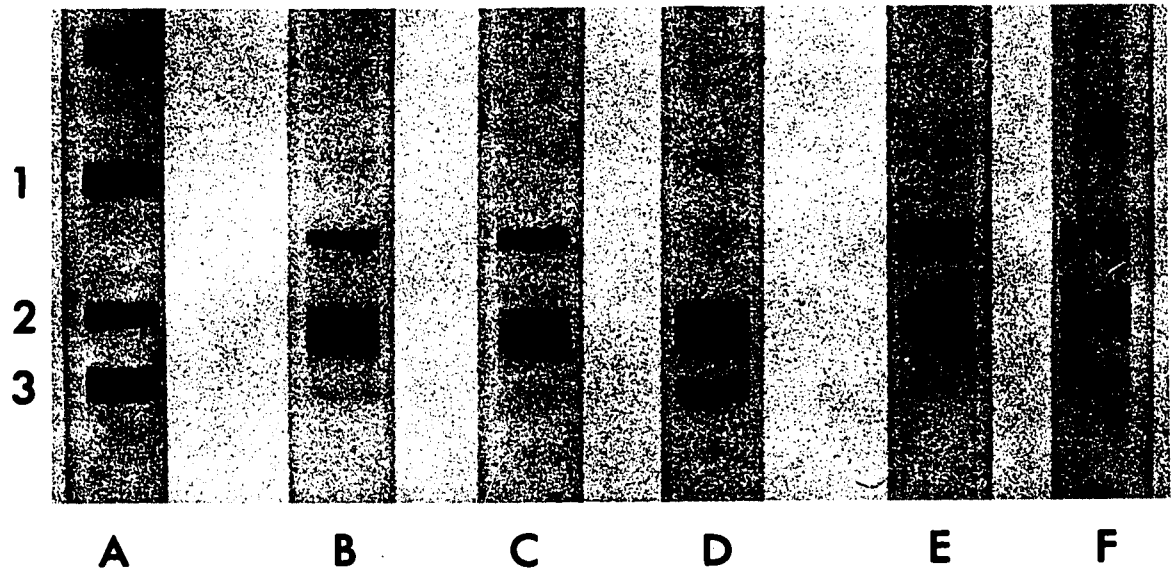


Figure 7. SDS-polyacrylamide gel electrophoresis of crude troponin (gel B), peaks 1 and 2 material from Sephadex G-200 treatment of crude troponin (gels C and D, respectively) and peaks 3 and 4 material from QAE-Sephadex treatment of peak 1 from Sephadex G-200 chromatography of crude troponin (gels E and F, respectively). About 10 µg of protein was applied in each case. Gel A contains a standard mixture of 1) bovine serum albumin, 2) chymotrypsinogen A, and 3) sperm whale myoglobin. Gels were 5% acrylamide.

weight 15,000 was often present, although in smaller amounts that the three major components (Murray and Kay, 1971). The SDS-polyacrylamide gel patterns were quite similar regardless of the preparative approach, although the bands varied in intensity. This is in agreement with Greaser and Gergely (1971) who compared different preparative methods by SDS-polyacrylamide gel electrophoresis. Throughout this work these protein components of troponin will frequently be referred to by their molecular weights.

2. Fractionation of Troponin by Gel Filtration

a. Sephadex G-200 chromatography

The initial attempt to fractionate troponin made use of Sephadex G-200 column chromatography in 0.5 M KCl, 0.067 M phosphate pH 7.0, 1 mM DTT. Void volumes were determined by passing Blue Dextran through the column. The elution profile was characterized by 3 or 4 retained peaks depending upon the approach used to prepare troponin. A small peak always appeared in the void volume position. The first two retained peaks (referred to henceforth as peaks 1 and 2) were always present, while the last two, which were present in much smaller amounts, were often not resolved. The elution profile in figure 8 resulted from Sephadex G-200 chromatography of troponin prepared by the method of Yasui et al. (1968). Troponin, resulting from a modified preparative approach, in which the pH 4.0 isoelectric precipitation of Yasui et al. (1968) was omitted, yielded a Sephadex G-200 profile in which peak 2 was by far the largest component present. At low KCl concentrations, peak 1 material tended to precipitate between 40 and 60% saturated ammonium sulfate while the precipitate between 60 and 70% saturated ammonium sulfate was richer in peak 2 material.

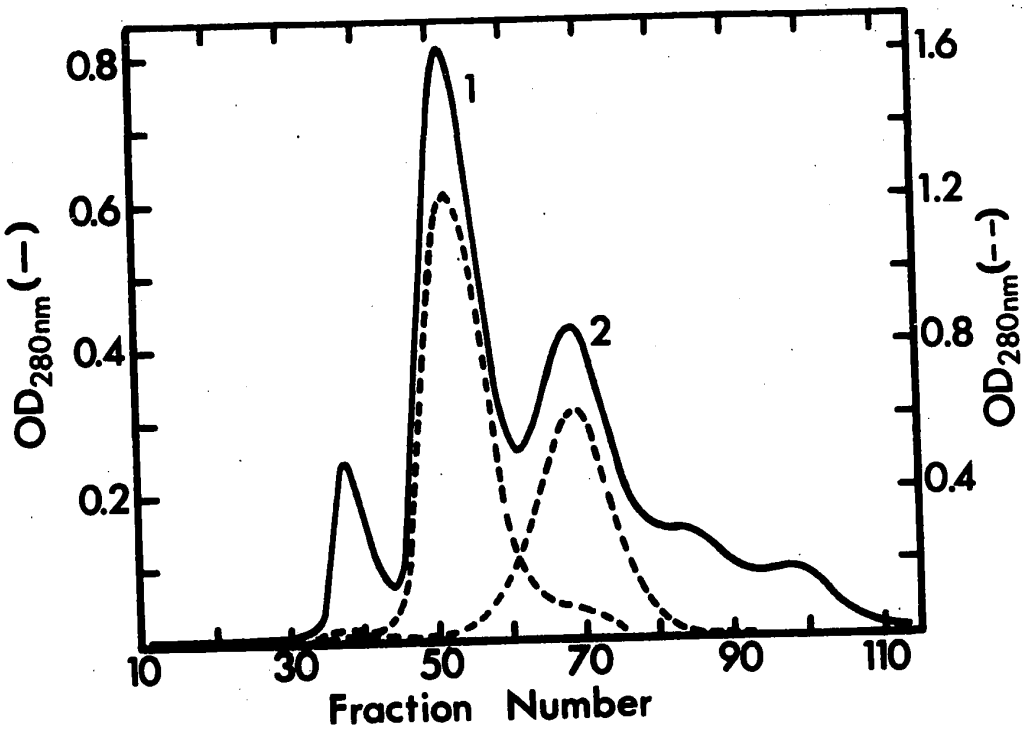


Figure 8. Sephadex G-200 chromatography of crude troponin. About 100 mg. protein in 4 ml. buffer (0.05 M phosphate, pH 7, 0.5 M KCl, 0.5 mM DTT) was applied to a 2.5 cm. X 100 cm. column, equilibrated against the same buffer and was eluted at 14 ml./hr. Fraction size was 4.6 ml. Rechromatography is indicated by the broken line.

Troponin could then be separated by gel filtration. Only protein from peaks 1 and 2 displayed significant biological activity (page 57). To obtain further purification, these fractions were rechromatographed (figure 8).

b. Properties of material eluting in the first two peaks from Sephadex G-200 column chromatography

(i) Homogeneity

In order that a substance elute from a Sephadex G-200 column as a single retained peak, it may be composed of one component, several components which diffuse at similar rates, or several components which interact strongly to give one larger component. This reasoning is based on the assumption that no significant interaction occurs between the protein and the gel matrix. Peaks 1 and 2 from Sephadex G-200 chromatography had elution volumes of 1.35 - 1.45 x void volume and 1.8 - 2 x void volume, respectively, corresponding to elution volumes for globular proteins of molecular weights of 185,000 - 230,000 and 50,000 - 80,000 respectively (Leach and O'Shea, 1965; Determann and Michel, 1966). From this information very little can be concluded about the size of the components, since their shapes are unknown.

The SDS-polyacrylamide gel patterns (figure 7) of the material eluting in peaks 1 and 2 revealed that several distinct protein species were present. Peak 1 material contained 3 protein species. The molecular weights of these, as determined from SDS-polyacrylamide gels calibrated with standard proteins, were 40,000, 25,000, and 22,000. Peak 2 contained species of molecular weights 25,000, 22,000 and 15,000.

The homogeneity of the material eluting in the peak 1 and 2 positions was checked with respect to sedimentation properties. Typical Schlieren patterns obtained during the sedimentation of the two fractions in 0.5 M KCl, 0.067 M phosphate, pH 7.0, 1 mM DTT are shown in figures 9 and 10. Peak 1 material sedimented as a single boundary and a plot of $S_{20,w}$ versus protein concentration (figure 11) led to determination of a $S^{\circ}_{20,w}$ value of 4.4S for this material. This $S^{\circ}_{20,w}$ value was greater than that of troponin (3.7S) but less than that of relaxing protein (5.1S - Hartshorne and Mueller, 1969). Peak 2 material sedimented as a two component system with a major peak as well as 20 - 30% of a faster sedimenting species, and for this reason the relationship between $S_{20,w}$ value and protein concentration was not resolved. The $S_{20,w}$ of the major component at a protein concentration of 6.0 mg/ml was 3.0S. No correction was made here for the Johnston-Ogston effect.

(ii) Molecular weights by sedimentation equilibrium

The determination of the molecular weights of the materials from the first two peaks from Sephadex G-200 was undertaken with the sedimentation equilibrium approach. Plots of ln y versus r^2 where y is the protein concentration in fringe units and r is the distance from the center of rotation in centimeters, exhibited upward curvature in all cases, indicating heterogeneity. Several ln y vs r^2 plots for peak 1 material at different initial loading concentrations are shown in figure 12. When the various determinations were represented in the form of molecular weight vs concentration plots (figure 13), a small

Figure 9. Sedimentation profiles of peak 1 material from Sephadex G-200 column chromatography of crude troponin. Two Kel F cells were used, one with a wedge window. Protein concentrations were 3.0 mg./ml. (upper) and 5.0 mg./ml. (lower). Time of photograph after reaching speed is indicated. Solvent - 0.05 M phosphate pH 7.0, 0.5 M KCl, 0.5 mM DTT. Bar angle - 55°.

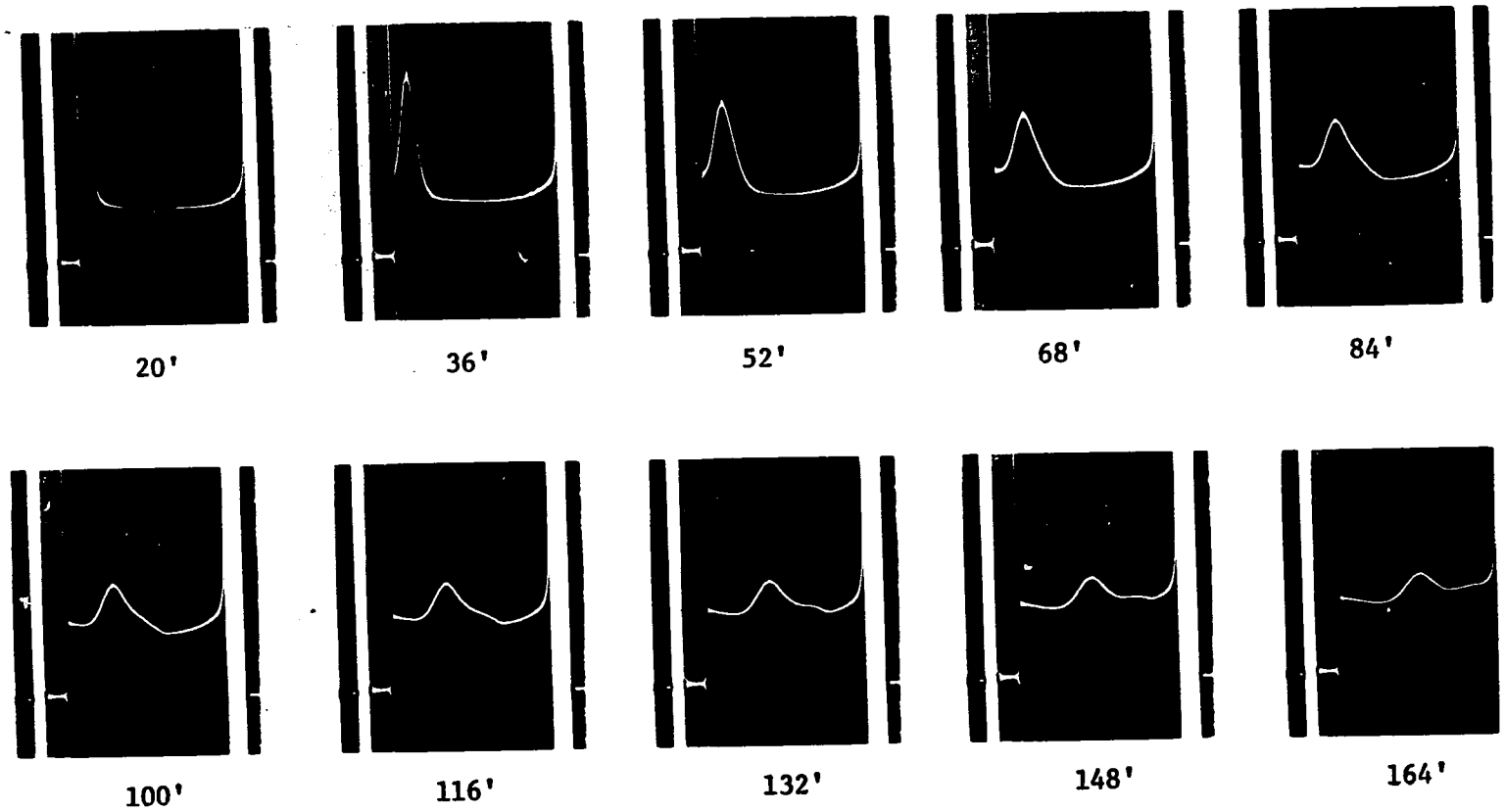


Figure 10. Sedimentation profiles of peak 2 material from Sephadex G-200 chromatography of crude troponin. Time of photograph after reaching speed is indicated. Protein concentration - 6.0 mg./ml. Solvent - 0.05 M phosphate pH 7.0, 0.5 M KCl, 0.5 mM DTT. Bar angle - 55°.

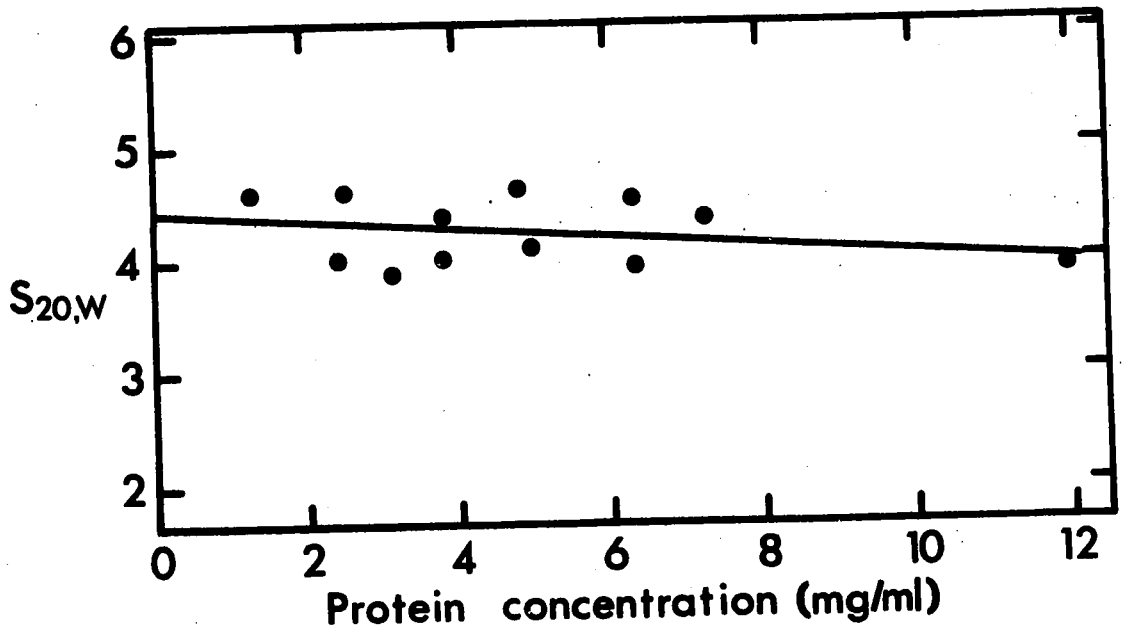


Figure 11. Concentration dependence of the $S_{20,w}$ value of peak 1 material from Sephadex G-200 chromatography of crude troponin. Solvent - 0.05 M phosphate pH 7, 0.5 M KCl, 0.5 mM DTT.

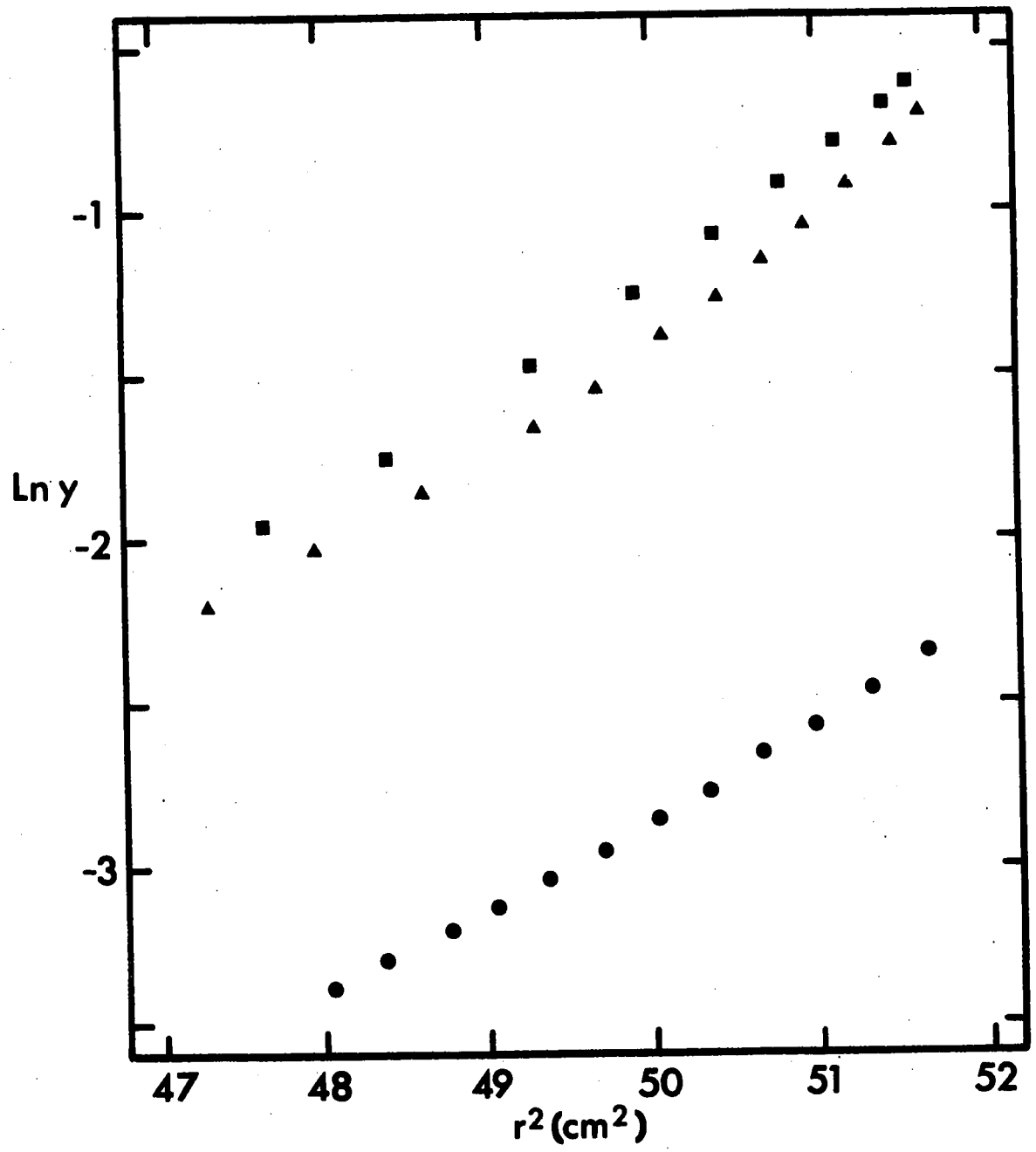


Figure 12. Sedimentation equilibrium ln y vs r^2 plots on peak 1 material from Sephadex G-200 chromatography of crude troponin. Initial loading concentrations were 0.52 mg./ml. (●), 1.87 mg./ml. (▲), and 2.48 mg./ml. (■). Speed - 8,000 rpm. Solvent - 0.05 M phosphate pH 7, 0.5 M KCl, 0.5 mM DTT.

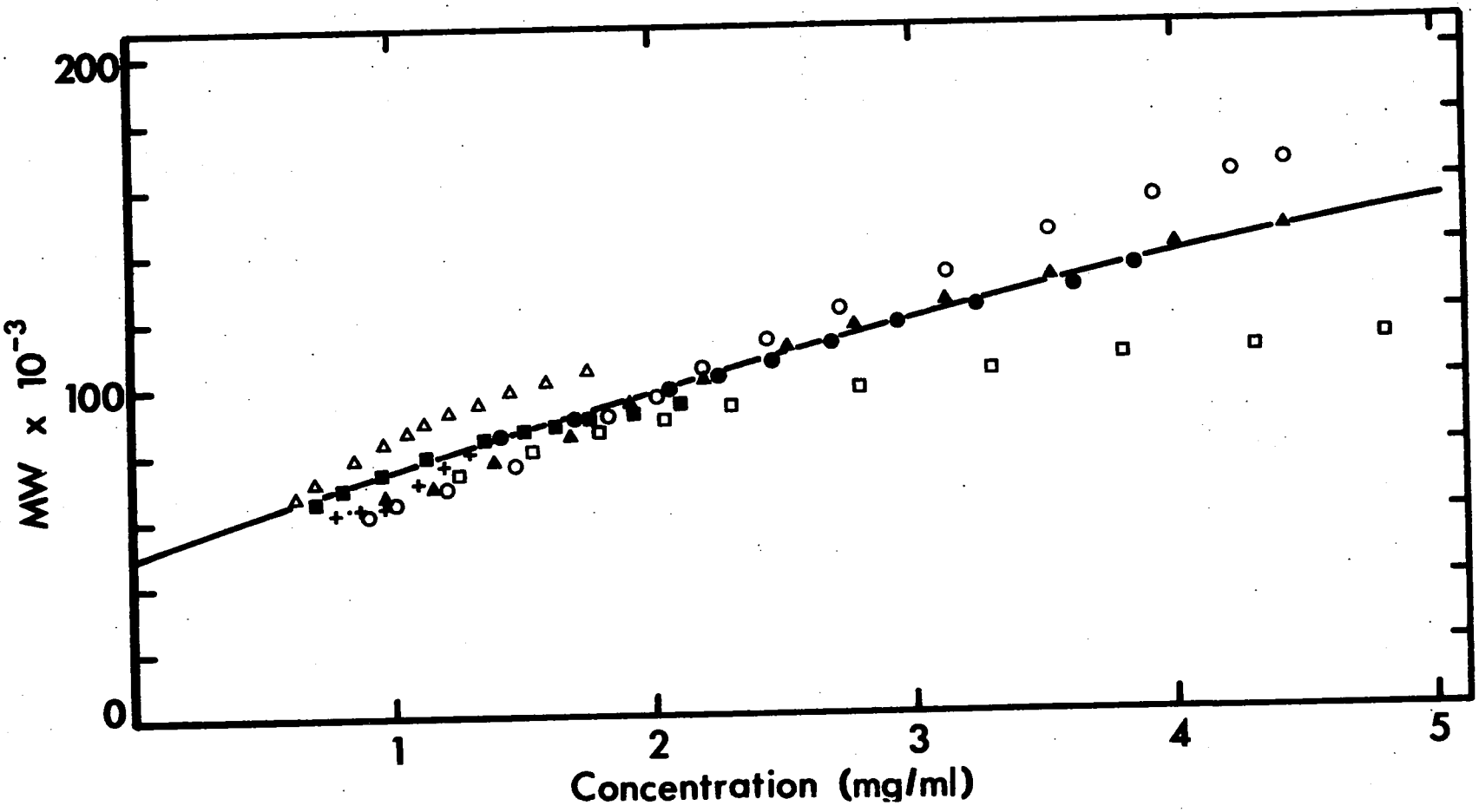


Figure 13. Concentration dependence of the molecular weight of peak 1 material from Sephadex G-200 chromatography of crude troponin. Different types of symbols represent different sedimentation equilibrium experiments. Speeds were 8,000 to 10,000 rpm. Solvent - 0.05 M phosphate pH 7, 0.5 M KCl, 0.5 mM DTT.

degree of overlap was observed. The data in figure 13 were treated with a computer program for determining the best polynomial fit and the curve generated was superimposed on the same figure. The computerized extrapolation of this curve gave a molecular weight of 49,000 at zero concentration. The molecular weight increased to 138,000 as the protein concentration increased to 4 mg/ml. Only one high speed sedimentation equilibrium experiment was carried out and for this reason no data are presented. The minimum molecular weight obtained by this method was 62,000. Protein species of 40,000, 25,000, and 22,000 appeared on SDS-polyacrylamide gel electrophoresis of this material. Under conditions similar to those for sedimentation equilibrium studies, perhaps all three components are involved in a complex of molecular weight 87,000 (40,000 + 25,000 + 22,000). If an equilibrium between such species does exist, the treatment of the molecular weight - concentration relationship is very complex indeed.

Typical ln y vs r^2 plots for Sephadex G-200 peak 2 material are presented in figure 14. Three different initial loading concentrations are included. Plots of molecular weights vs concentration for about 12 different experiments revealed no extensive overlap. Because of the difficulty in presenting this data in an intelligible way, the data is expressed as a curve only (figure 15) generated by a computerized least squares polynomial fit. The data fell within the dashed boundary. The molecular weight increased markedly with concentration. Extrapolation of the curve by computer analysis yielded a molecular weight of 33,000 at zero concentration. Minimum molecular weights of peak 2 material, as determined by the meniscus depletion method of Yphantis (1964), gave variable results. Values from 15,000 to 50,000 were obtained depending

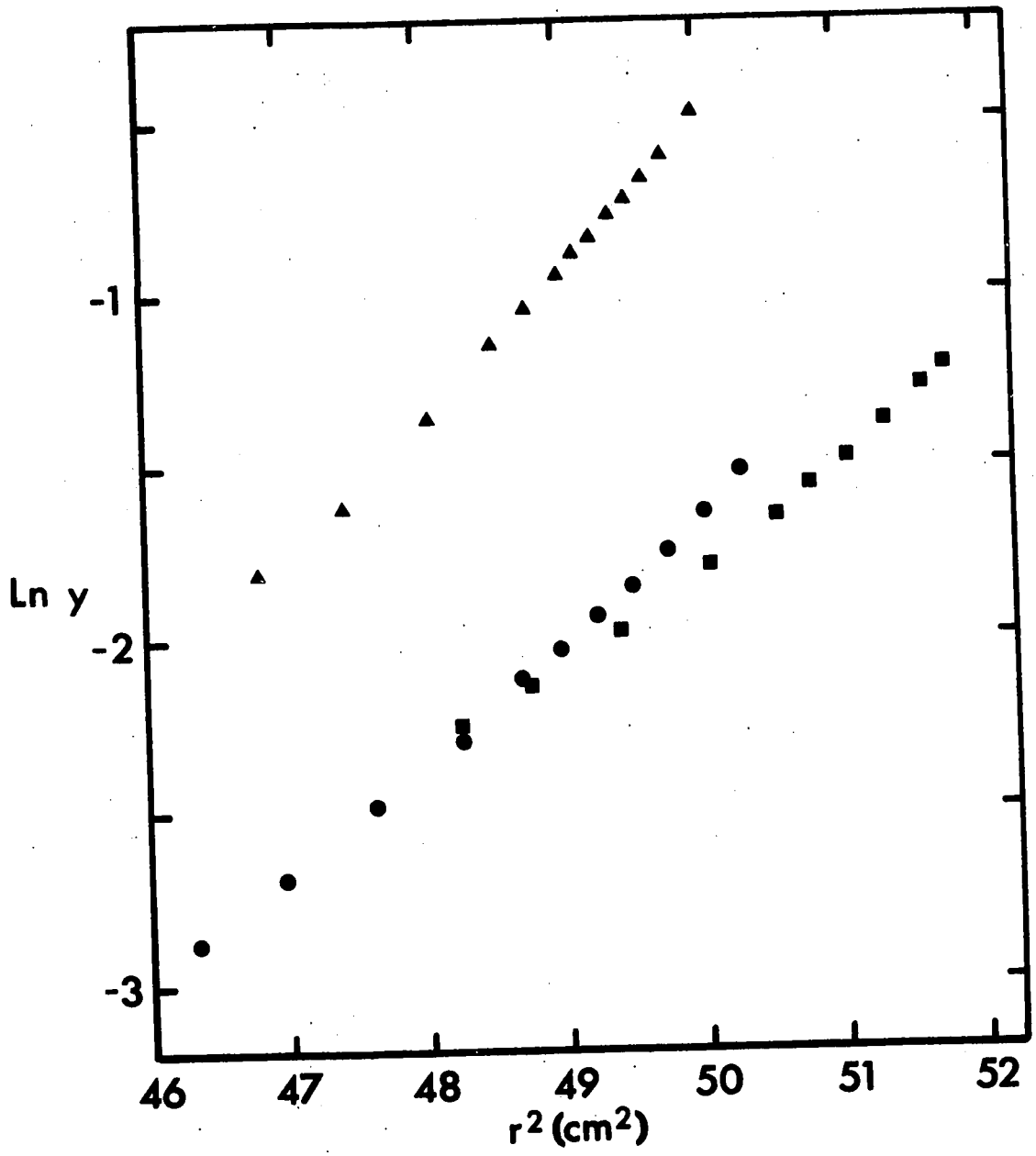


Figure 14. Sedimentation equilibrium ln y vs r^2 plots on peak 2 material from Sephadex G-200 chromatography of crude troponin. Initial loading concentrations were 0.99 mg./ml. (■), 1.53 mg./ml. (●), and 2.41 mg./ml. (▲). Speed - 12,000 rpm. Solvent - 0.05 M phosphate pH 7, 0.5 M KCl, 0.5 mM DTT.

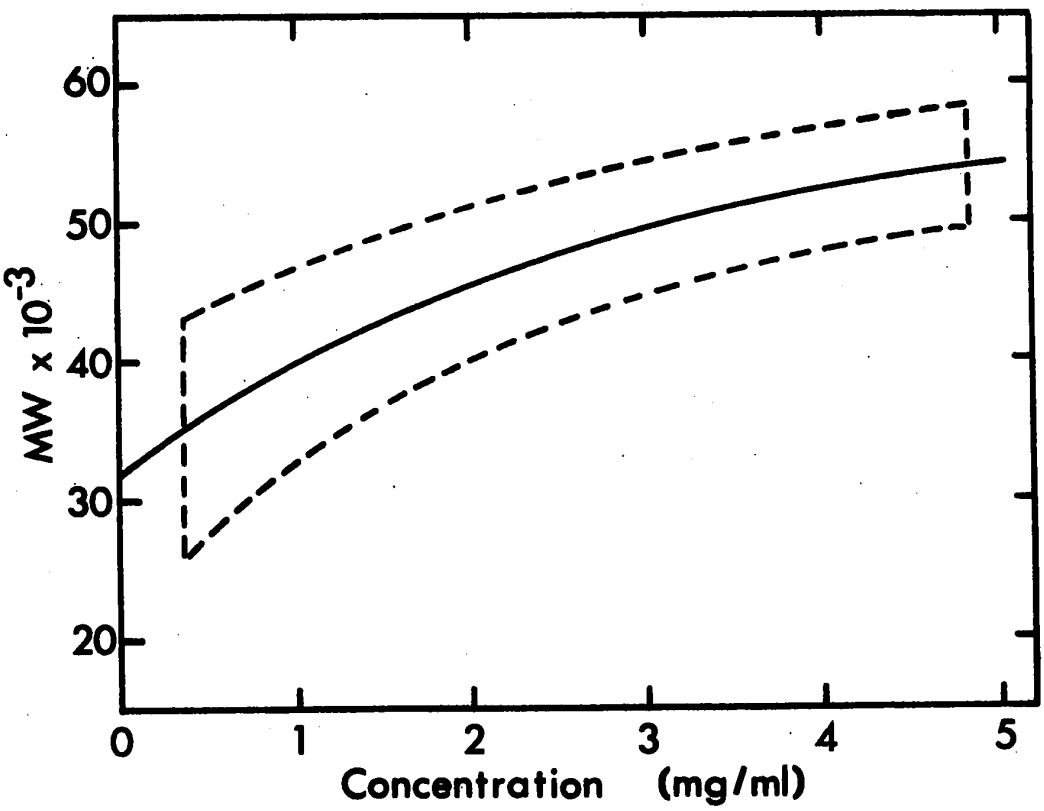


Figure 15. Concentration dependence of the molecular weight of peak 2 material from Sephadex G-200 chromatography of crude troponin. Data from 12 runs fell within the dashed boundary. The solid line represents the best polynomial fit of the data. Speeds were 10,000 to 14,000 rpm. Solvent - 0.05 M phosphate pH 7, 0.5 M KCl, 0.5 mM DTT.

on the preparation. It is noteworthy, however, that the preparation that gave a minimum molecular weight of 50,000 was shown from SDS-polyacrylamide gels to contain protein species of molecular weights 25,000 and 22,000. A preparation containing species of molecular weights 25,000, 22,000 and 15,000 from SDS-polyacrylamide gels, gave a high speed molecular weight of 15,000. This would imply interaction between species of molecular weights 25,000 and 22,000 to give a complex of about 47,000 molecular weight.

Interaction between species of molecular weights 25,000 and 22,000 may be stabilized by disulfide bonds, since in the absence of DTT, SDS-polyacrylamide gels of peak 2 material exhibited a band at 50,000 molecular weight, as well as those at 25,000 and 22,000, which broke down in the presence of DTT to enhance the species of molecular weights 25,000 and 22,000.

(iii) UV absorption and circular dichroism

Peaks 1 and 2 from Sephadex G-200 were compared with respect to far UV absorption spectra. The $E^{1\%}_{1\ cm}$ values (figure 16) of these two fractions were not very different. Both spectra are typical of proteins, with λ_{max} at 277-278 nm. One problem that existed throughout this work was that of determining concentrations. Nucleic acids were often isolated along with the protein components. The amount of nucleotide bound, and thus the $E^{1\%}_{1\ cm}$, varied slightly from preparation to preparation. Also, some of the protein components of troponin have abnormally low tyrosine contents and the method of Lowry et al. (1951) was not completely satisfactory. This problem was overcome in part by the use of an ultracentrifugal synthetic boundary cell run with interference optics to determine the concentration of the material. Even this method depended on the assumption of a dn/dc value of 0.185 ml/g.

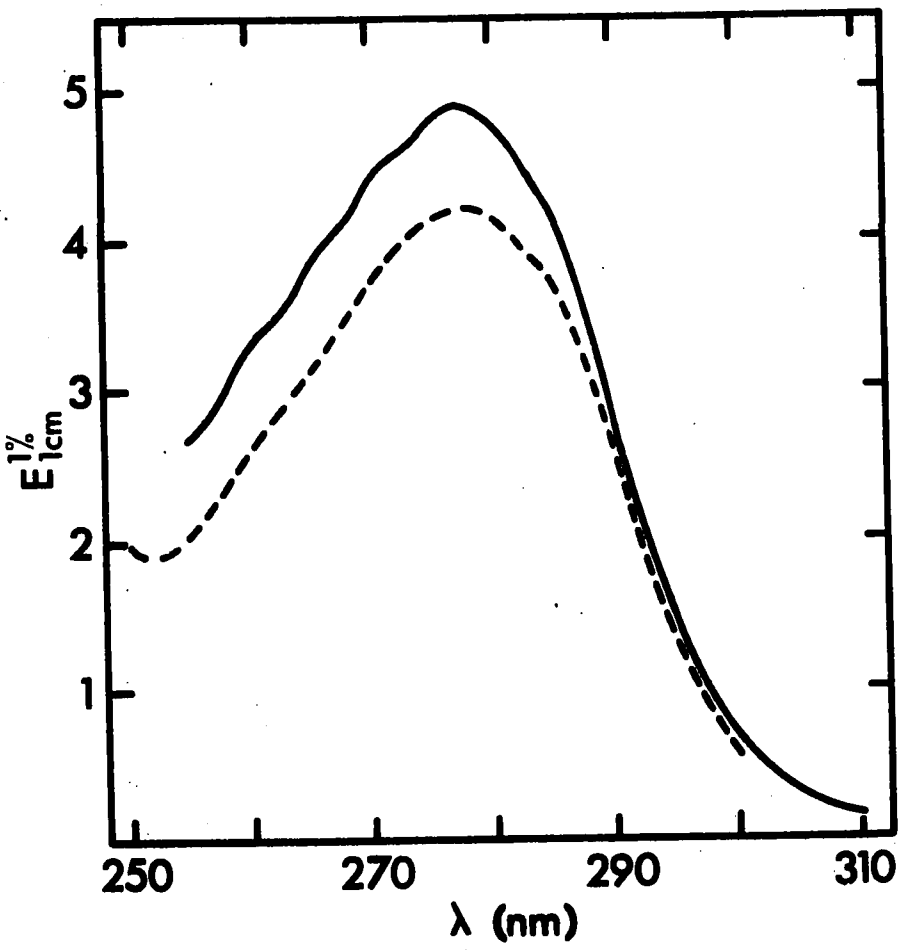


Figure 16. UV absorption spectra of peaks 1 and 2 material from Sephadex G-200 column chromatography of crude troponin. The spectrum for peak 1 material is represented by the dashed line and that for peak 2 material by the solid line. Solvent - 0.05 M phosphate pH 7, 0.5 M KCl, 0.5 mM DTT.

Differences in structure were investigated by circular dichroism. Far UV circular dichroism spectra for peaks 1 and 2 are shown in figure 17. Some differences in apparent helical content existed. By using polyglutamic acid at pH values 3.8 and 9.3 as models for 100% random coil and 100% α-helix respectively (Cassim and Yang, 1967), the apparent helical contents could be calculated. From the $[\theta]_{221\ nm}$ values of -20,400 and -16,400 deg·cm^2/decimole for peaks 1 and 2 material, peak 1 material was found to be 56% α-helical and peak 2 material 47% α-helical.

c. Attempts at further fractionation by ion exchange chromatography

Troponin was obviously composed of complexes or mixtures of protein but the main objective was to isolate the individual components present. Various ion exchangers were employed in an effort to further fractionate peaks 1 and 2 material from Sephadex G-200. In order not to belabor failures, two examples of typical ion exchange chromatographies will be presented. The first involved the use of QAE-Sephadex A-50 to fractionate peak 1 material from Sephadex G-200. The buffer employed was 50 mM tris pH 7.6, 0.5 mM DTT with a linear gradient of KCl to 0.5 M. Several distinct peaks resulted (figure 18). Proteins from the various peaks were compared in terms of their SDS-polyacrylamide gel profiles (figure 7). Peak 1 was not analyzed. Peak 2 contained essentially 15,000 molecular weight material. Peak 3 had components of molecular weights 40,000, 25,000, and 22,000 (gel E). Peak 4 was made up of components of molecular weights 40,000, 25,000, 22,000 and 15,000 (gel F). This approach appeared to be unsuccessful as

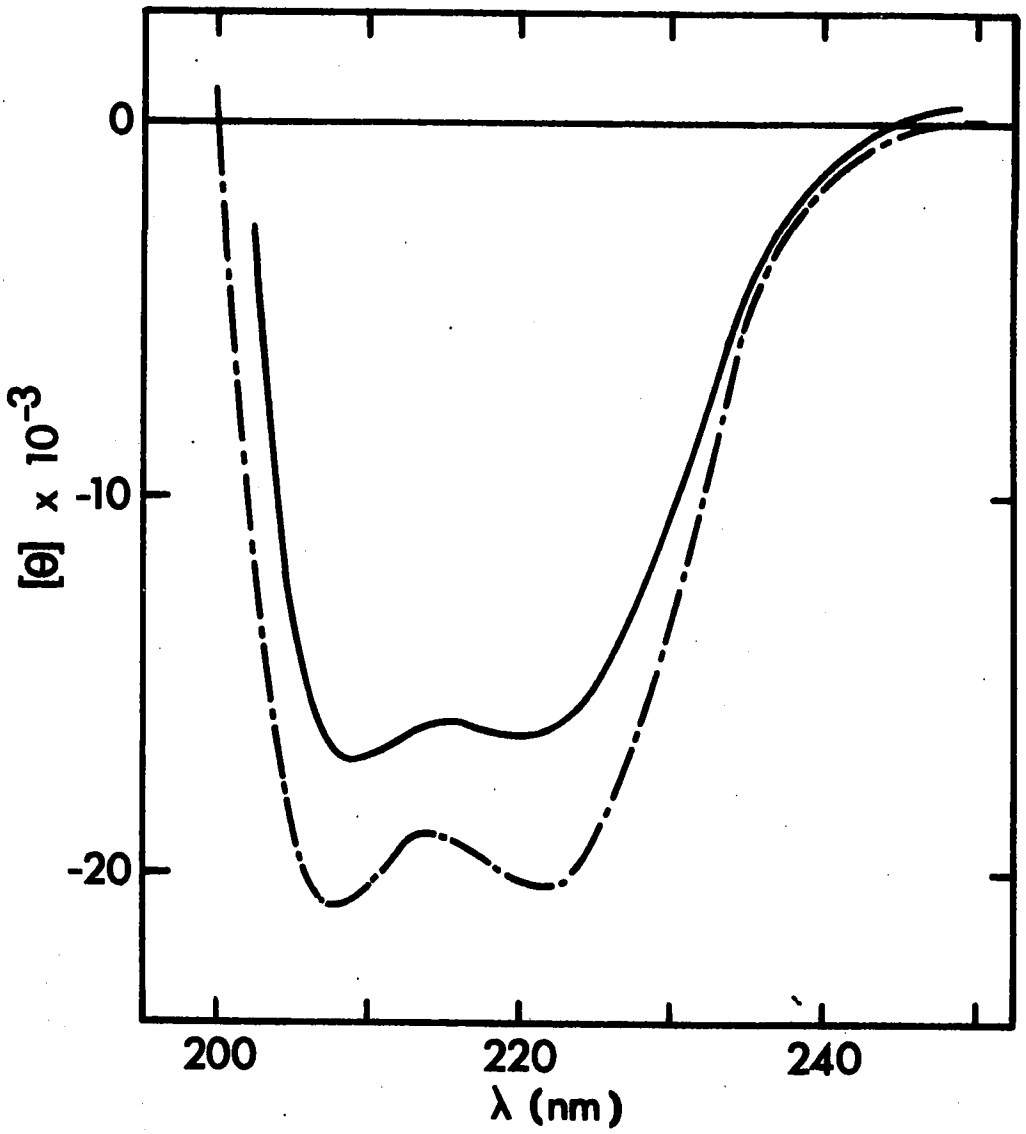


Figure 17. Far UV CD spectra of peaks 1 and 2 material from Sephadex G-200 column chromatography of crude troponin. The spectrum for peak 1 material is shown as the broken line and that for peak 2 material as the solid line. Solvent - 0.05 M phosphate pH 7, 0.5 M KCl, 0.5 mM DTT.

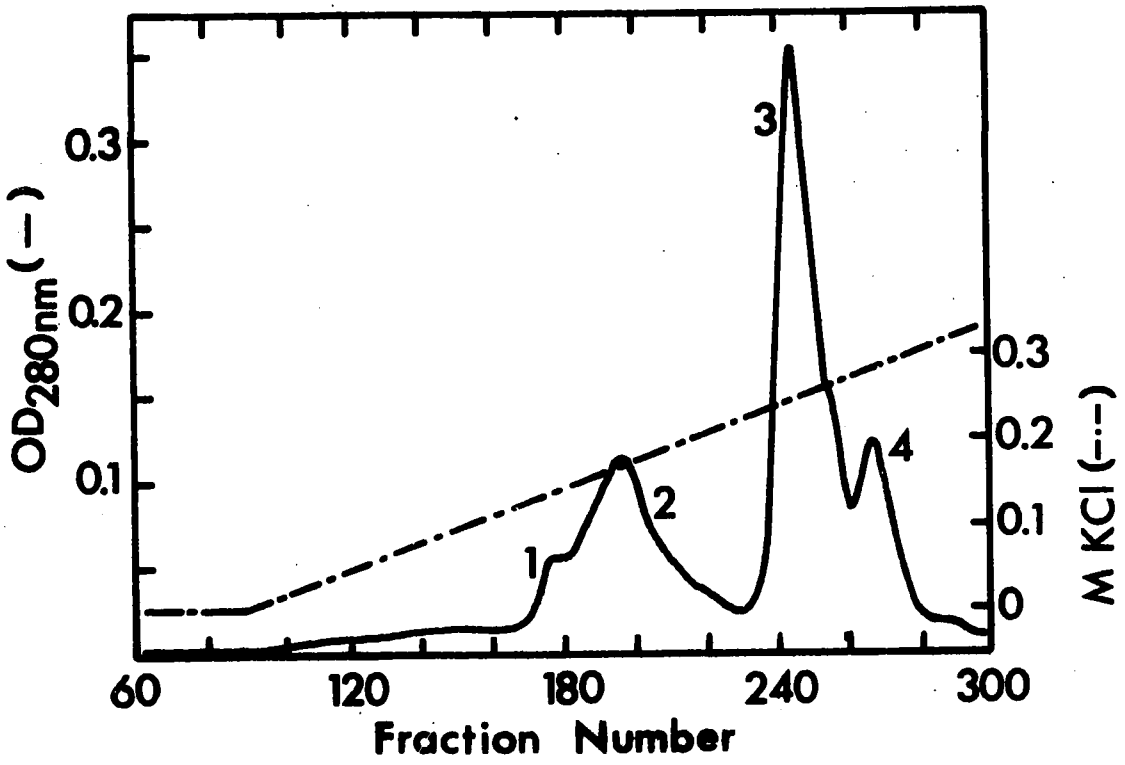


Figure 18. QAE-Sephadex A-50 column chromatography of peak 1 material from Sephadex G-200 chromatography of crude troponin. Approximately 150 mg. protein in 10 ml. 50 mM tris pH 7.6, 0.5 mM DTT was applied to a 2.5 cm. X 40 cm. gel bed equilibrated with 50 mM tris pH 7.6, 0.5 mM DTT and was eluted with a linear gradient of KCl.

a fractionation procedure. However, the fact that the three major protein species (of molecular weights 40,000, 25,000, and 22,000) elute together on both Sephadex G-200 and on QAE-Sephadex A-50 suggests that they are a definite complex. The apparent increase in the amount of material of molecular weight 15,000 was quite mysterious and it was thought to arise from proteolytic degradation of one of the other components.

The second peak from Sephadex G-200 was also chromatographed on QAE-Sephadex A-50 (figure 19). Protein was applied to a QAE-Sephadex A-50 column in 50 mM tris pH 7.6, 0.5 mM DTT, and eluted in the same buffer containing 0.2, 0.5, and 1.0 M KCl. Several distinct peaks resulted. The major peak, which eluted at a KCl concentration of just over 0.2 M, was found to consist of two protein species of molecular weights 25,000 and 22,000 by SDS-polyacrylamide gels. The initial peak contained most of the material of 15,000 molecular weight. These results would imply that a complex between species of molecular weights 25,000 and 22,000 exists which is stable during this ion exchange chromatography.

3. Fractionation of Troponin by the Hartshorne-Mueller Approach

Because of the difficulty encountered in separating the protein species of troponin, it became obvious that harsher preparative means were required. At about this time Hartshorne and Mueller (1968) suggested a methodology for fractionating troponin. They treated troponin with 1.2 M KCl, 0.1 N HCl, which resulted in precipitation of a component referred to as troponin A. After neutralizing the supernatant and dialyzing it at low ionic strength, another precipitate was obtained. This was referred to as troponin B.

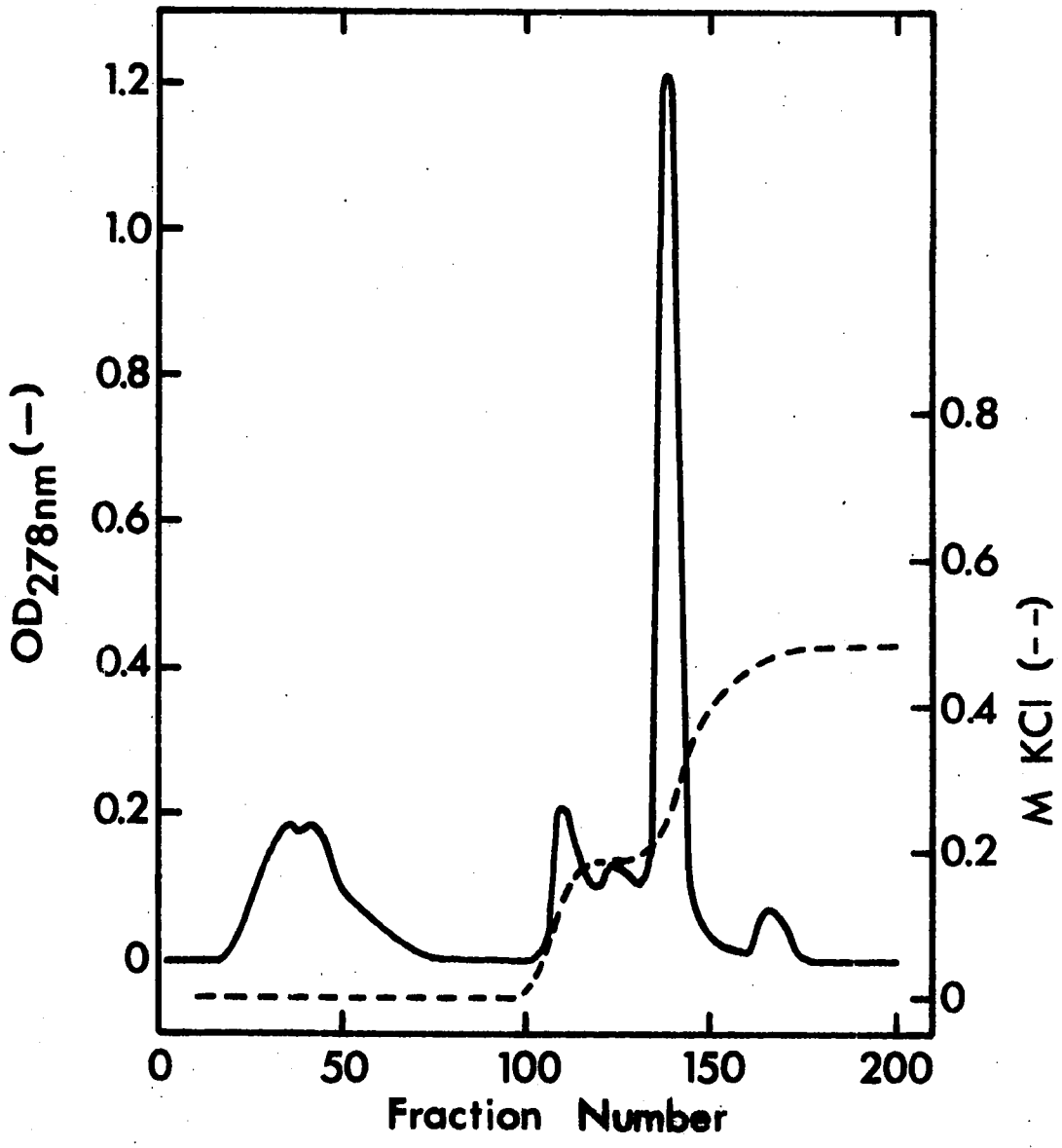


Figure 19. QAE-Sephadex A-50 column chromatography of peak 2 material from Sephadex G-200 chromatography of crude troponin. Approximately 150 mg. protein in 10 ml. 50 mM tris pH 7.6, 0.5 mM DTT was applied to a 2.1 cm. X 20 cm. column of QAE-Sephadex equilibrated with 50 mM tris pH 7.6, 0.5 mM DTT and was eluted with 0.2 M, 0.5 M, and 1.0 M KCl in a stepwise gradient.

In an attempt to isolate the proteins of troponin, the above approach was employed. However, instead of discarding the supernatant remaining after removal of troponin B, it was saved and was referred to as troponin B supernatant. Troponin B was insoluble in low ionic strength media at neutral pH and was dialyzed vs 0.05 mM sodium acetate buffer, pH 4.0, before lyophilization.

The protein components of troponin A, troponin B, and troponin B supernatant were determined from SDS-polyacrylamide gels (figure 20). Troponin A was mainly one component of molecular weight 22,000. Troponin B was found to contain species of molecular weights 40,000, 25,000, and 22,000. Troponin B supernatant contained a major component of molecular weight 25,000 and minor ones of molecular weights 40,000 and 15,000. This method offered an obvious improvement over gel filtration and ion exchange chromatography in the presence of non-denaturing solvents, in that species of molecular weights 25,000 and 22,000 could be isolated in relatively pure form.

4. Superprecipitation as an Indicator of Biological Activity

The necessity to associate the various isolated components of troponin with a known biological activity was realized. The superprecipitation technique of Ebashi (1961) was chosen as a means of elucidating the role of troponin and its constitutents in the actomyosin system.

A typical superprecipitation experiment is presented in figure 21. Curve 1 demonstrates turbidity development of desensitized actomyosin in the presence or in the absence of Ca^{2+}. Curves 2 and 3 point out the inhibitory action of Sephadex G-200 peak 2 material in

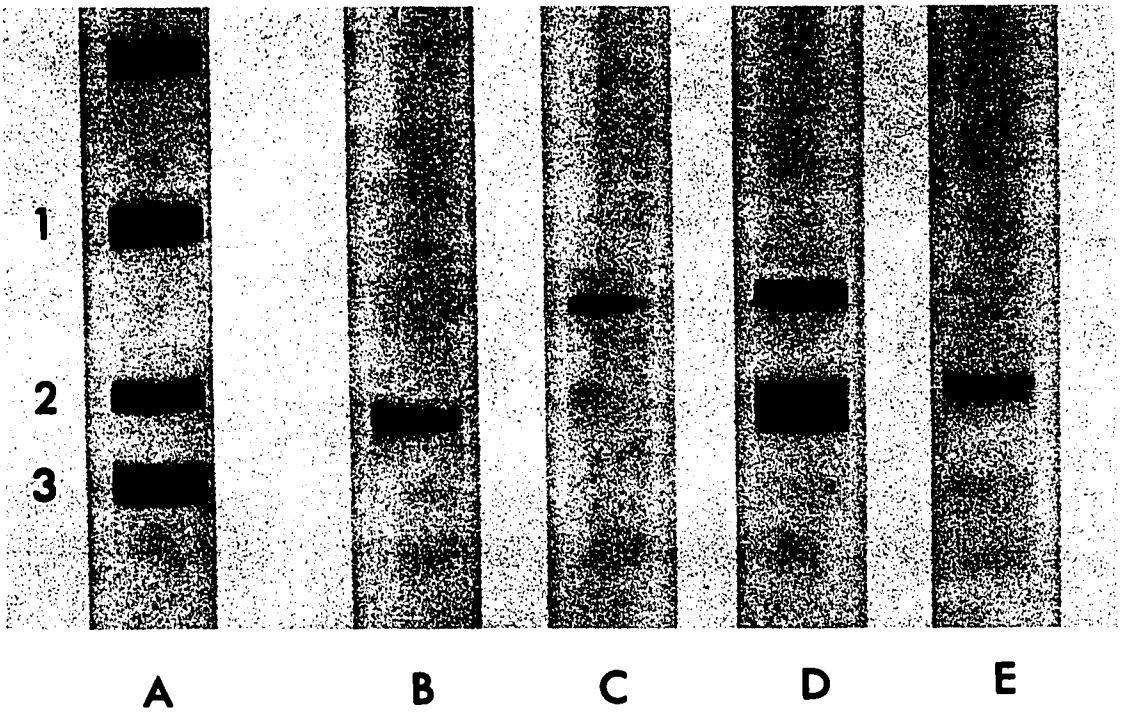


Figure 20. SDS-polyacrylamide gel electrophoresis of protein fractions resulting from Hartshorne and Mueller treatment of crude troponin. Gels B,C, and E contain 5-10 µg troponin A, troponin B, and troponin B supernatant respectively. Gel D contains 20 µg troponin B. Gel A contains a standard mixture of: 1) bovine serum albumin, 2) chymotrypsinogen A, and 3) sperm whale myoglobin. Gels were 5% acrylamide.

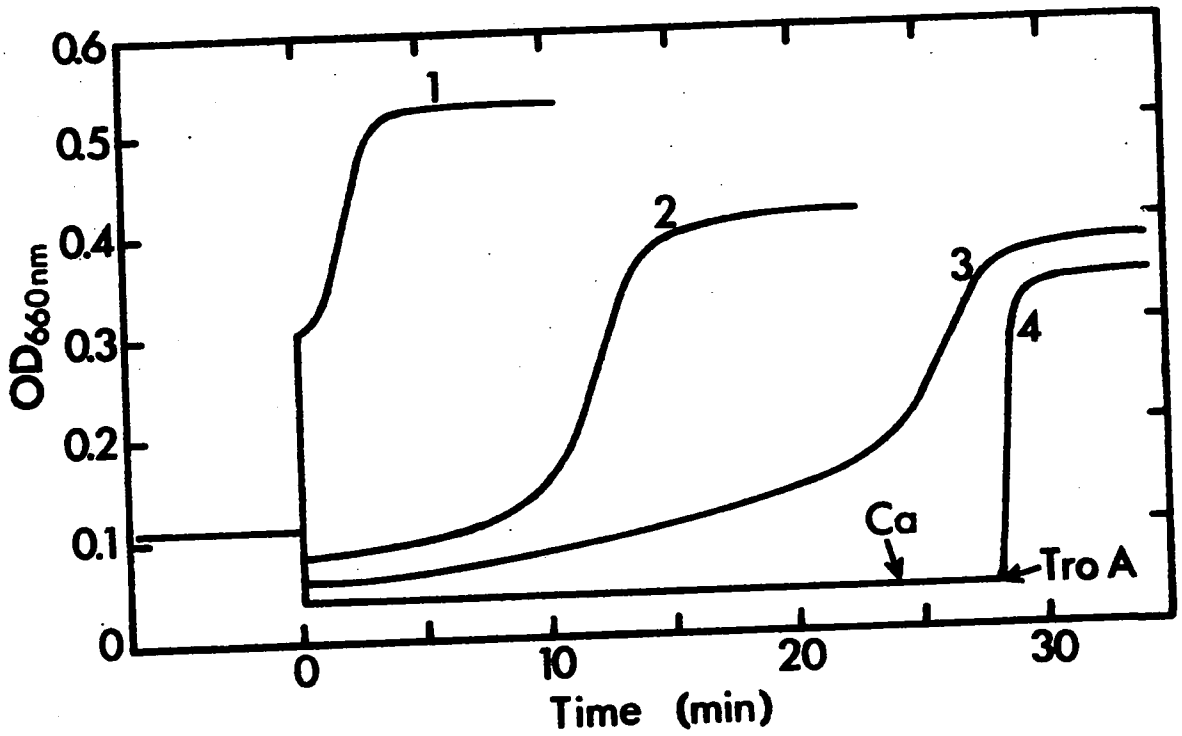


Figure 21. The effect of various troponin fractions on the superprecipitation of desensitized actomyosin. Curve 1 represents the control with or without 0.1 mM $CaCl_2$. Curves 2 and 3 resulted when 16 and 33 µg./ml. respectively of peak 2 material from Sephadex G-200 were added to the system. Curve 4 shows the effect of addition of 50 µg./ml. troponin B. $CaCl_2$ (at a final concentration of 0.1 mM) and troponin A (50 µg./ml.) were added at the times indicated. In each case ATP was added at time 0.

the absence of free Ca^{2+}, as manifested by a delay in the onset of turbidity. The inhibition did not require the addition of tropomyosin. However, Ebashi and Kodama (1966) have shown that limited trypsin treatment may destroy troponin but not tropomyosin, so some tropomyosin may in fact be present. Troponin B gave a Ca^{2+}-insensitive inhibition of superprecipitation which was overcome in the presence of Ca^{2+} by addition of troponin A (curve 4).

The extent of inhibition of superprecipitation was quantitated by measuring the time required to attain maximum turbidity development. In general, difficulties arose in quantitating superprecipitation unless the same preparation of actomyosin was used for all comparative determinations. The inhibitory activity of several of the fractions previously isolated is expressed in table 1. Also presented in this table are the % compositions of the various components of troponin. These were determined by measuring the areas under the peaks in scans of SDS-polyacrylamide gels. The assumption was made that Coomassie Blue stained all proteins to the same degree. As can be seen, the extent of inhibitory activity varies directly as the amount present of the species of molecular weight 25,000. This protein has thus been implicated as the essential inhibitory factor of the troponin system. Worthy of note is the fact that both peak 1 material from Sephadex G-200 and crude troponin contained species of 40,000, 25,000 and 22,000 in approximately equimolar ratios.

TABLE 1

CORRELATION OF COMPOSITION OF THE VARIOUS TROPONIN FRACTIONS WITH THEIR BIOLOGICAL ACTIVITY*

	% Composition of Components of molecular weight			Biological Activity
	40,000	25,000	22,000	
Crude troponin	43	27	25	+++
Peak 1 from Sephadex G-200	50	25	24	++
Peak 2 from Sephadex G-200	< 2	50	25	++++
Peak 3 from QAE-Sephadex treatment of peak 1 material from Sephadex G-200	48	26	26	+++
Peak 4 from QAE-Sephadex treatment of peak 1 material from Sephadex G-200	39	20	31	++
Troponin A (H&M)	< 2	< 2	90	0
Troponin B	51	35	10	—
Troponin B supernatant	5	85	2	+++++

* % compositions were determined planimetrically from areas under the peaks of densitometric scans of SDS acrylamide gels. Biological activities are expressed in time (+ represents about 4 min.) of inhibition of superprecipitation of desensitized actomyosin by 16 µg/ml protein.

B. Troponin A

While attempts were underway to obtain pure inhibitory factor and troponin A, an ether fire occurred in the department. Because of this, the method of treating fresh muscle was altered. The fresh muscle, instead of being subjected to the Bailey (1948) series of organic solvents, was given several low ionic strength washes (Ebashi et al., 1971), after which the muscle residue was treated as outlined in the 'Materials and Methods' section. From this point, yields of troponin were lower and contaminating proteins were more obvious. Troponin B supernatant was no longer 85% inhibitory factor, but instead near 40% inhibitory factor.

Although the isolation of inhibitory factor was unsuccessful, relatively pure troponin A was prepared by three times repetition of the Hartshorne and Mueller (1968) low pH precipitation step. Efforts were shifted to troponin A. At a later stage, troponin A was fractionated by ion exchange chromatography in 6 M urea. For convenience the two preparative approaches will be discussed simultaneously.

The objective was to characterize physicochemically homogeneous troponin A both in the presence and in the absence of Ca^{2+}.

1. Isolation and Purification

Troponin A was prepared by two approaches, which are described below.

Troponin A prepared by the Hartshorne and Mueller (1968) method was 50 - 80% pure as judged from densitometric scans of SDS-polyacrylamide gels. However, by repeating the low pH precipitation

twice more, 90 - 95% pure troponin A could be obtained. Troponin A prepared in this way will subsequently be referred to as Troponin A (H&M). SDS-polyacrylamide gels of troponin A (H&M) appeared as in figure 20. A densitometric scan of a typical gel is presented in figure 22. Small amounts of other protein species were present.

Troponin A was also obtained by DEAE-Sephadex A-25 ion exchange chromatography in 6 M urea. Approximately 200 mg crude troponin A or troponin was dissolved in 10 - 20 ml 50 mM tris pH 8.0, 6 M urea, 1 mM DTT, and was dialyzed against 1000 ml of the same buffer. The protein solution was then applied to a 2.1 cm x 15 cm column of DEAE-Sephadex A-25 equilibrated against the same buffer, and eluted with a linear gradient of KCl to 0.5 M. Such a chromatography is illustrated in figure 23. Troponin A represented but a small fraction of the total optical density units present, however it constituted greater than 1/3 by weight of the total protein applied to the column. The last peak eluted from the column contained nucleic acids. The nucleic acid exhibited hyperchromicity upon addition of pancreatic ribonuclease but not deoxyribonuclease I, indicating that it is an RNA. Upon analytical ultracentrifugation in 50 mM tris pH 7.6, this RNA sedimented as a single boundary of 4-5S at an $OD_{260\ nm}$ of 1. Troponin A prepared in this way will subsequently be referred to as troponin A (urea). SDS-polyacrylamide gel electrophoresis of troponin A (urea) yielded only one protein band (figure 24). A densitometric scan of the gel in figure 24 is presented in figure 25. Contamination by any other protein was not obvious.

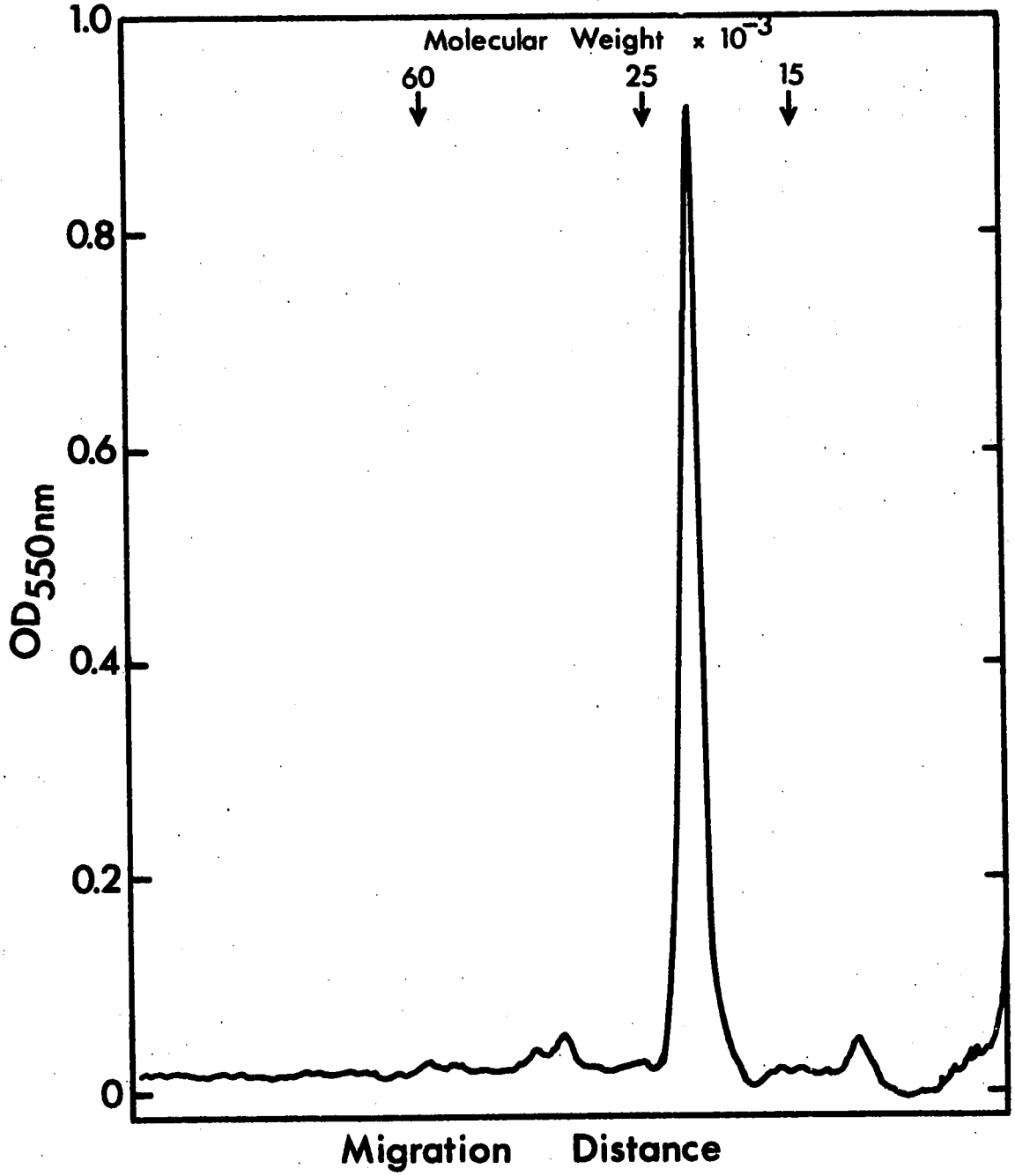


Figure 22. Densitometric scan of an SDS-polyacrylamide gel containing troponin A (H&M). The relationship between protein molecular weight and migration distance, as determined from gels of standard proteins, is also shown.

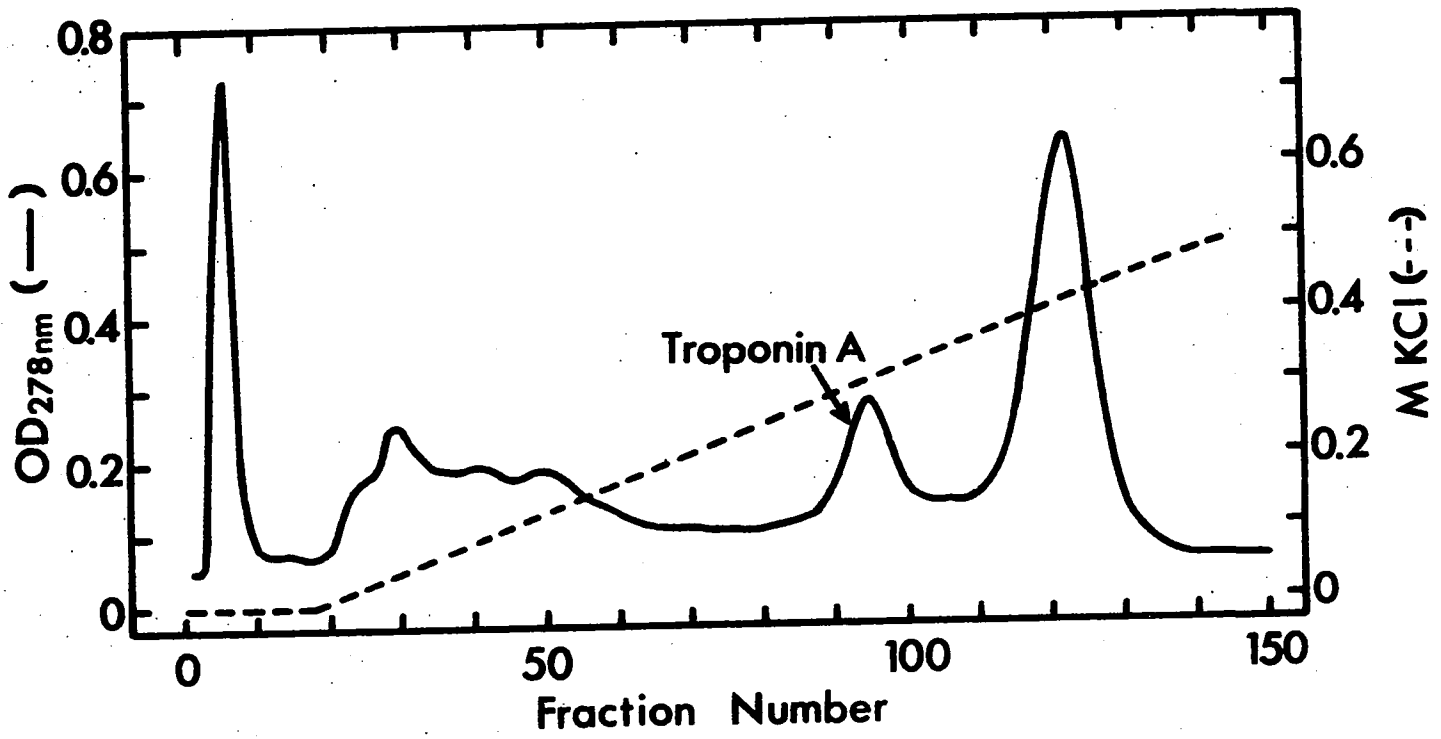


Figure 23. DEAE-Sephadex A-25 column chromatography of crude troponin A. About 180 mg. protein in 10 ml. 50 mM tris pH 8.0, 6 M urea, 0.5 mM DTT was applied to a gel bed (2.1 cm. X 16 cm.) equilibrated against the same buffer and was eluted at 21 ml./hr. with a linear gradient of KCl to 0.6 M. Each fraction contained 6.9 ml.

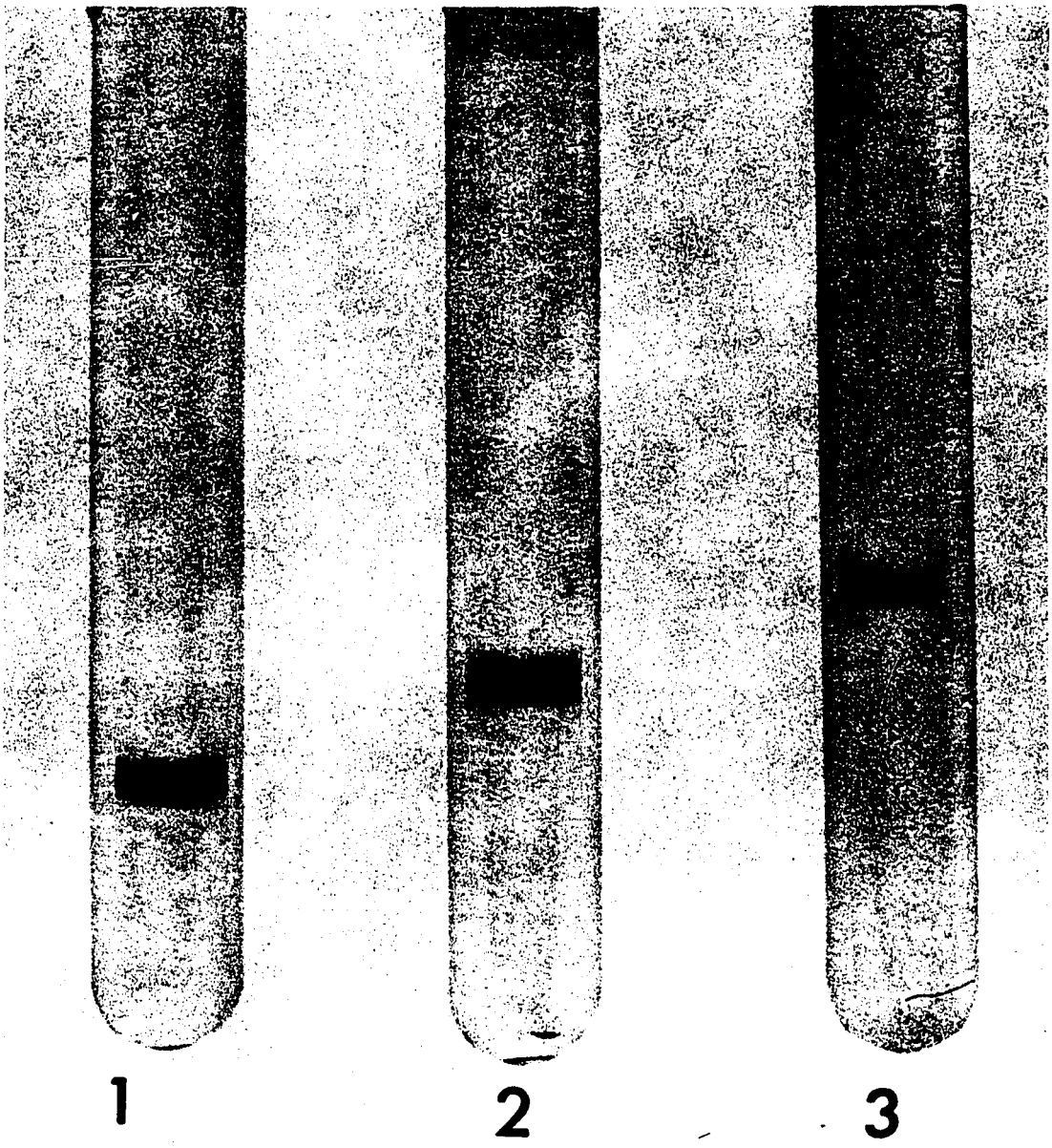


Figure 24. SDS-polyacrylamide gel electrophoresis of troponin A (urea). Gel 1 contained 10 µg lysozyme, gel 2 contained 25 µg troponin A (urea) and gel 3 contained 10 µg chymotrypsinogen A. Gels were 10% acrylamide.

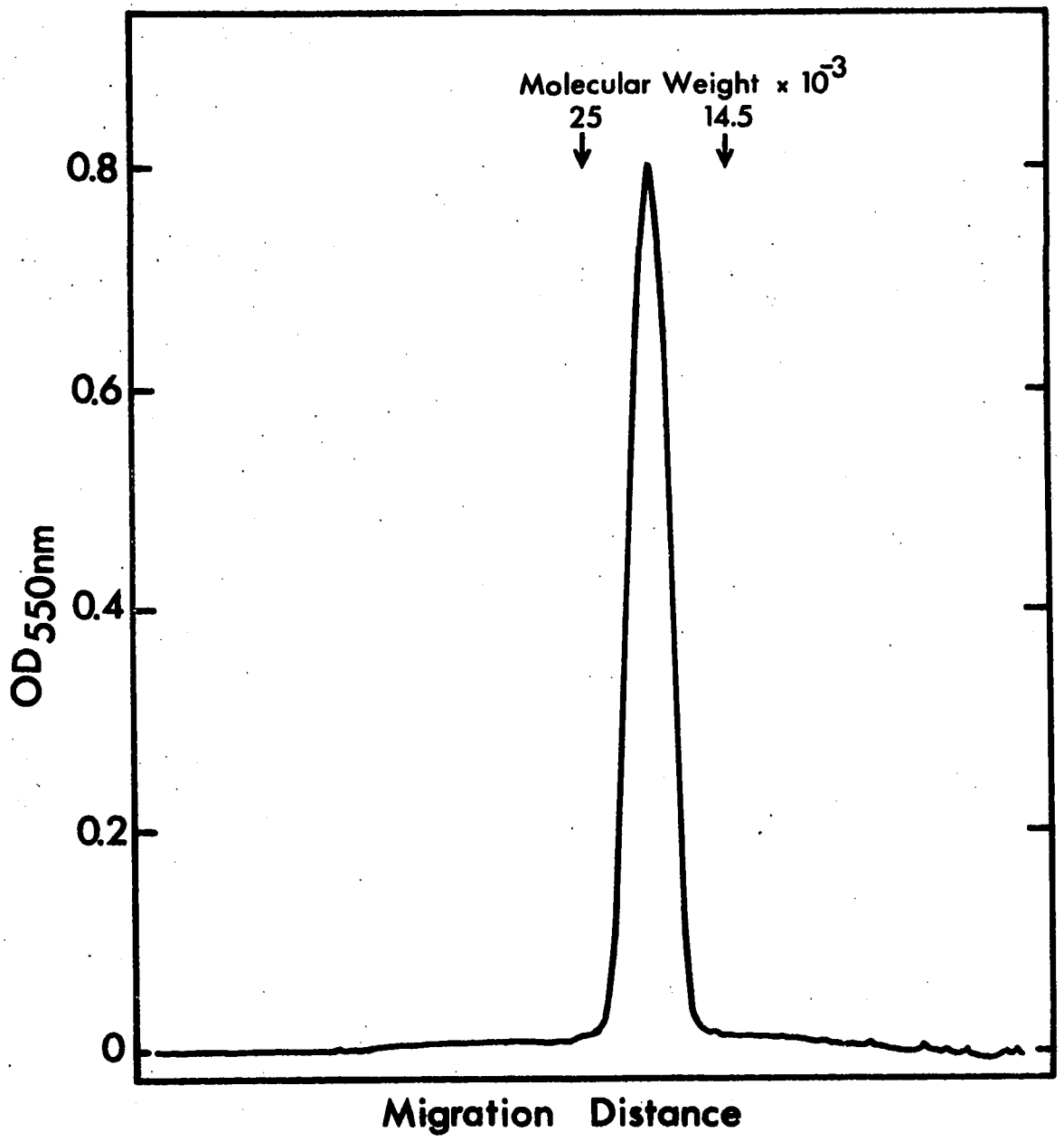


Figure 25. Densitometric scan of an SDS-polyacrylamide gel containing troponin A (urea). The relative migration distances of the standard proteins lysozyme (MW-14,500) and chymotrypsinogen A (MW-25,000) are also indicated.

2. Biological Activity

a. Superprecipitation

The biological activity of troponin A, as measured by the superprecipitation technique, will be mentioned only very briefly since it was not quantitated in any way. Both troponin A preparations displayed an activity similar to that of troponin A in figure 21. In all cases troponin A in the presence of Ca^{2+} overcame the Ca^{2+}-insensitive inhibition of superprecipitation caused by troponin B.

b. Dye binding

Polyanions have the ability to cause shifts in the absorption spectra of certain cationic dyes. Heparin, chondroitin sulfate, and polyglutamic acid at low concentrations shift the absorption maximum of toluidine blue to lower wavelengths. This effect is termed metachromasia. Szent-Gyorgyi and Kaminer (1963) realized that if relaxing protein bound Ca^{2+} strongly, negatively charged groups must be involved, which could cause similar spectral shifts in toluidine blue. Such was the case, and the term 'metin' was coined for the metachromatic component of muscle. Both tropomyosin, the major component of metin, and troponin, the minor component of metin, exhibited metachromasia, the latter to the greater extent (Azuma and Watanabe, 1965, 1965a).

Since troponin A was known to bind Ca^{2+} more strongly than any other myofibrillar protein, its metachromatic properties were investigated. Changes in spectral patterns were accounted for in terms of the theory of Bradley and Wolf (1959). They suggested that dye

molecules stack in solution, and the greater the degree of stacking, the lower will be the λ_{max} of the dye. Polyanions at high dye/polyanion ratios promote stacking of dye molecules. At low dye/polyanion ratios little stacking occurs. Spectral shifts to lower wavelengths occur because the pi electron systems of the stacked dye molecules interact strongly.

The spectrum of toluidine blue was run at each of several troponin A concentrations in 5 mM tris pH 7.6 (figure 26). As the concentration of troponin A was increased, the absorbance at 634 nm decreased. At the same time an absorption band became obvious at about 570 nm. At even higher troponin A concentrations the effect was reversed, that is, the λ_{max} returned to higher wavelengths and the absorbance at 634 nm increased. Plots of $OD_{634\ nm}$ and λ_{max} of the shorter wavelength transition versus troponin A concentration illustrate these relationships (figure 27). These changes were interpreted in terms of the Bradley and Wolf model. At lower concentrations of troponin A, stacking of the dye molecules was promoted due to the high dye/protein ratio. At low dye/protein ratios unstacking of the dye molecules occurred.

The relationship between the change in $OD_{634\ nm}$ and concentration of troponin A is presented in figure 28. The titration end points were determined by extrapolation of the linear limbs of the titration curve (Stone and Bradley, 1967). The end point is not as well defined as in the case of the titration of certain mucopolysaccharides with a variety of dyes. This implies a rather weak binding of toluidine blue to troponin A. The titration end point corresponds to dye/protein ratios of about 40/1 for troponin A (urea)

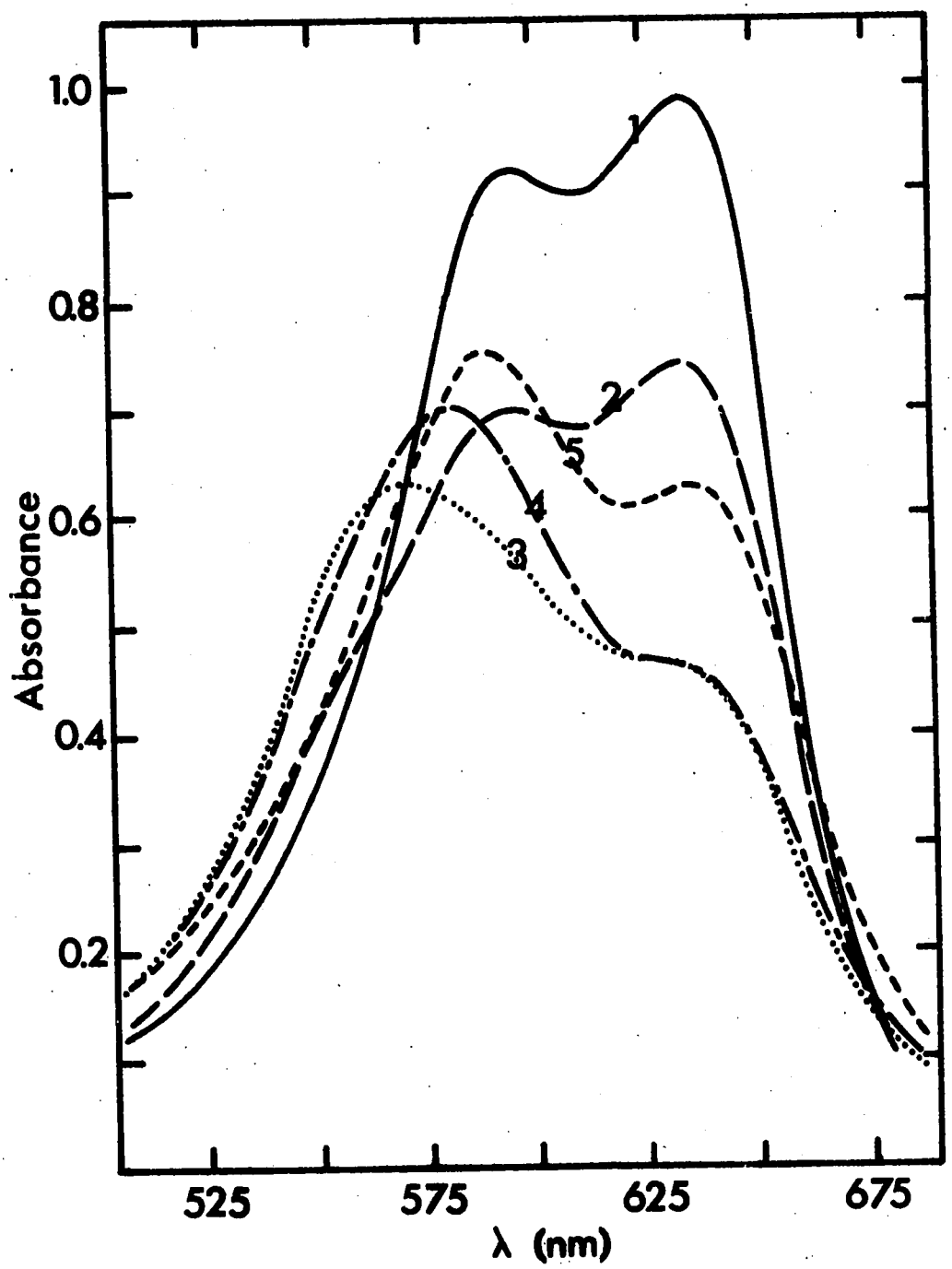


Figure 26. The effect of troponin A (H&M) on the absorption spectrum of toluidine blue. Spectrum 1 resulted from a solution of 2.2×10^{-5} M toluidine blue in 5 mM tris pH 7.6. Spectra 2,3,4 and 5 resulted when 0.025 mg./ml., 0.1 mg./ml., 0.5 mg./ml., and 1.8 mg./ml., respectively, of troponin A (H&M) were added.

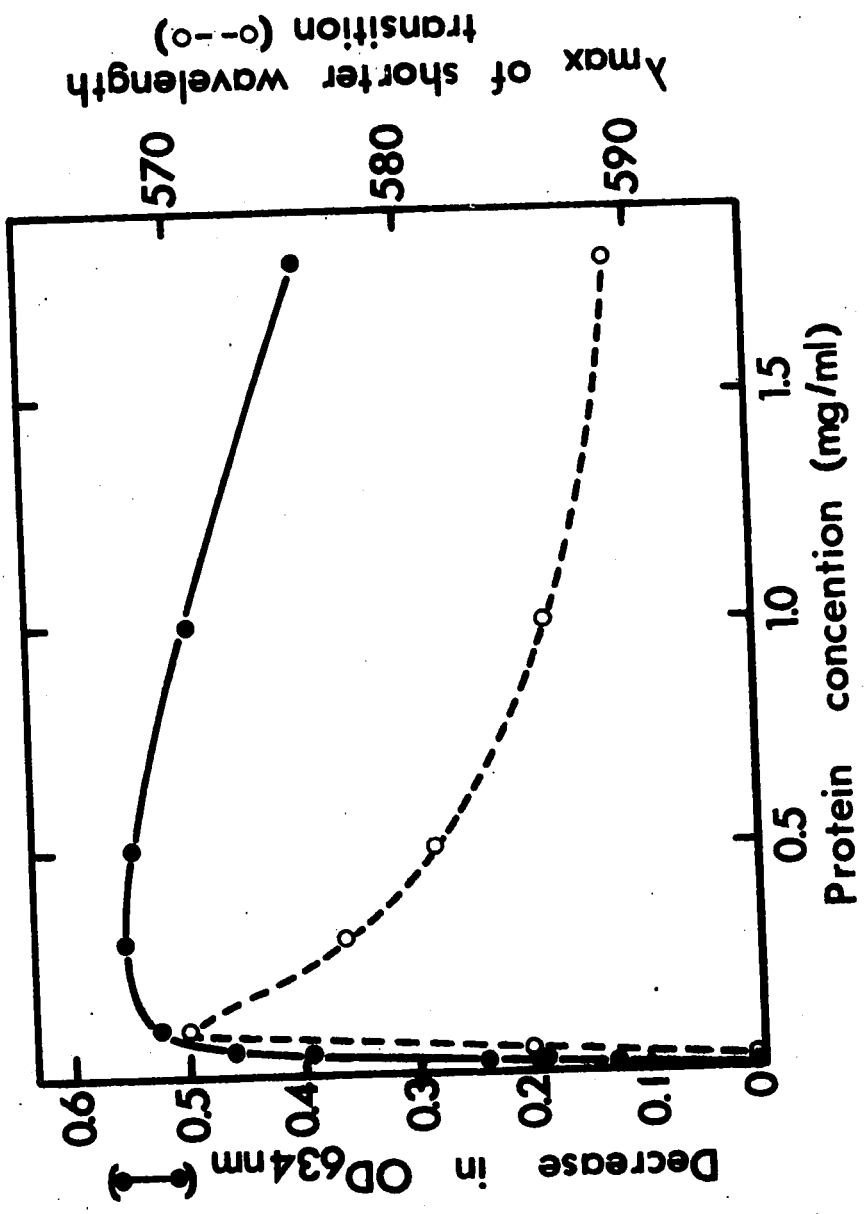


Figure 27. The $OD_{634\ nm}$ and the λ_{max} of the shorter wavelength transition of toluidine blue as a function of troponin A (H&M) concentration. Toluidine blue concentration was 2.2×10^{-5} M. Solvent - 5 mM tris pH 7.6.

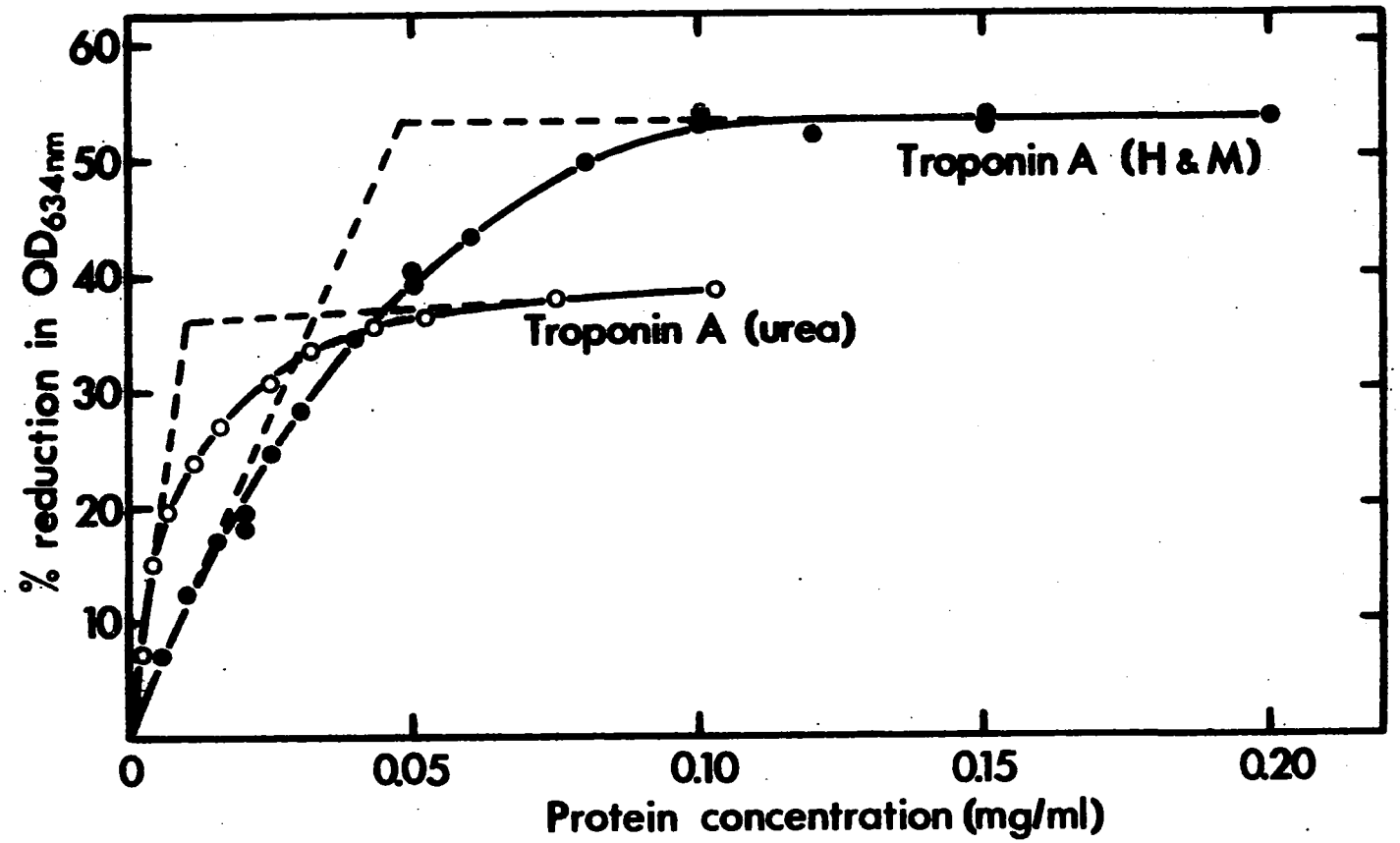


Figure 28. The change in $OD_{634\ nm}$ of toluidine blue with addition of troponin A (urea) and troponin A (H&M). Solvents were 5 mM tris pH 7.6 (●) and 5 mM tris pH 7.6, 1 mM EGTA (○). Toluidine blue concentration - 2.2×10^{-5} M. Determination of end points is denoted by the broken lines.

and about 12/1 for troponin A (H&M). The titration of troponin A (urea) was carried out in 5 mM tris pH 7.6, 1 mM EGTA, while that of troponin A (H&M) was in 5 mM tris pH 7.6. The addition of 1 mM EGTA to toluidine blue solutions caused no significant spectral changes. However, EGTA does serve to remove most of the bound Ca^{2+} from troponin A. If each Ca^{2+}-binding site of troponin A is similar to that of carp myogen, being composed of four side chain carboxyl groups (Nockolds et al., 1972), one would expect EGTA to release several carboxyl groups from coordination with Ca^{2+}. Toluidine blue presumably interacts with troponin A carboxyl groups. Addition of EGTA then would be expected to allow more dye molecules to interact with each troponin A molecule. This does appear to be the case. Although the data cannot be precisely quantitated at this stage, it does suggest that a correlation exists between the ability of troponin A to bind Ca^{2+} and its ability to bind toluidine blue.

3. Hydrodynamic Properties

a. Sedimentation velocity

The sedimentation properties of both troponin A preparations were investigated. In all cases single symmetrical peaks were observed with Schlieren optics. Characteristic $S_{20,w}$ versus protein concentration plots for troponin A (H&M) in 50 mM tris pH 7.6 and in 1 M KCl, 50 mM tris pH 7.6 are shown in figure 29. The difference in ionic strength was reflected by differences in the slopes of these plots. As was expected the slope was greater at the low ionic strength due to the greater interaction between the sedimenting protein molecules, although $S^{\circ}_{20,w}$ values were similar in the two cases.

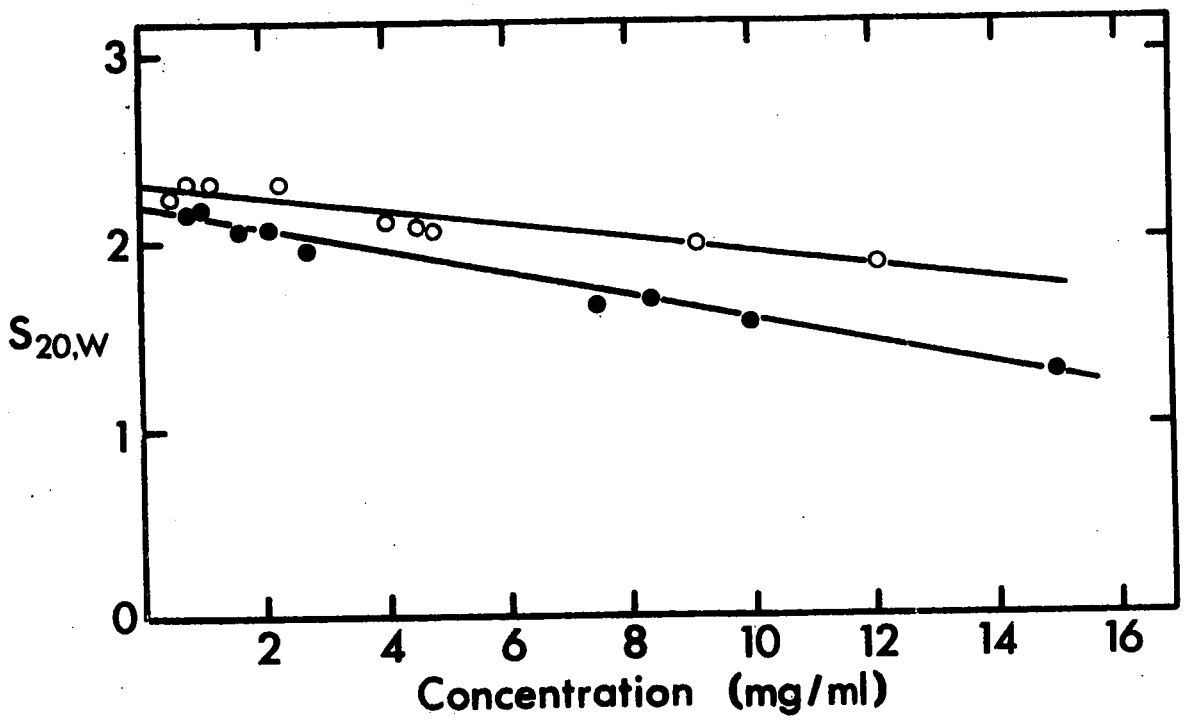


Figure 29. Concentration dependence of the $S_{20,w}$ values of troponin A (H&M). Solvents - 50 mM tris pH 7.6 (●) and 50 mM tris pH 7.6, 1 M KCl (○).

Sedimentation profiles of troponin A (urea) in 50 mM tris pH 7.6, 5×10^{-5} M $CaCl_2$, and in 50 mM tris pH 7.6, 1 mM EGTA are shown in figure 30. The concentration dependence of $S_{20,w}$ values of troponin A (urea) in the presence and absence of Ca^{2+} was determined (figure 31). Of importance is the fact that addition of 5×10^{-5} M $CaCl_2$ to troponin A (urea) causes an increase of 17% in the $S^{\circ}_{20,w}$ value, with no concomitant alteration in molecular weight (see below), implying a compacting of molecular conformation. A summary of $S^{\circ}_{20,w}$ values as well as slopes of $S_{20,w}$ vs concentration plots is presented in table 2.

b. Molecular weight

(i) SDS-polyacrylamide gel electrophoresis

By employing proteins of known molecular weights, the molecular weight of troponin A was determined in the usual fashion. Troponin A (H&M) was consistently found to have a molecular weight of 22,000. The molecular weight of troponin A (urea) was between 19,000 and 20,000.

(ii) Sedimentation equilibrium

High speed sedimentation equilibrium studies on troponin A resulted in linear plots of ln y vs r^2. A typical plot is shown in figure 32. Troponin A (H&M) was shown, from analysis of 7 runs, to have a minimum molecular weight of 22,200 ± 500 in 50 mM tris pH 7.6. Troponin A (urea) was found from analysis of 7 high speed runs to have a minimum molecular weight of 22,200 ± 600. The solvent in this case was 50 mM tris pH 7.6 with free $CaCl_2$ concentrations ranging from 10^{-5} to 5×10^{-4} M. In the presence of 10^{-4} M EGTA, 3 distinct experiments gave a minimum molecular weight for troponin A (urea) of

Figure 30. Sedimentation profiles of troponin A (urea) in the presence of Ca^{2+} and in the presence of EGTA. Two Kel F cells were used, one with a wedge window. Protein concentrations were 6 0 mg./ml. in both cases. Solvents - 50 mM tris pH 7.6, 5 X 10^{-5} M $CaCl_2$ (upper) and 50 mM tris pH 7.6, 10^{-3} M EGTA (lower). Time of photograph after reaching speed is indicated. Bar angle - 55^{o}.

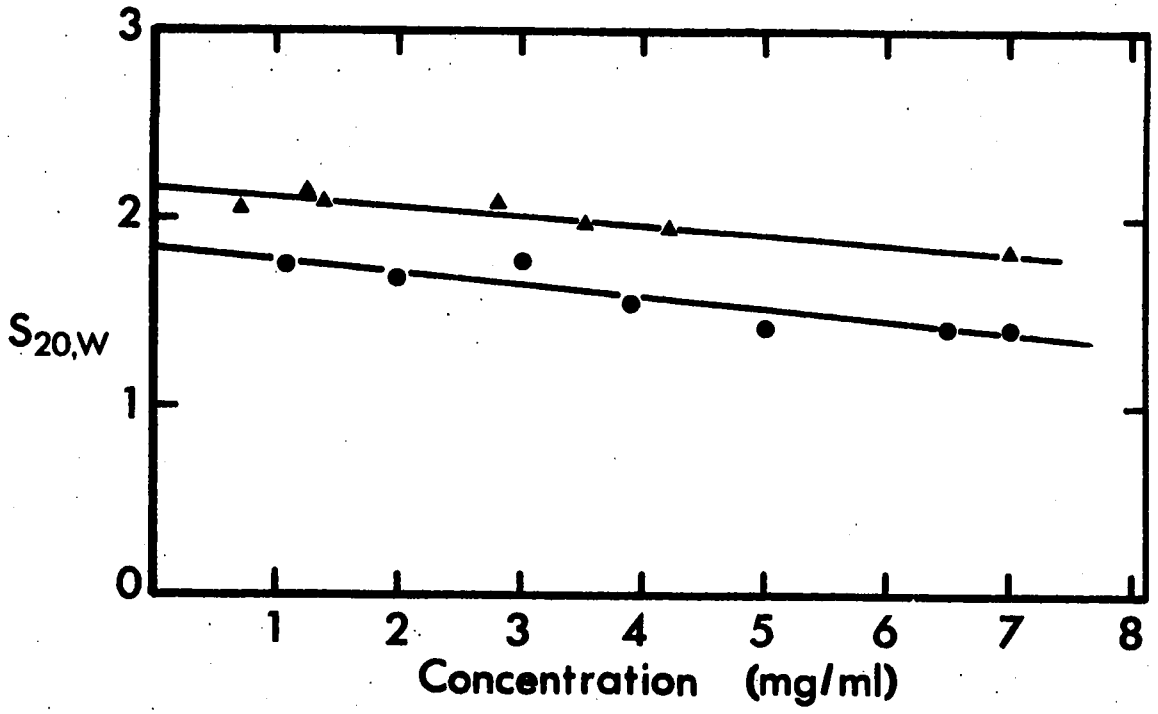


Figure 31. Concentration dependence of the $S_{20,w}$ of troponin A (urea) in the presence of Ca^{2+} and in the presence of EGTA. Solvents - 50 mM tris pH 7.6, 5 X 10^{-5} M $CaCl_2$ (▲) and 50 mM tris pH 7.6, 1 mM EGTA (●).

TABLE 2

SEDIMENTATION DATA ON TROPONIN A

	Buffer	$S^{\circ}_{20,w}$	Slope of $S_{20,w}$ vs. concentration plot
troponin A (H&M)	50 mM tris pH 7.6	2.20	-0.62
"	" , 1 M KCl	2.30	-0.37
troponin A (urea)	" , 10^{-3} M EGTA	1.84	-0.67
"	" , 10^{-5} M $CaCl_2$	2.15	-0.45

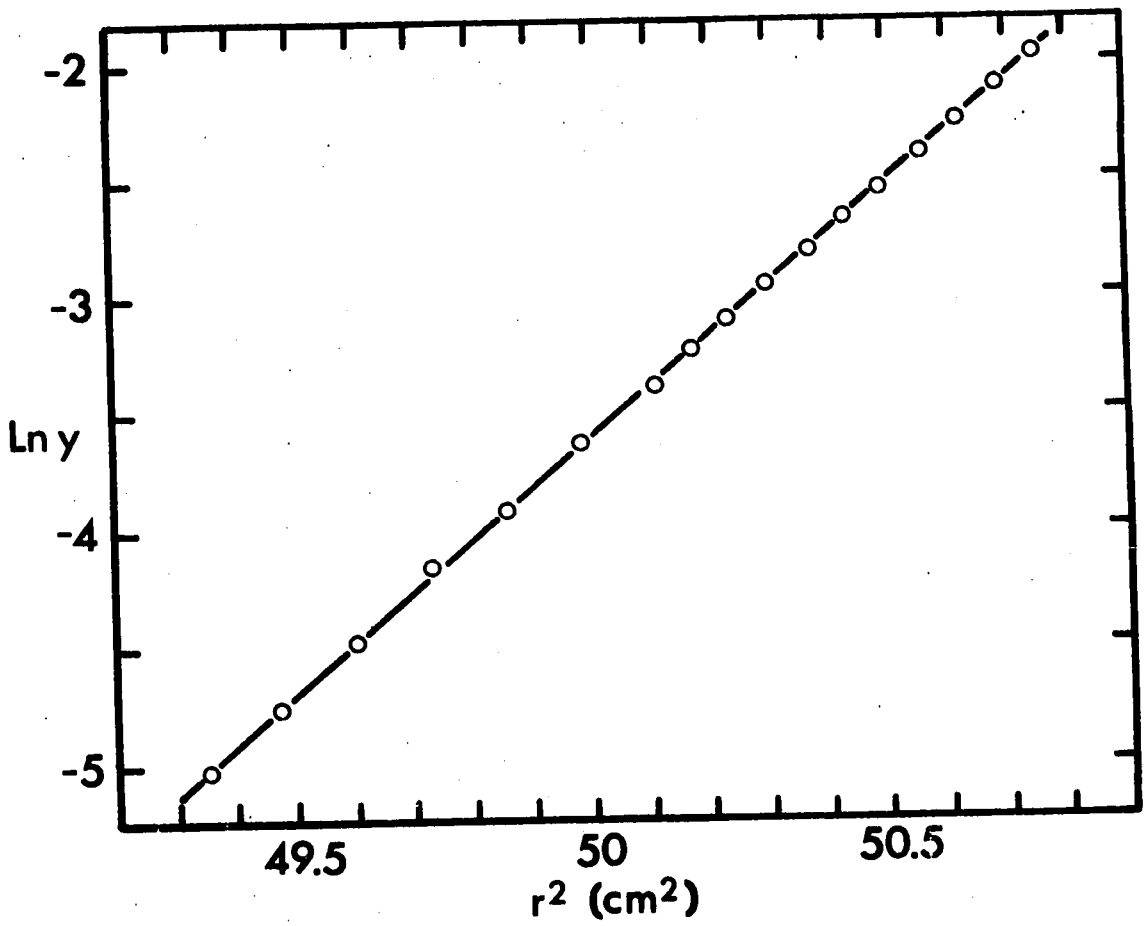


Figure 32. High speed sedimentation equilibrium ln y vs r^2 plot for troponin A (H&M). Initial protein concentration - 0.5 mg./ml. Speed - 40,000 rpm. Solvent - 50 mM tris pH 7.6.

21,800 ± 300. Results from high speed experiments are summarized in table 3. These high speed experiments were carried out at 40,000 to 44,000 rpm with equilibration times of about 24 hours.

Low speed sedimentation equilibrium runs were performed to establish the relationship between the molecular weight and the troponin A concentration. Typical ln y vs r^2 plots from low speed experiments on troponin A (urea) are shown in figure 33. The buffers are indicated in the figure legend. At free Ca^{2+} concentrations of 10^{-5} M, 5×10^{-5} M, and 10^{-4} M, the molecular weight vs concentration plots (figure 34) were similar and showed only evidence of slight aggregation. In 10^{-3} M EGTA the molecular weights of troponin A (urea) were about 2,000 lower. With addition of 10^{-3} M $CaCl_2$, aggregation of troponin A (urea) was apparent (figure 34). Plots of molecular weight vs concentration at different initial loading concentrations did not overlap. Aggregation to weight-average molecular weights of approximately 40,000 occurred.

In the case of troponin A (H&M), the $CaCl_2$ level was not controlled, but was probably less than 10^{-5} M. Typical plots of ln y vs r^2 are shown in figure 35. The solvent system was 50 mM tris pH 7.6. Plots of molecular weight vs concentration (figure 36) from several different low speed experiments show the tendency of this troponin A preparation to aggregate at higher concentrations. Superimposed on the figure is a line representing the best polynomial fit of the data. Molecular weights in excess of 60,000 are evident. The high weight-average molecular weights may be due to high molecular weight contaminants in the preparation, or to complexing of troponin A with low molecular weight contaminants such as inhibitory factor.

TABLE 3

HIGH SPEED SEDIMENTATION EQUILIBRIUM

MOLECULAR WEIGHTS OF TROPONIN A

Troponin A (H&M)

Run #		Molecular Weight
1.	50 mM tris pH 7.6	22,000
2.	"	22,000
3.	"	22,400
4.	"	22,700
5.	50 mM tris pH 7.6, 1 M KCl	22,700
6.	"	22,200
7.	"	21,900
	average	22,200

Troponin A (urea)

8.	59 mM tris pH 7.6	22,000
9.	50 mM tris pH 7.6, 10^{-5} M $CaCl_2$	21,200
10.	"	22,250
11.	50 mM tris pH 7.6, 5 x 10^{-4} M $CaCl_2$	22,700
12.	"	22,200
13.	50 mM tris pH 7.6, 10^{-4} M $CaCl_2$	22,150
14.	"	22,900
	average	22,200
15.	50 mM tris pH 7.6, 1 mM EGTA	21,500
16.	"	22,100
17.	"	21,900
	average	21,800

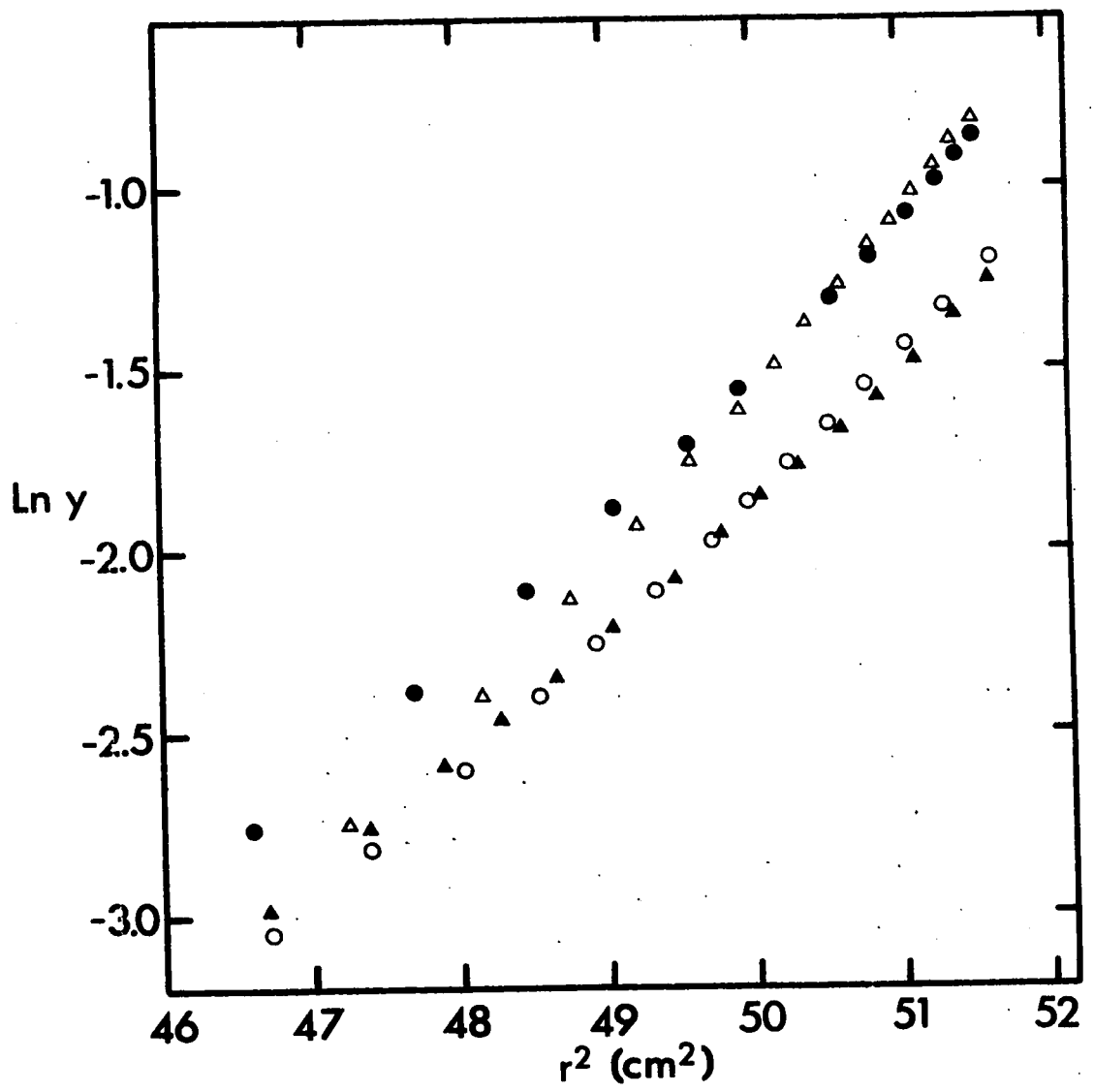


Figure 33. Low speed sedimentation equilibrium ln y vs r^2 plots for troponin A (urea). Operating conditions are tabulated below:

	▲	○	△	●
Initial loading concentration (mg./ml.)	1.04	1.11	1.75	1.54
Speed (rpm)	14,000	16,000	18,000	18,000
Solvent - 50 mM tris pH 7.6 plus..	10^{-3}M $CaCl_2$	10^{-5}M $CaCl_2$	10^{-4}M $CaCl_2$	10^{-3}M EGTA

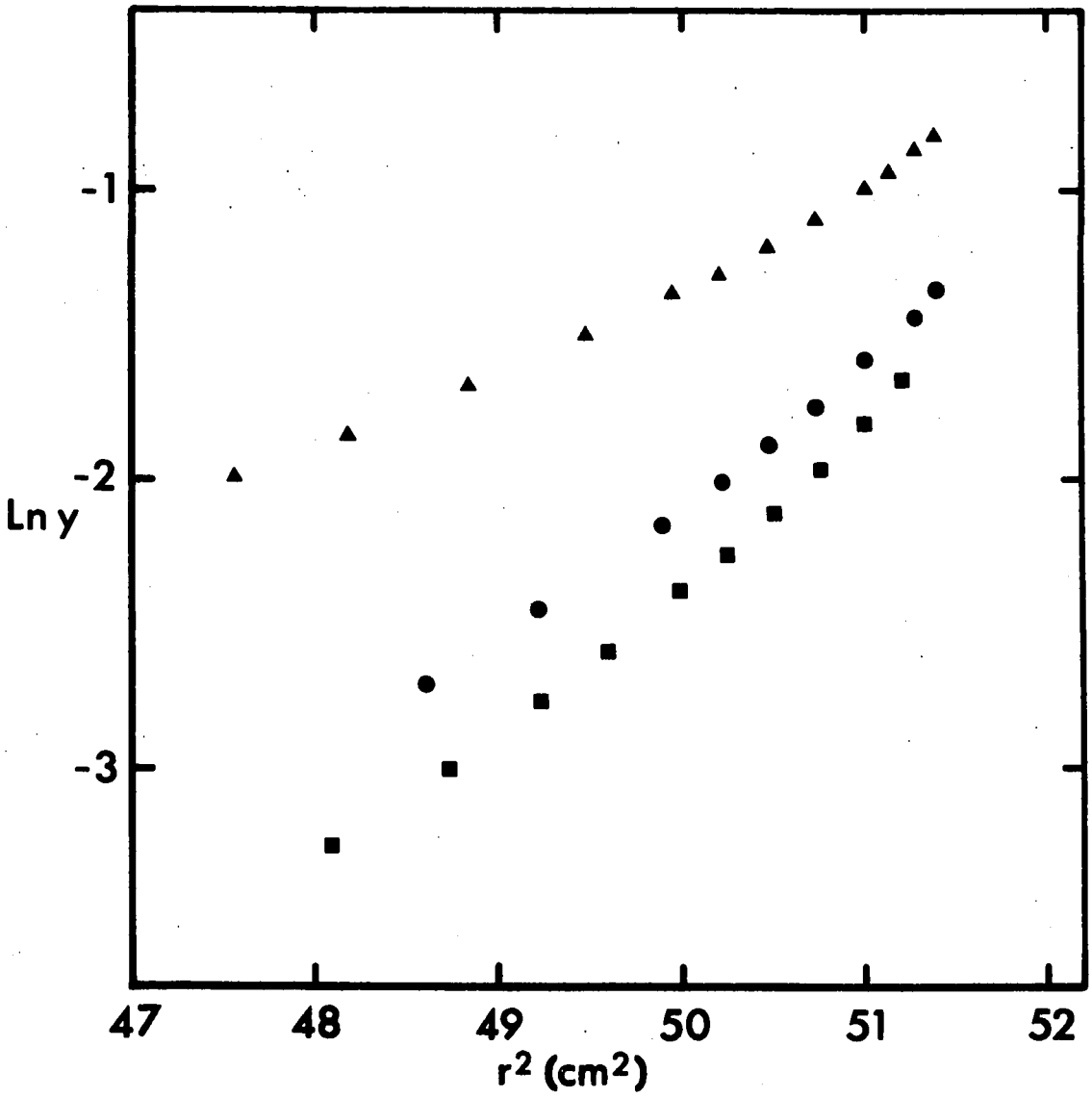


Figure 35. Low speed sedimentation equilibrium ln y vs r^2 plots for troponin A (H&M). Initial protein concentrations were 0.75 mg./ml. (■), 1.0 mg./ml. (●), and 1.9 mg./ml. (▲). Speeds were 18,000 (■,●) and 14,000 (▲) rpm. Solvent - 50 mM tris pH 7.6.

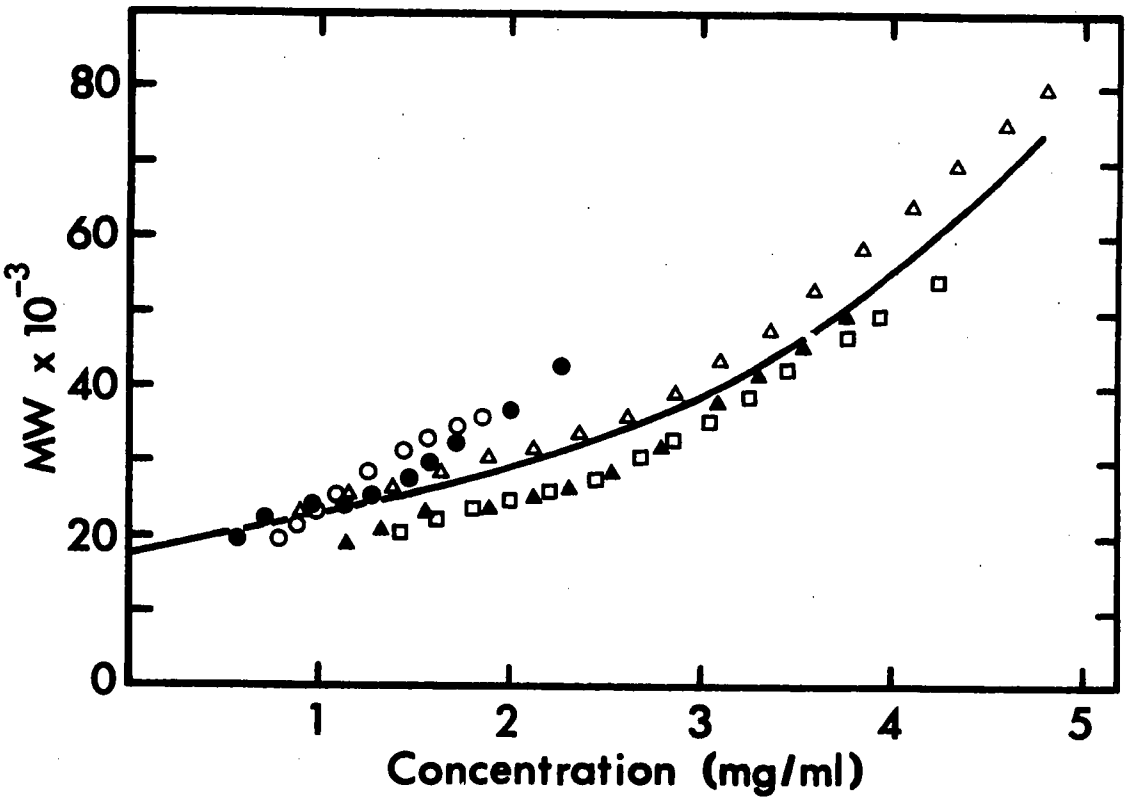


Figure 36. Concentration dependence of the molecular weight of troponin A (H&M). Solvent - 50 mM tris pH 7.6. Each series of points represents a separate sedimentation equilibrium experiment.

In summary, troponin A has a minimum molecular weight of 22,200 which in the case of troponin A (urea), increases to 24,000 - 26,000 at protein concentrations of about 4 mg/ml in a solvent containing 50 mM tris pH 7.6 and Ca^{2+} concentrations of less than 10^{-4} M. Above this Ca^{2+} level, more extensive aggregation of the protein occurs.

c. Viscosity

In order to obtain additional information concerning molecular shape, measurements of the viscosity of troponin A were undertaken. Plots of reduced viscosity (η_{sp}/c) vs concentration for troponin A (urea) in 50 mM tris pH 7.6 with either 10^{-5} M free Ca^{2+} or 1 mM EGTA are shown in figure 37. The Ca^{2+} effect is clearly obvious. In the presence of 10^{-5} M $CaCl_2$, the intrinsic viscosity ([η]) was 0.033 dl/g, while in the absence of Ca^{2+} [η] was 0.085 dl/g. Troponin A becomes less asymmetric in the presence of Ca^{2+}.

The plot of η_{sp}/c vs concentration for troponin A (H&M) in 50 mM tris pH 7.6, 1 M KCl (figure 37) deviates from linearity. If this deviation is real, it can be explained by aggregation at higher concentrations, which has already been suggested from molecular weight studies.

d. Troponin as a prolate ellipsoid of revolution

Hydrodynamic properties of proteins reflect the overall size and shape of the molecules. Since the hydrodynamic properties of troponin A vary with the amount of Ca^{2+} present, it is of interest to represent this protein in terms of equivalent hydrodynamic models in the presence and in the absence of Ca^{2+}. When interpreting hydrodynamic

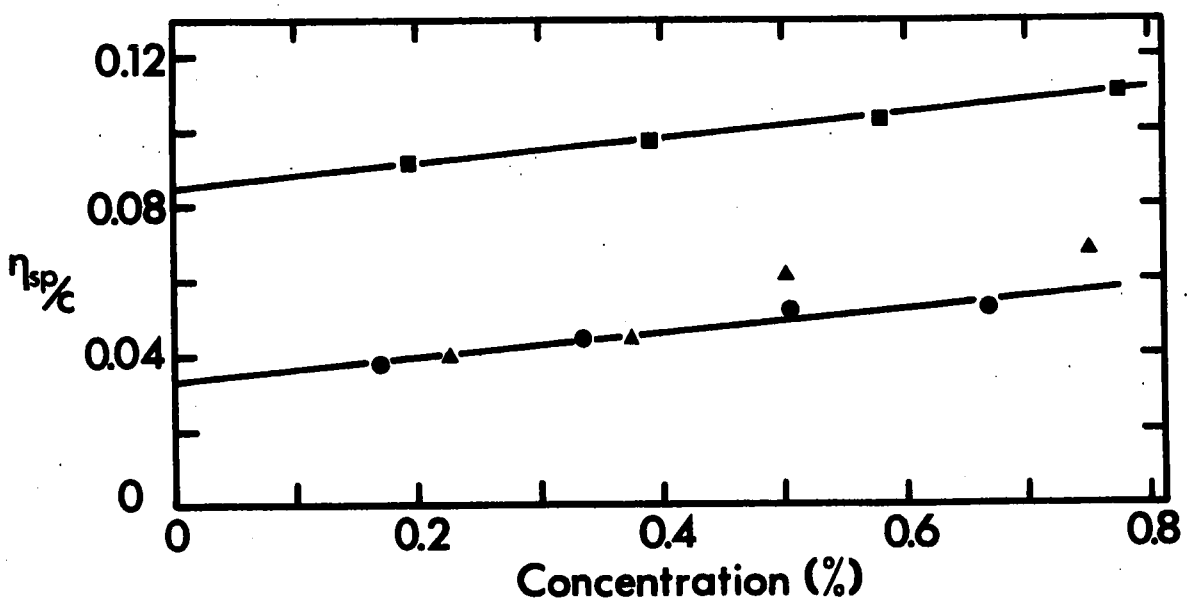


Figure 37. The concentration dependence of the reduced viscosity, η_{sp}/c, of troponin A. The symbols ■ and ● are for troponin A (urea) in 50 mM tris pH 7.6, 1 mM EGTA and in 50 mM tris pH 7.6, 10^{-5} M $CaCl_2$, respectively. The symbol ▲ is for troponin A (H&M) in 50 mM tris pH 7.6, 1 M KCl.

parameters in terms of equivalent models, limitations exist. For example, the degree of hydration is not precisely known, and furthermore the proposed model may not resemble the actual protein structure in solution.

Scheraga and Mandelkern (1953) have made available an equation which incorporates several hydrodynamic parameters to yield a value, β, which is a function of the axial ratio of the molecule but not of its effective particle volume. It is represented thus:

$$\beta = \frac{N \times S^{\circ}_{20,w} \times [\eta]^{1/3} \times \eta_o}{M^{2/3} \times (1 - \bar{v}\rho)}$$

where N = Avogadro's number

$S^{\circ}_{20,w}$ = intrinsic sedimentation coefficient of the protein

$[\eta]$ = weight intrinsic viscosity of the protein

η_o = solvent viscosity

$\bar{v}$ = partial specific volume of the protein

ρ = solvent density

M = molecular weight of the protein

β-values were calculated for troponin A (urea) in 50 mM tris pH 7.6 in the presence of 10^{-5} M free Ca^{2+} and in the presence of 10^{-3} M EGTA. Table 4 summarizes the information used in the calculations. β values were 2.00×10^6 in the presence of 10^{-5} M $CaCl_2$ and 2.34×10^6 in the presence of 10^{-3} M EGTA. Values for the viscosity increment, γ, and the axial ratio of a prolate ellipsoid of revolution equal in value to troponin A (urea) in the presence of 10^{-3} M EGTA were

TABLE 4

CALCULATION OF β-VALUES FOR TROPONIN A (UREA)

Parameters in each of two buffers:

	50 mM tris pH 7.6, 10^{-5} M $CaCl_2$	50 mM tris pH 7.6, 10^{-3} M EGTA
$S_{20,w}$	2.15×10^{-3} sec	1.84×10^{-13} sec
$[\eta]$	3.32 ml/g	8.48 ml/g
M	22,200	22,200
$\bar{v}$	0.73 ml/g	0.73 ml/g
η_o	0.01022 poise	0.01022 poise
ρ	1.0015 g/ml	1.0015 g/ml
β	2.00×10^6	2.34×10^6

obtained with use of the calculated β-value from tables presented by Scheraga (1961). In 10^{-3} M EGTA the viscosity increment was 9.67 and the axial ratio was 7.7 for an equivalent ellipsoid of revolution.

The viscosity increment, γ, is related to the intrinsic viscosity, [η], and the effective volume, V_e, by the expression:

$$V_e = \frac{[\eta]}{\gamma}$$

The effective volume of troponin A (urea) in 50 mM tris pH 7.6, 10^{-3} M EGTA, calculated from the above equation, was 0.877 ml/g. Hydration increased the apparent specific volume by 0.147 ml/g.

Difficulty arises in obtaining accurate axial ratios for equivalent ellipsoids of revolution from low β-values. The β-value for troponin A (urea) in the presence of 10^{-5} M $CaCl_2$ was 2.0×10^6, which is below the theoretical minimum value of 2.12×10^6. Creeth and Knight (1965) have shown that several proteins including bovine serum albumin, human γ-globulin, and thyroglobin exhibit β-values of below 2.12×10^6. Such values may occur, if one assumes for a hydrodynamic model, a rigid string of spherical beads (Holtzer and Lowey, 1963), or a partially draining model (Holcomb and Van Holde, 1962). A low β-value may also be attributed to inaccuracy of the sedimentation, viscosity and/or molecular weight data. The viscosity increment is somewhat more sensitive to changes in axial ratio than is the β-value. The viscosity increment for troponin A (urea) was calculated using an effective volume of 0.877 ml/g and an intrinsic viscosity of 3.32 ml/g. A value corresponding to an axial ratio of 3.1 was obtained.

The length of a prolate ellipsoid of revolution is calculable from an equation of Yang (1961):

$$L = 6.82 \times 10^{-8} ([\eta]M)^{1/3} (p^2/\gamma)^{1/3},$$

where L is the length in cm, p is the axial ratio, and the other parameters are as previously specified.

The dimensions of a prolate ellipsoid of revolution equivalent in volume to troponin A (urea) in the absence and presence of Ca^{2+} are shown below:

	$-Ca^{2+}$	$+Ca^{2+}$
axial ratio	7.7	3.1
length	154 Å	84 Å
diameter	20 Å	27 Å

The length of troponin A (urea) decreased nearly 50% with addition of only 10^{-5} M $CaCl_2$. As indicated earlier, caution must be exercised when interpreting such data in terms of the real dimensions of the molecule in solution. However, the fact remains that the addition of Ca^{2+} does cause an overall compacting of the molecular structure of troponin A (urea).

4. Amino Acid Analysis

The amino acid analysis of troponin A was unusual in some respects. The analyses of troponin A (H&M) and troponin A (urea) are summarized in table 5. The results are expressed in moles amino acid

TABLE 5

AMINO ACID COMPOSITION OF TROPONIN A (H&M) AND TROPONIN A (UREA)

	Troponin A (H&M)		Troponin A (urea)	
	residues per 10^5 g protein	moles per mole protein	residues per 10^5 g protein	moles per mole protein
lysine	58.2	12.9	52.7	11.7
histidine	8.2	1.8	7.0	1.56
arginine	36.8	8.1	36.0	8.0
aspartic acid	114.1	25.1	113.1	25.1
threonine	32.7	7.2	30.6	6.8
serine	38.1	8.4	37.4	8.3
glutamic acid	149.1	33.9	167.6	37.2
proline	18.5	4.1	14.5	3.22
glycine	67.7	14.9	65.8	14.6
alanine	71.8	15.8	67.1	14.9
cysteine	-	-	5.54*	1.23*
valine	44.1	9.7	52.3	11.6
methionine	45.9	10.1	46.8 (45.9*)	10.4 (10.2*)
isoleucine	48.6	10.7	47.3	10.5
leucine	58.6	12.9	48.2	10.7
tyrosine	13.4	3.0	9.5	2.11
phenylalanine	49.8	11.0	48.6	10.8
tryptophan	-	-	nil**	nil**

* Determined after performic acid oxidation (Moore, 1963).

** Determined by the procedure of Oprenska-Blauth et al. (1963).

per 10^5 g protein and in moles amino acid per mole of protein. The experimentally obtained value of 22,200 was used as the molecular weight of troponin A. The two preparative approaches yielded proteins very similar in amino acid composition. Troponin A was found to be a very acidic protein, containing 3 times as many acidic residues as basic ones. It contained histidine, proline, and tyrosine in small amounts, and tryptophan was not present. One mole cysteic acid was found per mole of oxidized troponin A (urea) from amino acid analysis and the colorimetric method of Sedlak and Lindsay (1968) yielded 0.98 moles free sulfhydryl per mole protein. This indicates that native troponin contains 1 mole of cysteine per mole protein. The molar ratio of phenylalanine to tyrosine was 5.5 for troponin A (urea). This high ratio is reflected in the way in which phenylalanine dominates the UV spectrum of this protein (figure 38).

The partial specific volume of troponin A was determined by making use of theoretical residue specific volumes listed by Kouba (1965) and the weight fraction of each amino acid residue calculated to be present in troponin A (urea). The precise computation of partial specific volume is outlined in table 6, from which a value of 0.726 ml/g was established.

Addition of partial residue weights (table 6) and subtraction of the weight of water lost upon peptide bond formation allowed calculation of a minimum molecular weight of 21,461 for troponin A (urea). This value is in good agreement with the value of 22,200 obtained from high speed sedimentation equilibrium experiments.

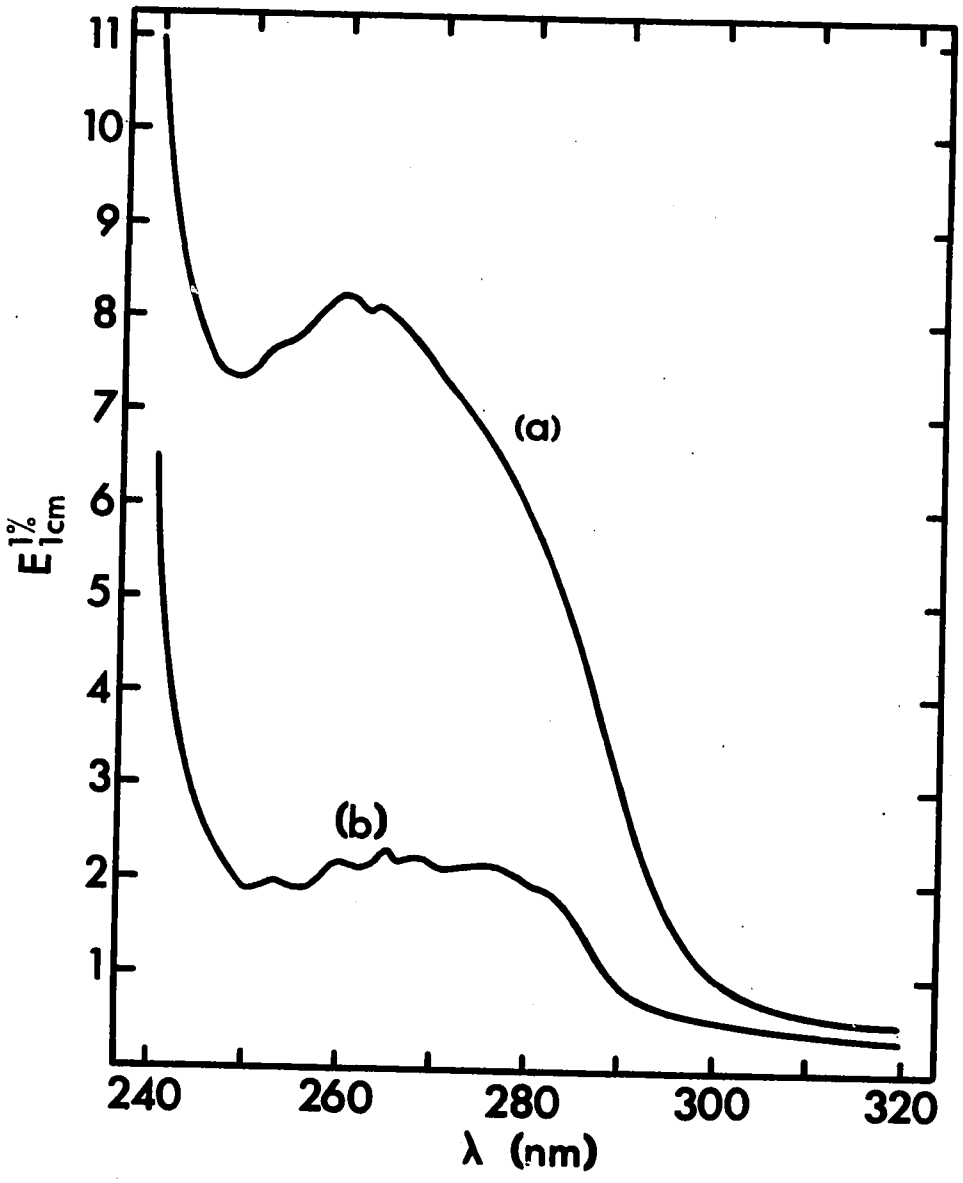


Figure 38. UV absorption spectra of troponin A. Spectra of troponin A (H&M) and troponin A (urea) are indicated by (a) and (b), respectively. Solvent - 50 mM tris pH 7.6.

TABLE 6

CALCULATION OF PARTIAL SPECIFIC VOLUME OF TROPONIN A (UREA) FROM THE AMINO ACID ANALYSES

	Nearest Whole Number of Residue per 22,200 g protein	Residue Molecular Weight	Partial Weight	Residue Specific Volume	Residue Weight (%)	Residue Weight (%) x Residue Specific Volume
lysine	12	146.2	1754	0.82	7.05	5.78
histidine	2	155.2	310	0.67	1.25	0.84
arginine	8	174.2	1394	0.70	5.61	3.92
aspartic acid	25	133.1	3328	0.59	13.38	7.90
threonine	7	119.1	834	0.70	3.35	2.35
serine	8	105.1	841	0.63	3.38	2.13
glutamic acid	37	147.1	5443	0.67	21.89	14.67
proline	3	115.1	345	0.76	1.39	1.05
glycine	15	75.1	1127	0.64	4.53	2.90
alanine	15	89.1	1137	0.74	5.38	3.98
valine	12	117.2	1406	0.86	5.65	4.86
methionine	10	149.2	1492	0.75	6.00	4.50
isoleucine	11	131.2	1443	0.90	4.80	5.22
leucine	11	137.2	1509	0.90	6.07	5.46
tyrosine	2	181.2	362	0.71	1.46	1.04
phenylalanine	11	165.2	1817	0.77	7.31	5.63
cysteine	1	121.2	121	0.63	0.49	0.31
tryptophan	nil					
total	190		24863		99.99	72.54

$$\text{partial specific volume} = \frac{\Sigma \text{ (residue weight \% x residue specific volume)}}{\Sigma \text{ (residue weight \%)}} = 0.726$$

5. Optical Properties

a. UV absorption

The UV absorption spectra of troponin A (H&M) and troponin A (urea) are shown in figure 38. The $E^{1\%}_{1\ cm}$ values were calculated after measuring protein concentrations by ultracentrifugal synthetic boundary runs with interference optics. Spectra were not altered by changing the levels of Ca^{2+}. Troponin A (urea) had a spectrum similar to that expected from its amino acid content (table 5). The low $E^{1\%}_{1\ cm}$ in the 250 - 290 nm region reflected the low tyrosine and tryptophan levels. The influence of phenylalanine was manifested as a multiplicity of fine structure in the 250 - 275 nm region. When measuring concentrations by absorption, the $E^{1\%}_{1\ cm}$ 277.5 nm was taken as 2.3. The spectrum of troponin A (H&M) was characterized by the presence of a considerable amount of nucleic acid. The fine structure due to phenylalanine was still obvious and the $E^{1\%}_{1\ cm}$ 260 nm was about 8. The amount of nucleotide bound to troponin A (H&M) varied and therefore so did the $E^{1\%}_{1\ cm}$.

b. Spectrophotometric titration

Spectrophotometric titration of troponin A was undertaken to determine whether or not the tyrosine hydroxyl groups ionized normally. Troponin A (urea) contained two moles tyrosine per mole. If both tyrosines were exposed completely to solvent, they would be expected to ionize similarly with pK values of 9 - 10. Partially or completely buried tyrosines would have a higher pK value.

Neutral solutions of troponin A (urea) were added to buffered solutions and the change in $OD_{295\ nm}$ was monitored with time (figure 39). The solution pH values were checked after the reaction was complete. At certain pH values absorbance changes occurred in the first few minutes. In all cases changes in $OD_{295\ nm}$ were complete by 1 hour. Completely exposed tyrosines should ionize instantly and show no time dependence, while buried tyrosines should show time dependent ionizations. The change in $OD_{295\ nm}$ after 1 minute and after 1 hour were plotted versus pH in figure 40. Above pH 10 the ionization of the tyrosines is time dependent. The number of tyrosines ionized as a function of pH is shown on the same figure. For this calculation the ionization of one mole tyrosine was assumed to cause an increase in $OD_{295\ nm}$ of 2300 (Kronman and Robbins, 1970). A total of 2 moles tyrosine ionized per mole of troponin A. This agrees well with the amino acid analysis which showed the presence of 2 moles tyrosine per mole troponin A. Figure 40 clearly indicates that the ionization of one of the tyrosines was time dependent and occurred at a higher pK value. One of the two tyrosines appeared to be exposed to external environment with a pK of 9.8. The other, with a pK of 11, was in some way masked, or buried, or at least less exposed to the external environment. Subsequent results will show that at pH values above 10 the apparent α-helical structure of troponin A tends to be destroyed. This change in structure most likely exposes the buried or partially buried tyrosine to external solvent.

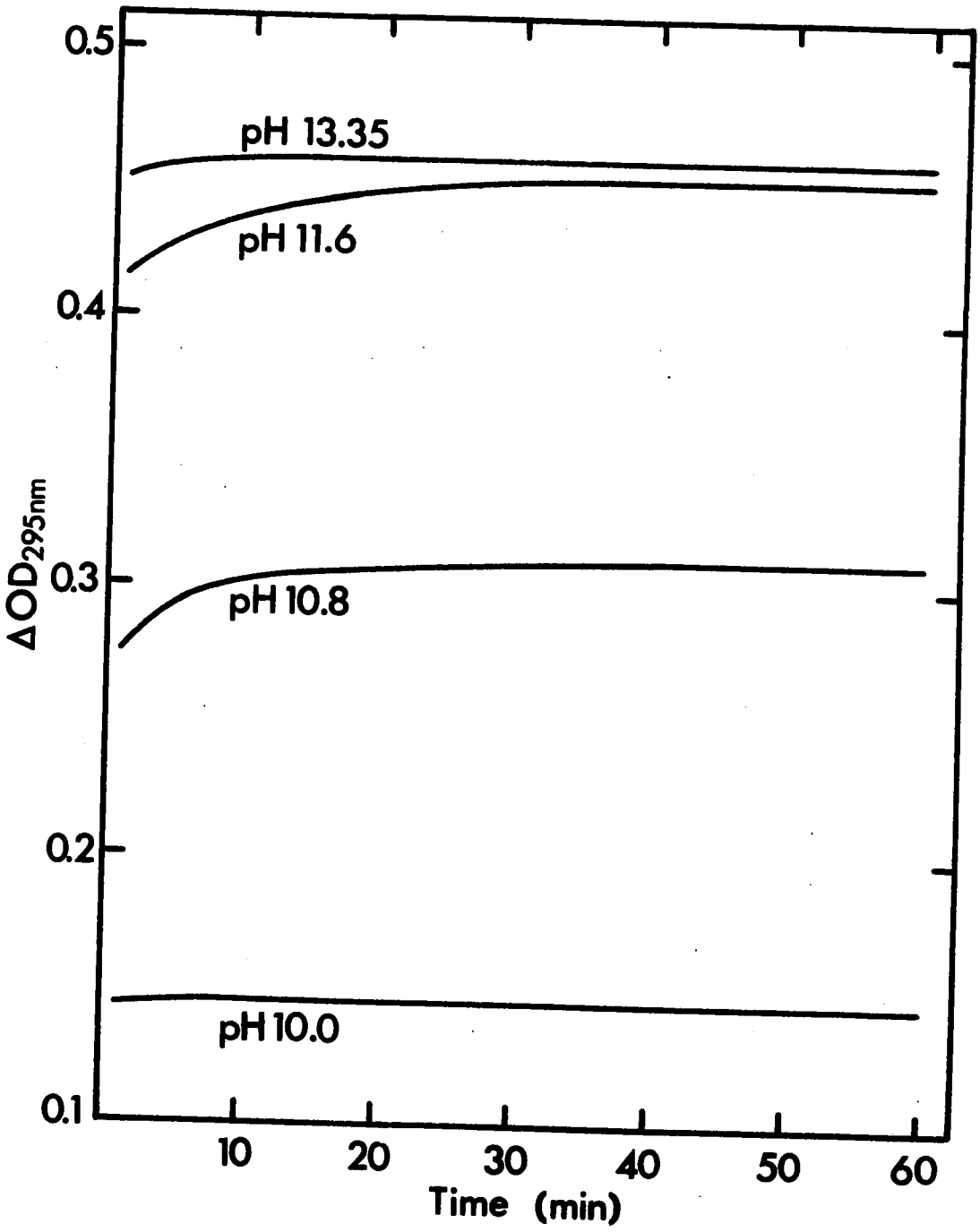


Figure 39. Time dependence of the ionization of the tyrosines of troponin A (urea). The change in $OD_{295\ nm}$ as a function of time is shown for several pH values. At time 0 the pH was adjusted, as described in the text, from 7 to the indicated values.

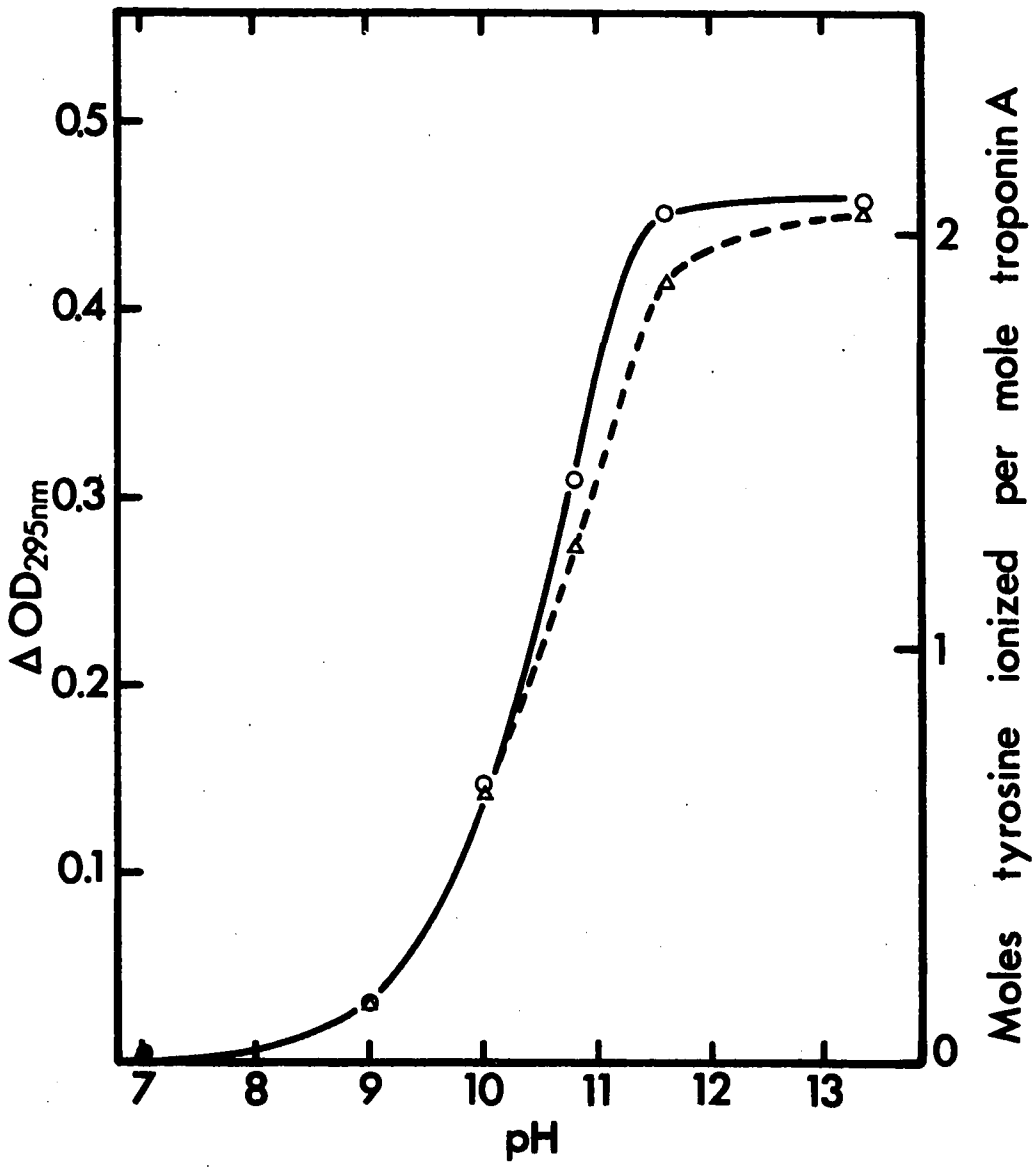


Figure 40. Spectrophotometric titration curve for the tyrosines of troponin A (urea). The data from the previous figure was plotted as $\Delta OD_{295\ nm}$ versus pH for times of 1 min. (Δ) and 1 hr. (O). The number of tyrosines ionized / mole troponin A (urea) is also shown. This was calculated assuming that the ionization of one mole of tyrosine causes an increase in $OD_{295\ nm}$ of 2300.

c. Circular dichroism

Far UV CD spectra of troponin A (H&M) are shown in figure 41. The $[\theta]_{221\ nm}$ value in 50 mM tris pH 7.6 is -13,000 deg·cm^2/decimole. The apparent % α-helix was estimated using polyglutamic acid at pH 3.8 as a prototype for 100% α-helix and the same polymer at pH 9.3 as a model for a random coil (Cassim and Yang, 1967). From the $[\theta]_{221\ nm}$ an apparent α-helical content of 39% was calculated. Alkaline pH values and 8 M urea each caused an increase in the amount of random coil, resulting in a reduction in $[\theta]_{221\ nm}$. During these experiments no effort was made to control Ca^{2+} levels.

Circular dichroism studies on troponin A (urea) revealed that the conformation of the protein depends to a great extent on the concentration of Ca^{2+} present. Figure 42 represents typical far UV CD spectra of troponin A (urea) in the absence and in the presence of Ca^{2+}. In the absence of Ca^{2+} or in the presence of EGTA, the $[\theta]_{221\ nm}$ was -10,000 ± 300 deg·cm^2/decimole, while the addition of 5 x 10^{-4} M $CaCl_2$ caused a 60% increase to -16,000 ± 400 deg.cm^2/decimole. This corresponds to an increase in apparent α-helix from 33 to 46%. The ellipticity band at about 207 nm underwent a corresponding change. In the absence of Ca^{2+} the $[\theta]_{207\ nm}$ was -13,000 deg·cm^2/mole corresponding to 23% α-helix. Addition of 5 x 10^{-4} M $CaCl_2$ caused an increase in $[\theta]_{207\ nm}$ to -16,500 deg·cm^2/decimole corresponding to 34% α-helix. These results indicate that the apparent helical content of troponin A increases significantly by about 50% upon the addition of 5 x 10^{-4} M $CaCl_2$. Ahmed et al. (1970) observed a similar coil to helix transition in tropocalcin with addition of similar quantities of

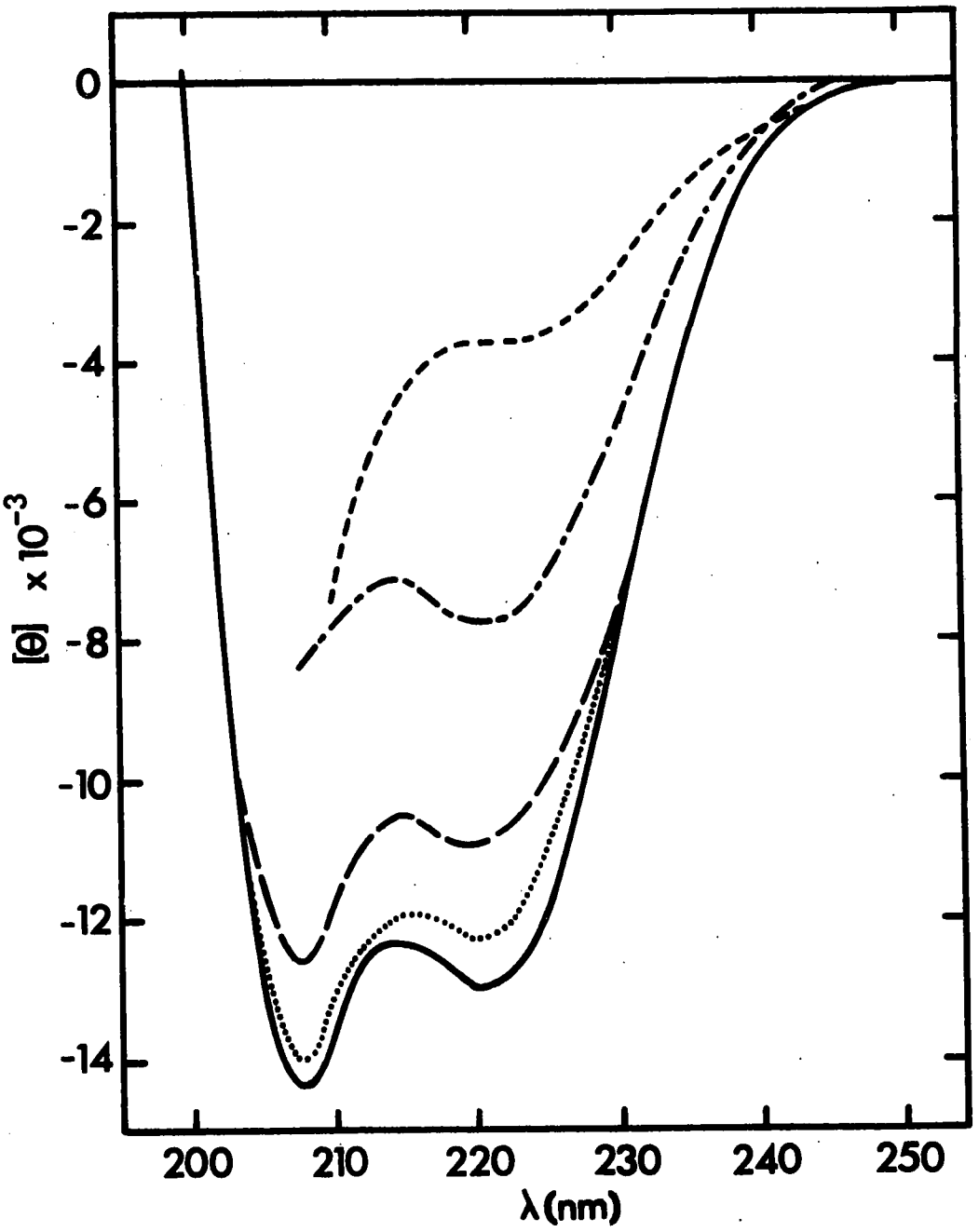


Figure 41. Far UV CD spectra of troponin A (H&M). By adding alkali to troponin A (H&M) in 50 mM tris pH 7.6 (———), pH values of 10.0 (·····), 11.0 (— —), and 12.8 (—·—) were obtained. The spectrum in 50 mM tris pH 7.6, 8 M urea is also presented (- - - -).

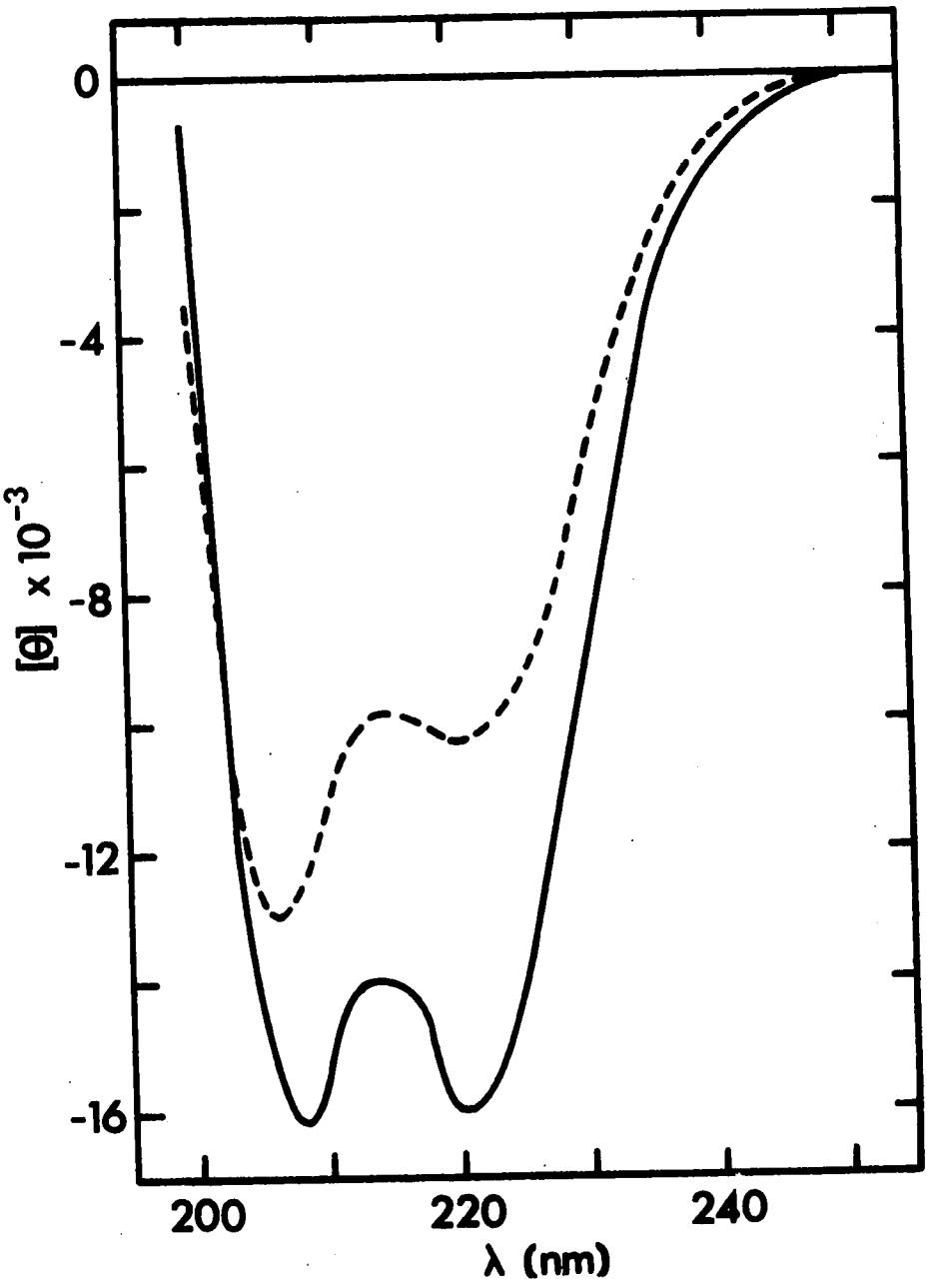


Figure 42. Far UV CD spectra of troponin A (urea) in the presence of EGTA and in the presence of Ca^{2+}. Solvents were 50 mM tris pH 7.6, 1 mM EGTA (– – –) and 50 mM tris pH 7.6, 10^{-5} M $CaCl_2$ (——).

Ca^{2+}. Tropocalcin, however, appears to be a different protein than troponin A, since neither magnitudes nor positions of far UV ellipticity bands coincide with those of our preparations of troponin A. Our findings concerning the conformational change in troponin A (Murray and Kay, 1972) were recently confirmed by Van Eerd and Kawasaki (1972), who found by optical rotatory dispersion an increase from about 19 to 24% apparent α-helical content with addition of small amounts of Ca^{2+}. They noted changes in fluorescence implying that Ca^{2+} caused the tyrosines of troponin A to enter into a more restricted environment.

Far UV CD spectra of troponin A (urea) at alkaline pH and in 6 M urea are shown in figure 43. Both pH 12.5 and 6 M urea caused large decreases in the $[\theta]_{221\ nm}$ values, indicating an α-helix to random coil conversion.

The interaction of Ca^{2+} with troponin A was studied by CD in more detail. Troponin A, isolated with no precautions taken to reduce Ca^{2+} levels, usually had some bound Ca^{2+}. This Ca^{2+} was removed by dialysis of troponin A (urea) against 500 volumes 10^{-4} M EGTA twice, followed by dialysis against three changes of 500 volumes 50 mM tris pH 7.6 to remove the EGTA. The Ca^{2+} level was then increased and the ellipticity band at 221 nm monitored. The results are expressed as % completion of the change in $[\theta]_{221\ nm}$ vs Ca^{2+} concentration (figure 44). The change was essentially 0% complete with no $CaCl_2$ added when $[\theta]_{221\ nm}$ was -10,000 deg·cm^2/decimole, and 100% complete at above 10^{-3} M $CaCl_2$ when $[\theta]_{221\ nm}$ was -16,000 deg·cm^2/decimole. The troponin A (urea) concentration for this experiment was 5.5×10^{-5} M (1.2 mg/ml). By assuming a binding constant of 10^6 M^{-1} (Hartshorne and Pyun, 1971) and

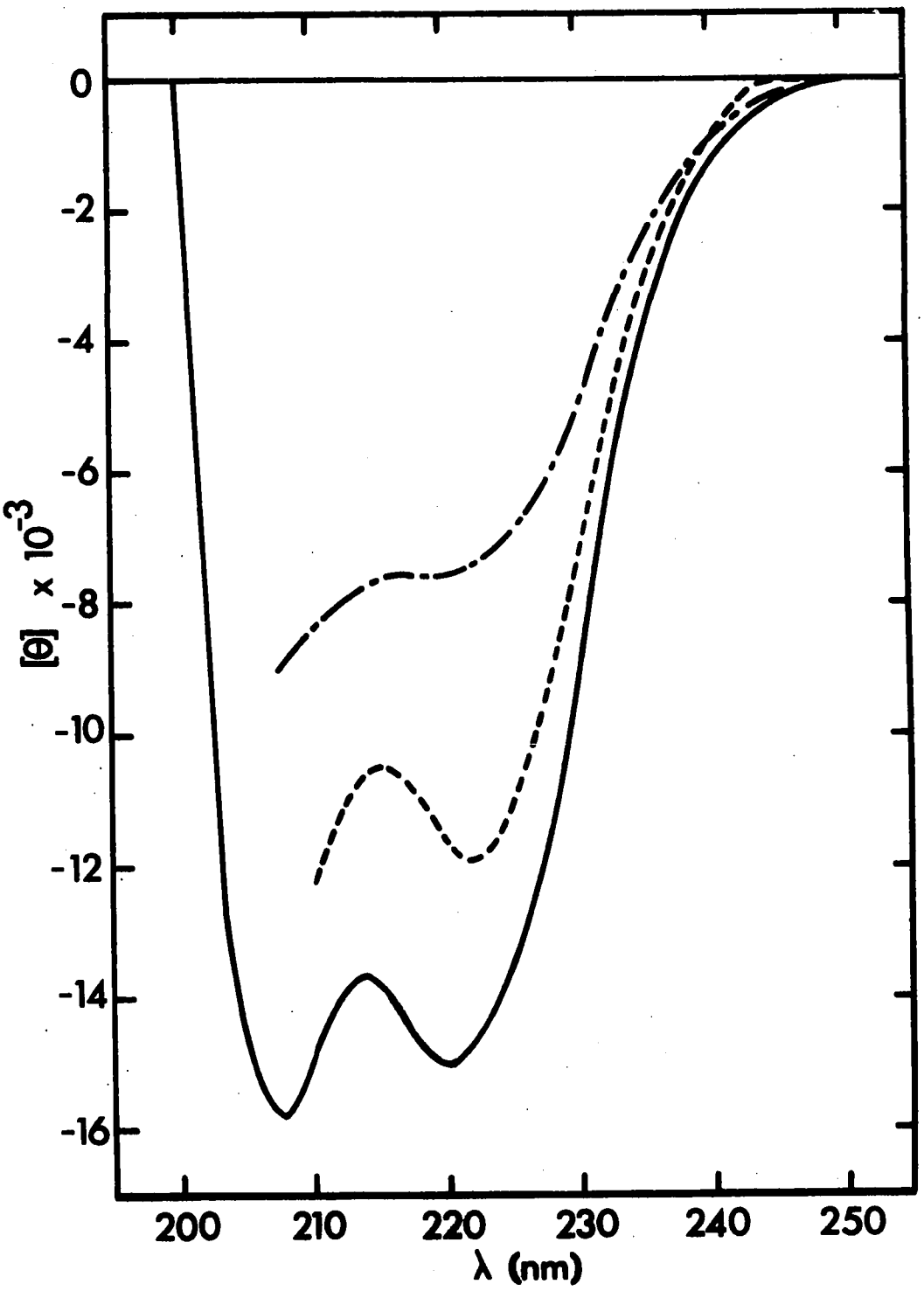


Figure 43. Far UV CD spectra of troponin A (urea) as affected by alkali and by urea. Spectra in 50 mM tris at pH 7.6 (———) and at pH 12 (- - - -) as well as that in 50 mM tris pH 7.6, 6 M urea (—·—) are presented here.

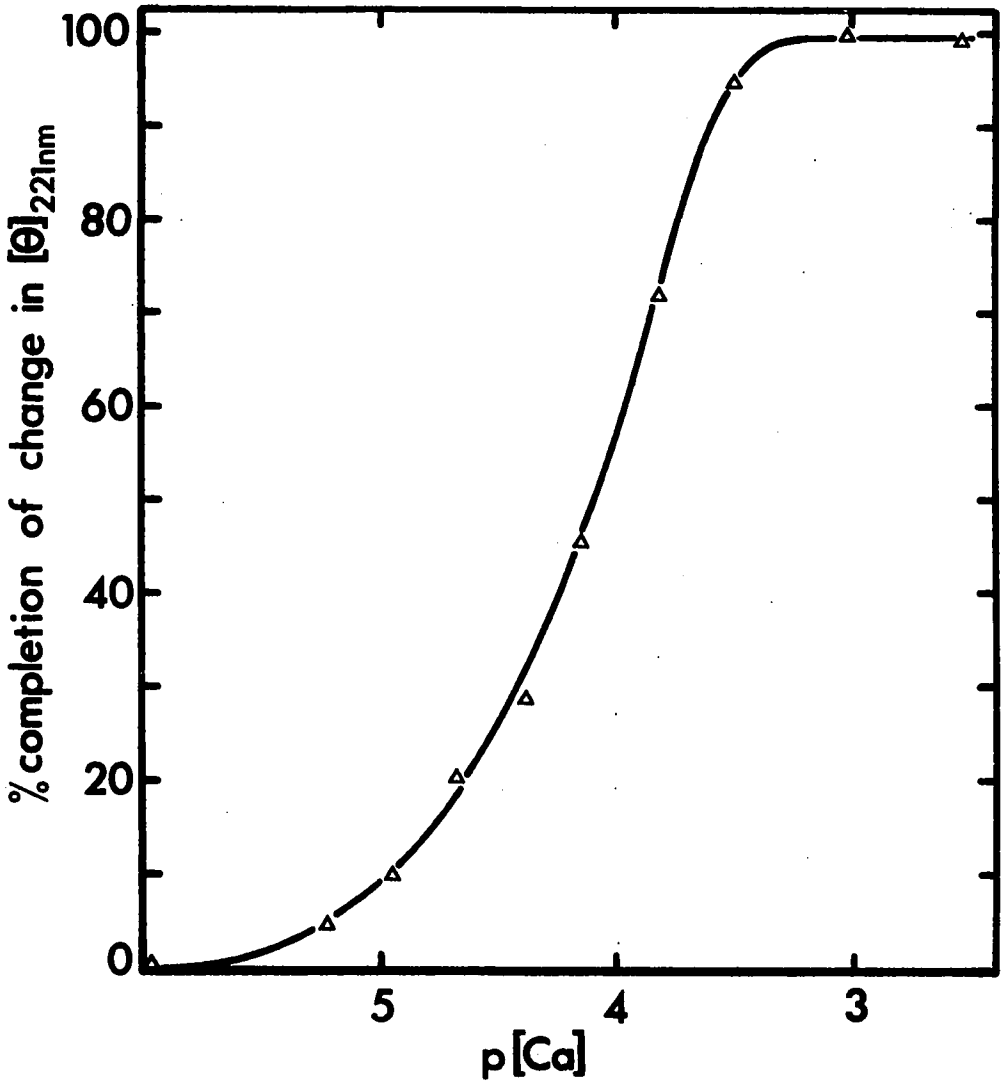


Figure 44. The extent of conformational change in troponin A (urea) with addition of Ca^{2+}. The negative logarithm of the total Ca^{2+} added is represented by p[Ca]. The conformational change is essentially 0% complete when no $CaCl_2$ has been added, at which time $[\theta]_{221\ nm}$ is -10,000 deg·cm^2/decimole, and 100% complete at above 10^{-3} M $CaCl_2$ when $[\theta]_{221\ nm}$ is -16,000 deg·cm^2/decimole. The troponin A (urea) concentration was 5.5×10^{-5} M.

that each troponin A molecule binds 3 molecules of Ca^{2+}, a theoretical curve for % Ca^{2+}-binding sites filled vs p[Ca] was constructed (figure 45). There is a close resemblance between the theoretical curve and that obtained experimentally. Hartshorne and Pyun (1971) found that troponin A binds not 3, but 2 moles Ca^{2+} per 22,200 g protein. The discrepancy may be due to incomplete removal of EGTA from troponin A before the addition of Ca^{2+} or to a decrease in the Ca^{2+}-binding constant of troponin A because of the harsh treatment with 6 M urea.

Rabbit white muscle contains about 0.07 μmoles troponin per gram (Ebashi et al., 1969). This corresponds to a concentration of troponin A of 1.5 mg per gram of muscle, which is comparable to the concentrations used in the CD experiments. The change observed occurred between total Ca^{2+} concentrations of 10^{-6} M and 5 x 10^{-4} M. However, if the Ca^{2+}-binding constant of troponin A is 10^{6} M^{-1} (Hartshorne and Pyun, 1971), the change occurs between free Ca^{2+} levels of 10^{-8} M and 10^{-5} M. During the excitation leading to contraction, the free Ca^{2+} level increases from 10^{-8} M or less to near 10^{-5} M (Ebashi et al., 1969). The observed conformational change could occur during contraction and may serve a regulatory function.

The above-mentioned conformational change is not reflected by gross changes in the near UV CD spectra of troponin A. The near UV CD spectra of troponin A (urea) in the presence and absence of Ca^{2+} are shown in figure 46. The contribution appears to be due largely to phenylalanine with ellipticity bands at 259 and 265 nm, since their sign and position are in complete agreement with the CD spectra of

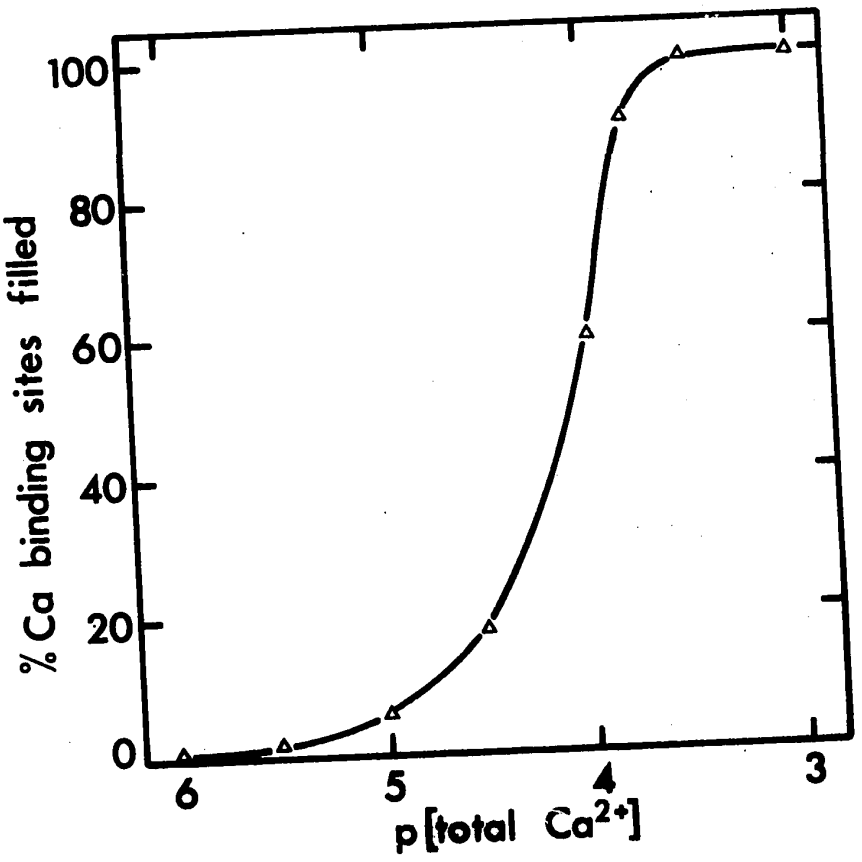


Figure 45. A theoretical Ca^{2+}-binding curve for troponin A. This curve was obtained by assuming that troponin A bound 3 moles Ca^{2+}/mole protein, each with a binding constant of 10^6 M^{-1}.

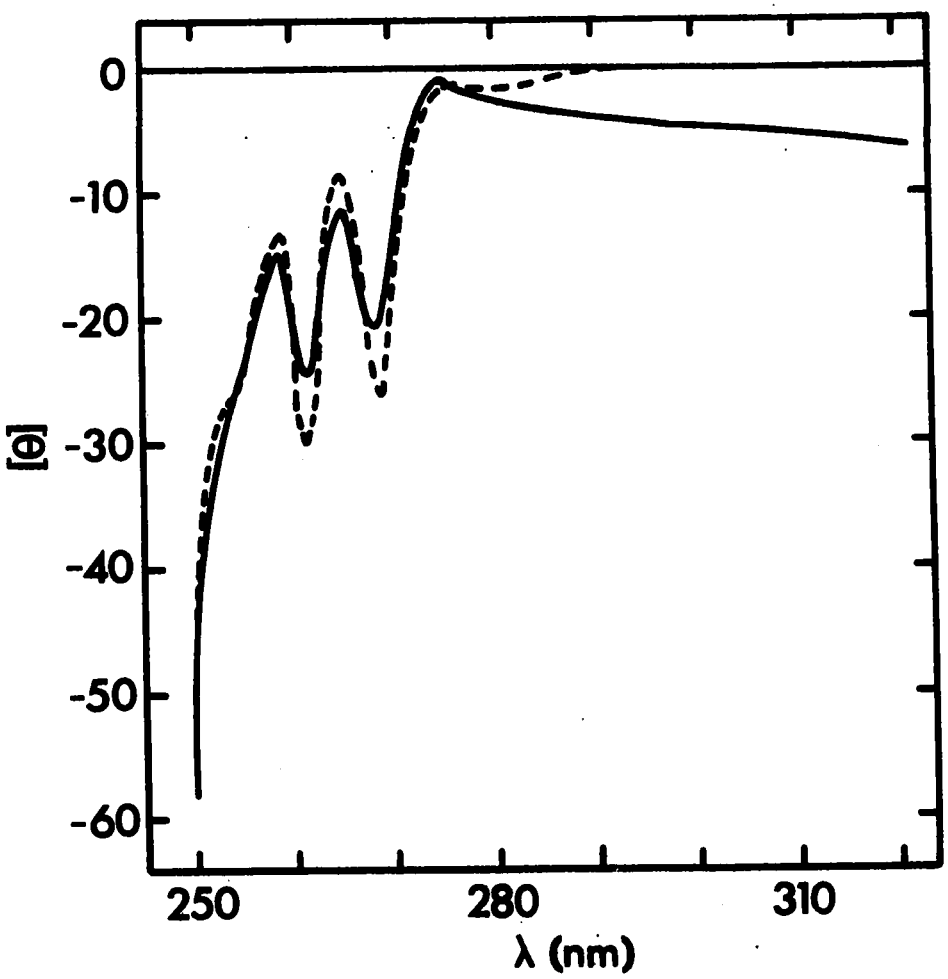


Figure 46. Near UV CD spectra of troponin A (urea) in the presence of EGTA and in the presence of Ca^{2+}. The solvent was 50 mM tris pH 7.6 containing either 1 mM EGTA (solid line) or 10^{-5} M $CaCl_2$ (dashed line).

acetyl-L-phenylalanine methyl ester (Goodman and Toniolo, 1968). Addition of Ca^{2+} caused only a slight sharpening of the ellipticity bands, with no alteration in their wavelength positions.

The effect of 6 M urea and alkaline pH on the near UV CD spectrum of troponin A (urea) is presented in figure 47. The solvent contained 10^{-5} M $CaCl_2$. Troponin A (urea) denatured considerably in 6 M urea (figure 43), however the aromatic CD was little affected. The [θ] values in the 250 - 270 nm region became less negative and although the phenylalanine fine structure became less well defined, it was still obvious. At alkaline pH the most prominent change in the aromatic CD spectrum was the appearance of a large band at 253 nm. This transition is due to the ionization of the tyrosine hydroxyl group (Ikeda et al., 1967; Ikeda and Hamaguchi, 1969; Sakei et al., 1970). The $[\theta]_{253\ nm}$ value increased until pH 11.6 and at higher pH decreased slightly. The plot of $[\theta]_{253\ nm}$ versus pH (figure 48) shows that the tyrosine hydroxyl groups ionized with a pK of 10.1. $[\theta]_{253\ nm}$ values were measured 2-3 hours after adjusting the pH and should have reached equilibrium. When the ionization of the tyrosines was followed by monitoring the $OD_{295\ nm}$ (figure 40), the titration curve resulting after time dependent changes had ceased, had an apparent pK of 10.4. The agreement between these two pK values led to the conclusion that the ionization of tyrosine hydroxyl groups of troponin A (urea) could be followed by observing changes in either $OD_{295\ nm}$ or $[\theta]_{253\ nm}$. The maximum change in $[\theta]_{253\ nm}$ was 142 deg·cm^2/decimole. Troponin A (urea) has been shown to contain 2 tyrosine residues (this study). If the contributions were similar from each ionized tyrosine, then each was responsible for an increase in $[\theta]_{253\ nm}$ of 71 dec·cm^2/decimole.

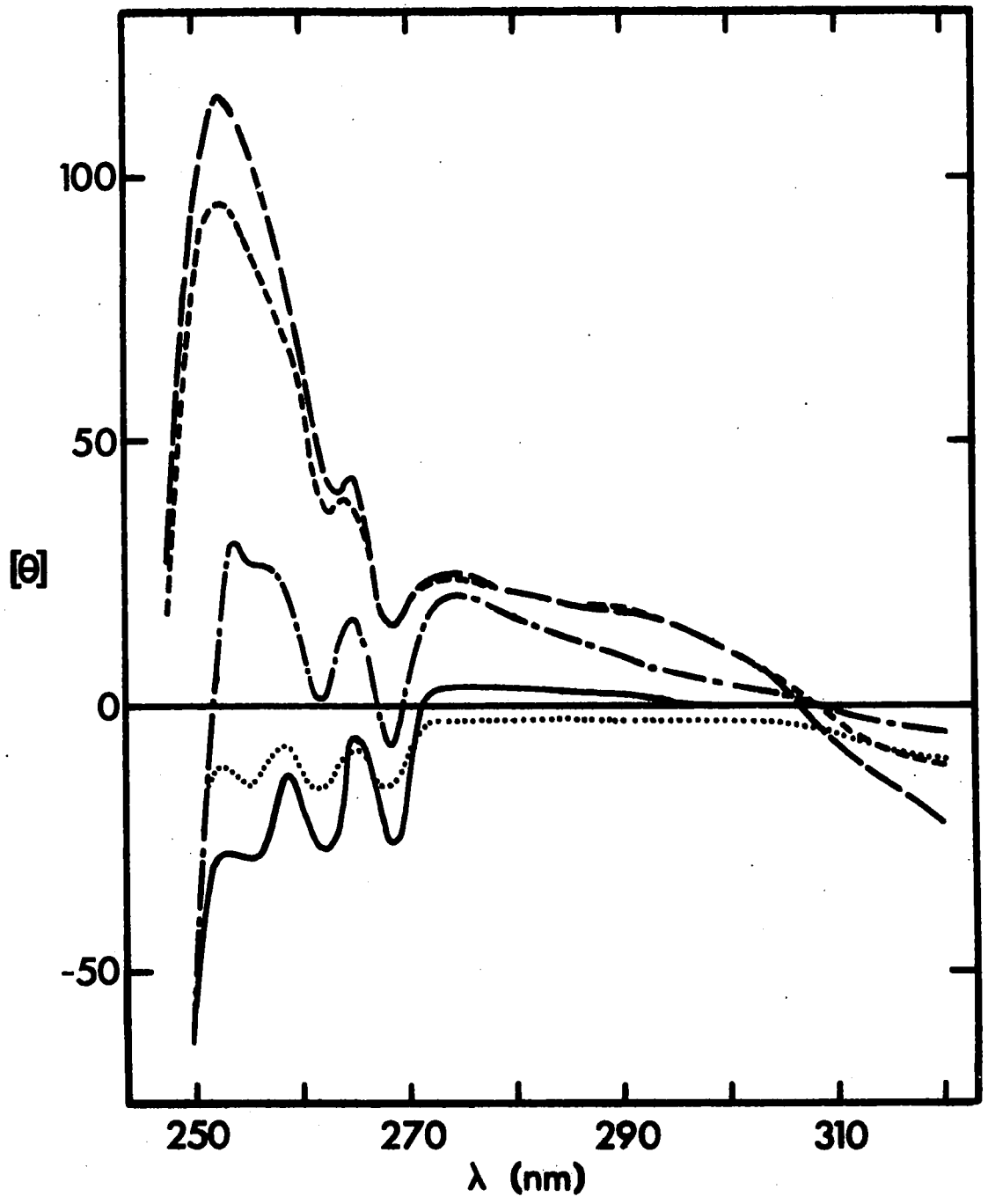


Figure 47. The effect of alkali and urea on the near UV CD spectrum of troponin A (urea). The solvent was 0.08 M phosphate, 0.8 M KCl at pH 7 (———), 0.08 M carbonate-bicarbonate, 0.8 M KCl at pH 10 (—·—·—), a mixture of 1 M KOH and 1 M KCl at pH 11.6 (— —) or at pH 13.3 (- - - -), or 50 mM tris pH 7.6, 6 M urea (.....).

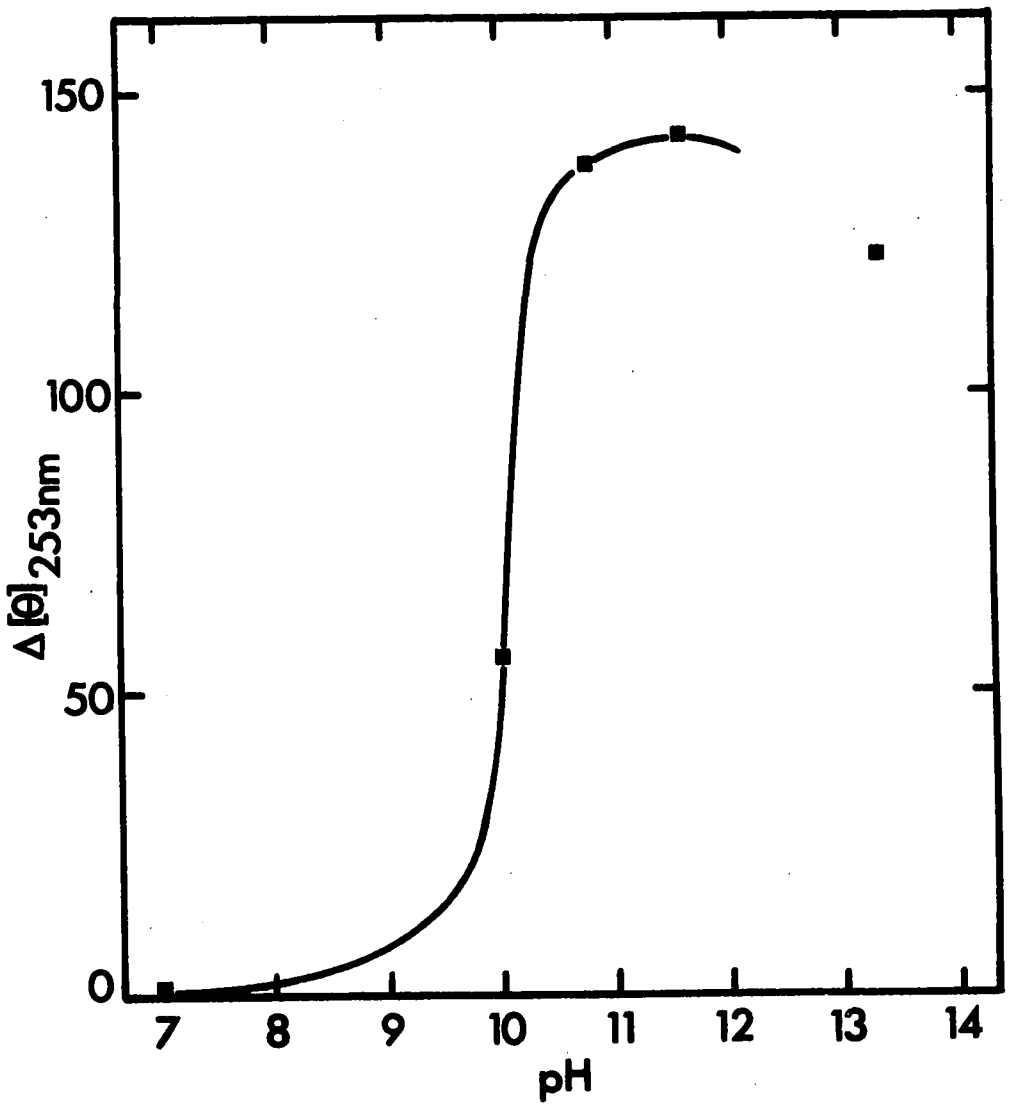


Figure 48. The effect of pH on the $[\theta]_{253\ nm}$ of troponin A (urea). Buffers contained 0.8 M KCl and either phosphate or carbonate at 0.08 M from pH 7 to 11, and mixtures of 1 M KCl and 1 M KOH at pH values above 11.

Near UV CD spectra of troponin A (H&M) are presented in figures 49A and 49B. At pH 7.6 the spectrum was much different from that of troponin A (urea). The fine structure due to phenylalanine was still present. A large positive band centered at about 265 nm, probably due to nucleic acid, dominated the spectrum. This was seen more clearly upon calculation of a difference spectrum by subtracting the ellipticities of troponin A (urea) at pH 7.6 from those of troponin A (H&M) at the same pH. A large positive band with its maximum at 266 nm, which was most likely a contribution from nucleic acid, resulted (figure 50). Changes in the aromatic CD spectrum of troponin A (H&M) with increasing pH were complicated by the presence of nucleic acid. At pH 13 a positive band at 254 was clearly present. Another band at 276 nm was also obvious. The magnitudes of both the ellipticity bands were time-dependent (figure 49B). In an effort to clarify the situation regarding the nucleic acid present, a difference spectrum was constructed by subtracting the ellipticity of troponin A (urea) at pH 13 from that of troponin A (H&M) at pH 13. The resulting difference spectrum (figure 50) indicated that at pH 13 the nucleic acid caused an ellipticity band at 275 nm. No attempt was made to account for the change in contribution from the nucleic acid as the pH was raised from 7.6 to 13.

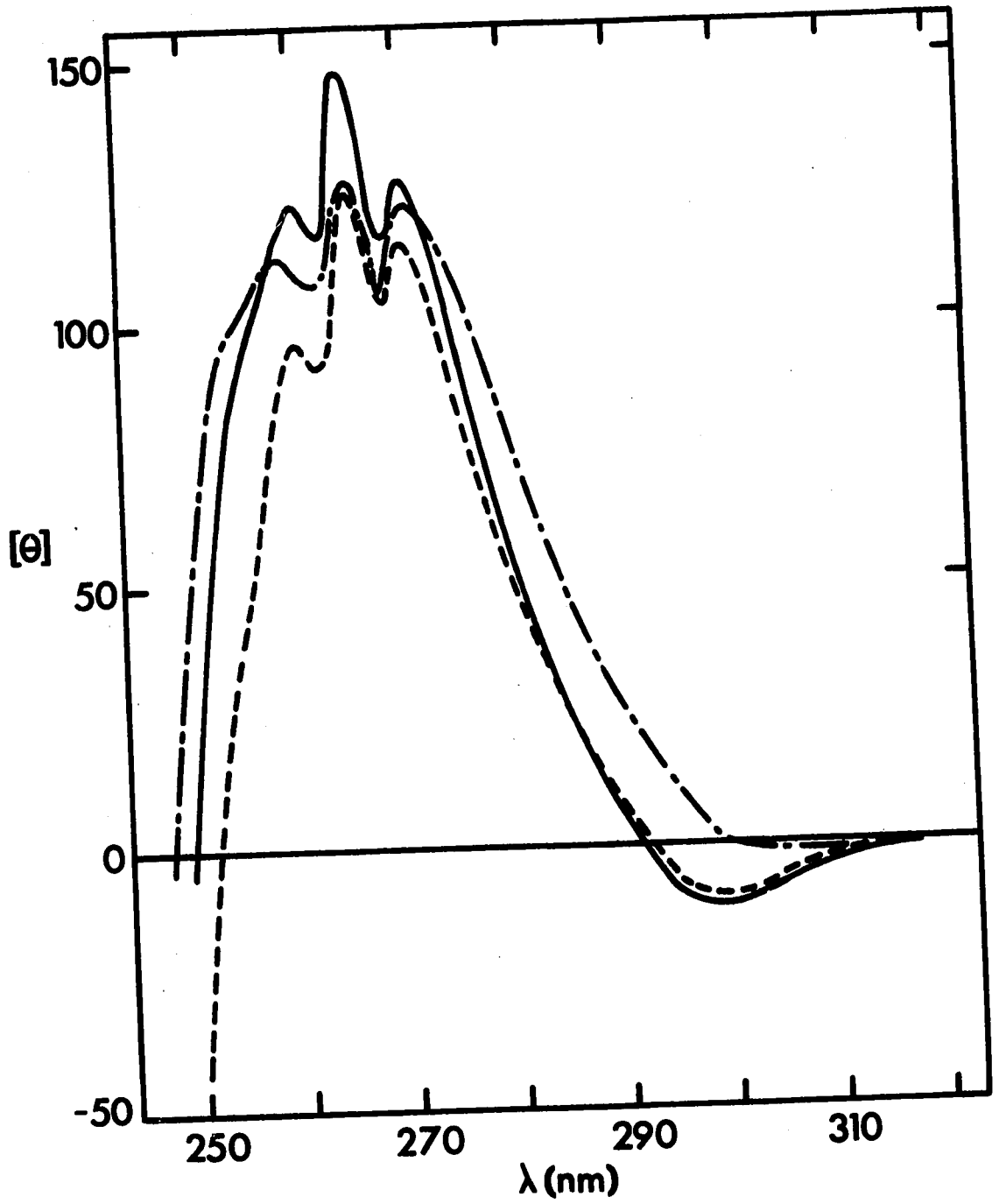


Figure 49A. The effect of pH on the near UV CD spectrum of troponin A (H&M). By adding KOH to troponin A (H&M) in 50 mM tris pH 7.6 (— — —), pH values of 10 (————) and 11.1 (—·—) were obtained.

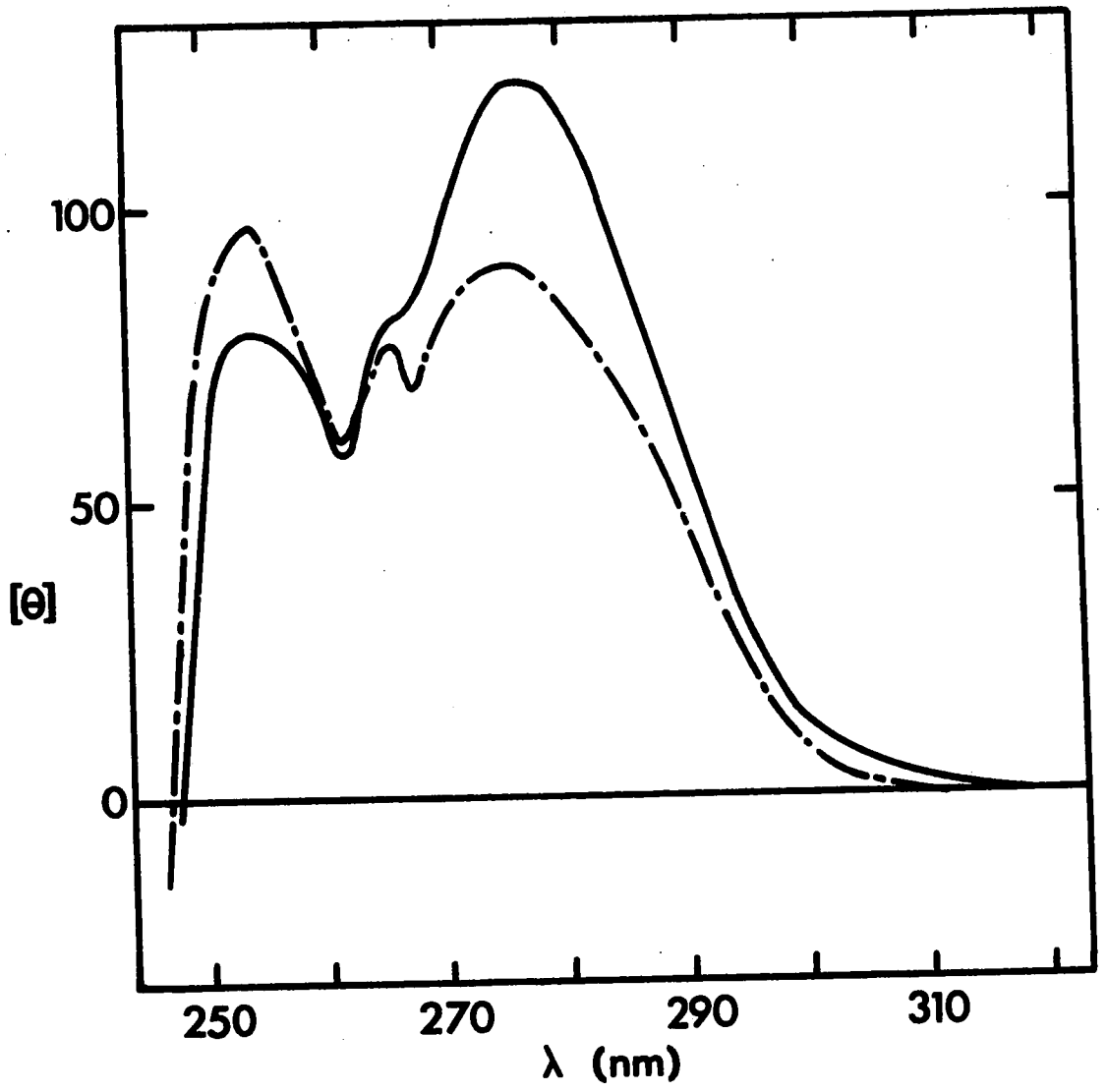


Figure 49B. The effect of pH on the near UV CD of troponin A (H&M). These spectra were run after 2 hr. (———) and after 24 hr. (—·—) at pH 12.75. The pH was adjusted by adding 1 M KOH to a solution of 50 mM tris at pH 7.6.

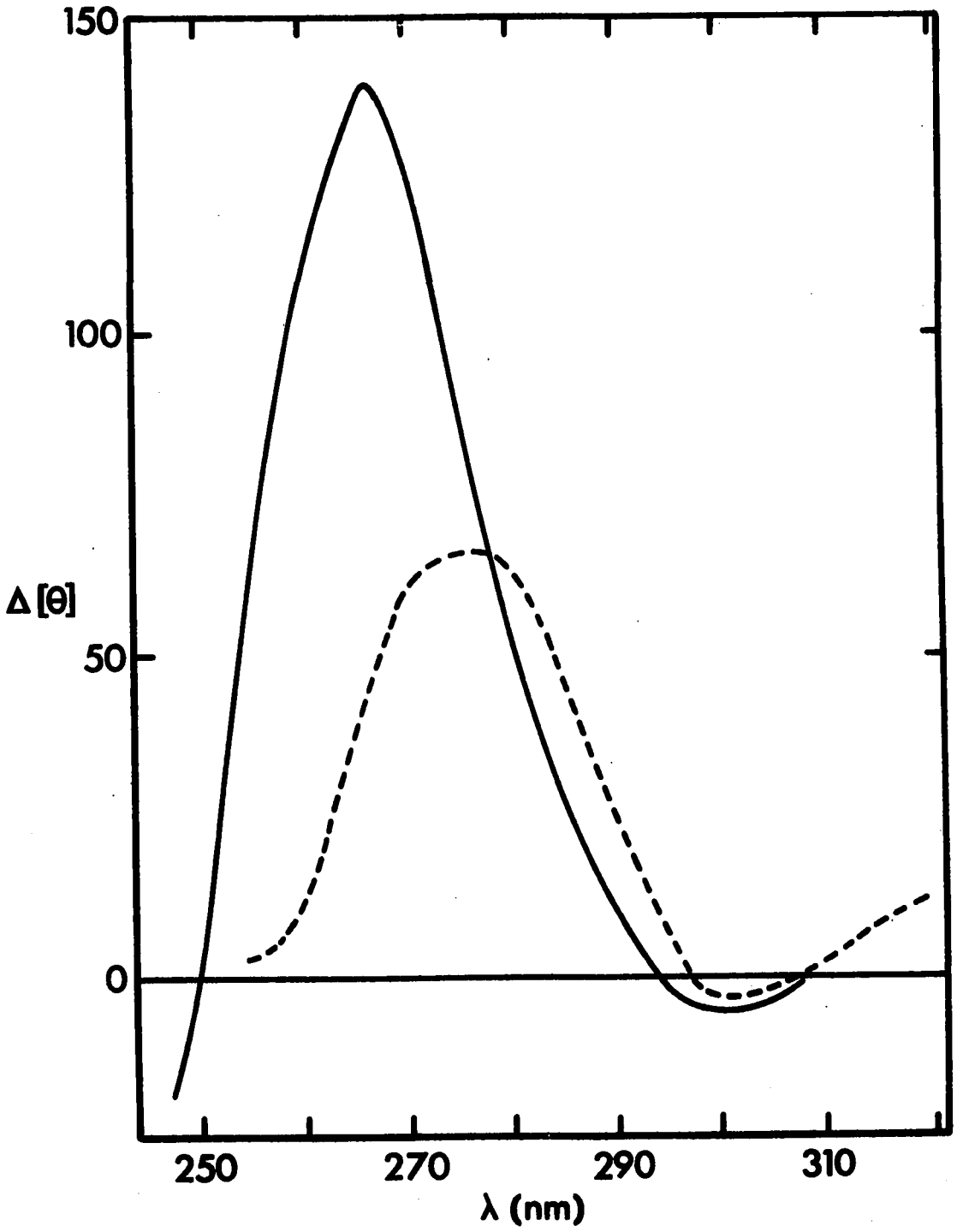


Figure 50. Difference spectra generated by subtracting the near UV ellipticities of troponin A (urea) at pH values of 7.6 and 13.3 from those of troponin A (H&M) at the same pH values. Ellipticity of troponin A (H&M) at pH 7.6 minus that of troponin A (urea) at pH 7.6 - (———). Ellipticity of troponin A (H&M) at pH 13.3 minus that of troponin A (urea) at pH 13.3 - (— — —).

IV. SUMMARY AND CONCLUSION

A. The Proteins of the Troponin System and Their Interaction

The difficulties encountered upon attempting to separate troponin into its protein constituents lead to the conclusion that these proteins interact strongly. The question then arises as to the specificity of protein interaction and the nature of the protein complex which is functional in the intact muscle. Integration of some of the information concerning troponin and its components permits speculation regarding the *in vivo* make-up of the tropomyosin-troponin system of proteins.

Troponin was found to contain proteins of molecular weights 40,000, 25,000 and 22,000 as well as traces of a component of molecular weight 15,000. The complex sedimented as single boundary with an $S^{\circ}_{20,w}$ value of 3.7S, and Sephadex G-200 column chromatography of this material resulted in two retained peaks displaying biological activity. Peak 1 material contained protein species of molecular weights 40,000, 25,000 and 22,000 from SDS-polyacrylamide gels. Further, it sedimented as a single boundary with a $S^{\circ}_{20,w}$ of 4.4S, and had a sedimentation equilibrium molecular weight which rose from 40,000 - 60,000 at zero protein concentration to 138,000 at a protein concentration of 4 mg/ml. Peak 2 material contained protein species of molecular weights 25,000 and 22,000 as well as variable amounts of a component of molecular weight 15,000 on SDS-polyacrylamide gels. It sedimented as a major component following 10 - 30% of a faster component, and had sedimentation equilibrium molecular weights which increased from 15,000 - 50,000 at zero protein concentration to 50,000 - 55,000 at a protein

concentration of 4 mg/ml. From circular dichroic spectra, the apparent α-helical content of peak 1 material was ∿ 50%, while that of peak 2 material was ∿ 40%.

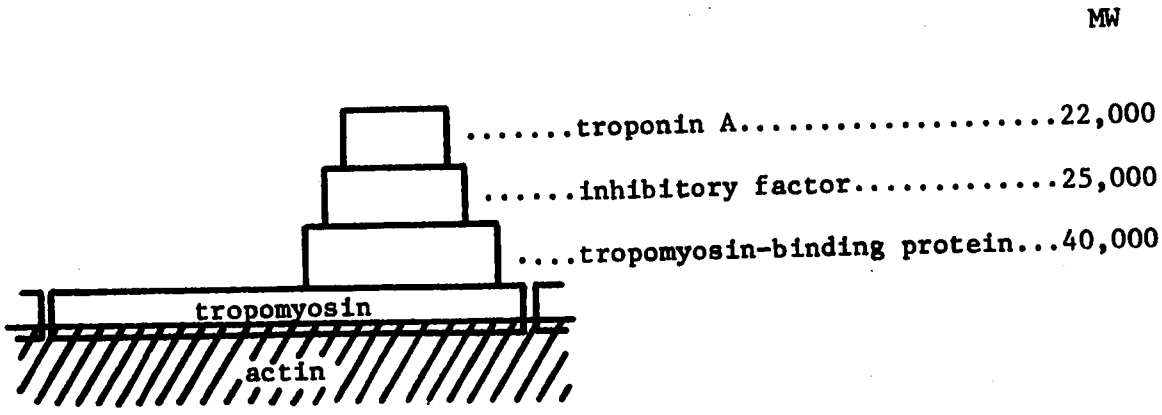
If the assumption is made that Coomassie blue binds similarly to all the components of troponin, then the protein components of molecular weights 40,000, 25,000, and 22,000 appear to be present in crude troponin and in peak 1 material from Sephadex G-200 in nearly equimolar ratios. The component of 15,000 molecular weight will not be discussed here, since it arose mainly during and after preparation of troponin. Peak 1 material from Sephadex G-200 sedimented as a single boundary, and eluted from both Sephadex G-200 and QAE-Sephadex as a single peak containing similar ratios of protein components. These three protein components seem to exist as a firmly bound complex.

Peak 1 material from Sephadex G-200 treatment of troponin, from which troponin A had been removed by the Hartshorne-Mueller procedure, contained mainly species of molecular weights 40,000 and 25,000. There is probably an interaction between these proteins, as suggested by Ebashi et al. (1969).

QAE-Sephadex column chromatography of peak 2 material from Sephadex G-200 yielded a complex of equimolar amounts of components of molecular weights 25,000 and 22,000. The minimum molecular weight of the complex as determined by high speed sedimentation equilibrium was 50,000. Although these two proteins interact in the presence of reducing agents, their interaction may be stabilized in vivo by a disulfide bond, since SDS-polyacrylamide gels of the complex displayed

a band at a molecular weight of 50,000 in the absence of DTT. In the presence of DTT this band disappeared to enhance species of molecular weights 25,000 and 22,000.

The biological roles of the proteins of molecular weights 25,000 and 22,000 have been elucidated. No role has been found for the protein of molecular weight 40,000. Ebashi et al. (1971) suggested that it acts as a binding link between tropomyosin and the troponin moiety. If the interactions described above are also preferred in vivo, troponin can be thought of as a chain of proteins with specific interactions between protein pairs. This is illustrated below:



B. Troponin A

Troponin A has been prepared by two different methods. The first approach involved three times repetition of the Hartshorne and Mueller high ionic strength, low pH precipitation steps and resulted in troponin A (termed troponin A (H&M)), which from SDS-polyacrylamide gel electrophoresis was near 90% pure. The second method involved DEAE-Sephadex A-25 column chromatography of crude troponin or crude troponin A in 6 M urea. The resulting troponin A (termed troponin A (urea)), was found from SDS-polyacrylamide gels to contain only one component. Both troponin A preparations were biologically active by the superprecipitation technique.

The hydrodynamic and optical properties of troponin A (urea) were found to be dependent on the Ca^{2+} concentration. Both troponin A preparations displayed single symmetrical Schlieren peaks upon sedimentation in a variety of conditions. Troponin A (H&M) had a $S^{\circ}_{20,w}$ value of 2.30S in 50 mM tris pH 7.6, 1 M KCl, and a $S^{\circ}_{20,w}$ value of 2.20S in 50 mM tris pH 7.6. Troponin A (urea) sedimented with a similar value of 2.15S in 50 mM tris pH 7.6, 5×10^{-5} M $CaCl_2$, but in 50 mM tris pH 7.6, 1 mM EGTA the $S^{\circ}_{20,w}$ value was 1.84S. This 17% increase in $S^{\circ}_{20,w}$ value with the addition of a small amount of Ca^{2+} implied a considerable compacting of the troponin A molecule.

The molecular weights of troponin A (H&M) and troponin A (urea), as determined by SDS-polyacrylamide gel electrophoresis, were 22,000 and 19,000 - 20,000 respectively. Both troponin A preparations had a minimum molecular weight of 22,200 from sedimentation equilibrium studies. Troponin A (H&M) aggregated in 50 mM tris pH 7.6 to a

molecular weight of 55,000 at a protein concentration of 4.0 mg/ml. The molecular weight of troponin A (urea) increased to 24,000 - 26,000 at a protein concentration of 4 mg/ml in a solvent containing 50 mM tris pH 7.6 and Ca^{2+} concentrations below 10^{-4} M. At 10^{-3} M $CaCl_2$, more extensive aggregation of the protein occurred.

The weight intrinsic viscosity of troponin A (urea) changed from 8.48 ml/g in the presence of 50 mM tris pH 7.6, 10^{-3} M EGTA to 3.32 ml/g in 50 mM tris pH 7.6, 10^{-5} M $CaCl_2$, implying that the addition of Ca^{2+} caused the troponin A molecule to become much more symmetrical in shape.

Combination of hydrodynamic parameters of troponin A (urea) allowed estimation of the axial ratios of prolate ellipsoids of revolution equal in volume to troponin A. In the presence of EGTA, the axial ratio was near 8:1 while in the presence of 10^{-5} M $CaCl_2$ the axial ratio was 3:1 or less. A large conformational change occurred at a Ca^{2+} concentration which caused little change in molecular weight. The change then must involve significant intramolecular rearrangement of the protein.

The UV absorption spectra of troponin A (H&M) and troponin A (urea) were quite dissimilar. The $E_{1\ cm}^{1\%}$ value of troponin A (urea) was less than 2.5 between 250 and 280 nm, whereas that of troponin A (H&M) was 8 with an absorption maximum near 260 nm. In both cases the fine structure due to phenylalanine was present, although it was more obvious in the spectrum of troponin A (urea).

Changes in secondary protein structure were investigated by circular dichroism. Troponin A (urea) was found to have an apparent α-helical content of ∿ 40% in the presence of 50 mM tris pH 7.6, 10^{-5} M $CaCl_2$. Upon increasing the pH to above 10 or upon addition of 6 M urea, the apparent α-helical content dropped drastically. Removal of Ca^{2+} by addition of 10^{-3} M EGTA to troponin A (urea) in 50 mM tris pH 7.6 caused a decrease in apparent α-helix to ∿ 28%. By addition of Ca^{2+} to Ca^{2+}-free troponin A (urea), the conformational change was found to occur between 10^{-6} M and 10^{-4} M total Ca^{2+}, or if the Ca^{2+}-binding constant is 10^6 M^{-1}, between 10^{-8} and 10^{-5} M free Ca^{2+}. Only very small changes in molecular weight occurred at these levels of Ca^{2+}, so the near 50% increase in apparent α-helix must be due to intramolecular rearrangement of troponin A. In other words, twenty to twenty-five amino acid residues per mole troponin A shift from random coil to the α-helical form. The conformational change was seen to result in a large decrease in axial ratio, indicating an alteration in polypeptide chain folding. Ca^{2+}-binding in carp myogen involves the coordination of Ca^{2+} to three aspartic carboxyl groups and one glutamic carboxyl group in a tetrahedral arrangement (Nockolds et al., 1972). In this case Ca^{2+} binds in a region where α-helix is absent. Most likely Ca^{2+} is bound to troponin A through carboxyl groups. Troponin A has 40 more acidic residues per mole than basic residues. In the absence of Ca^{2+}, high concentrations of non-protonated acidic residues probably tend to keep certain protein segments in a random, somewhat extended structure, as in the case of poly-glutamic acid (Cassim & Yang, 1967). The addition of Ca^{2+} perhaps

lessens or eliminates the repulsive interactions of key carboxyl groups allowing folding of the troponin A molecule in such a way as to enhance the formation of α-helical regions.

This conformational change may be involved in releasing the restraints imposed upon the actomyosin system by the inhibitory factor.

C. Regulation of the Contractile Process by Troponin

Troponin was shown to contain three proteins of molecular weights 22,000 (troponin A), 25,000 (inhibitory factor) and 40,000 (tropomyosin-binding protein). The preferred interactions among these proteins *in vitro* suggest a pattern of arrangement *in vivo* as described previously (page 118). Since these proteins interact so strongly, they probably contain centers of high charge density, and one must exercise caution when carrying out biological activity studies with only certain of these proteins present. The apparent activity might be a function of interaction of the troponin component with actin or myosin which does not occur *in vivo*. For example, although inhibitory factor inhibits the ATPase activity of synthetic actomyosin in the presence or in the absence of Ca^{2+} when it is added to the assay system with tropomyosin, after addition of troponin A, which binds strongly to inhibitory factor, a new type of control may exist which involves inhibitory factor only indirectly.

An attempt will be made to divulge a possible regulatory mechanism without explaining either how Ca^{2+} gets to myofibrillar proteins or how it is removed. Tropomyosin will be considered as a structural protein situated in the groove of the helical actin filament. It probably serves to stabilize actin structure and also to

bury charged areas of actin which might interfere with normal ATPase activity. In addition tropomyosin appears to serve as a foundation for the troponin structure. The protein of molecular weight 40,000 seems to function by enhancing the binding of tropomyosin to actin as well as by connecting tropomyosin and inhibitory factor. Several possible roles of troponin in controlling the contractile process in terms of conformational changes in troponin A are presented in table 7. Possibility number 1 (table 7) involves an inhibitory function for inhibitory factor while the other alternatives in table 7 suggest that troponin A is the basic regulatory protein. Of course any combination of these possibilities listed, as well as others, could occur in consort. The above speculations are only possible with the assumption that the conformational changes observed in troponin A upon the addition of Ca^{2+} also occur *in vivo*.

TABLE 7

REGULATORY ACTION OF TROPONIN IN TERMS OF CONFORMATIONAL CHANGES IN TROPONIN A

	$Ca^{2+} \leq 10^{-8}$ M	$Ca^{2+} \simeq 10^{-5}$ M
State of troponin A	equivalent ellipsoid of revolution: axial ratio 8:1 length 155 Å apparent α-helix ∿ 28%	equivalent ellipsoid of revolution: axial ratio 3:1 length 85 Å apparent α-helix ∿ 40%
ATPase activity	low	normal
Actin-myosin interaction at site giving normal ATPase activity	no	yes
Possible changes in troponin system to allow Ca^{2+} regulation of actomyosin ATPase activity	1. charge repulsion between inhibitory factor and myosin crossbridge. 2. troponin A sterically blocks actin-myosin interaction 3. troponin A covers actin-myosin interaction site	1. troponin A covers or changes inhibitory factor so that no repulsion occurs 2. troponin A shortens by nearly 50% and allows interaction 3. troponin A shortens considerably making interaction site available.

SUGGESTIONS FOR FURTHER RESEARCH

This thesis presents only a prelude of what will surely follow in the next 2 or 3 years. More adequate and more extensive physicochemical characterization of troponin A is a necessity. Most troponin A studies were carried out in 50 mM tris at pH 7.6. Hydrodynamic and optical parameters should be determined at higher ionic strengths and at different pH values, in particular, those corresponding to physiological conditions. More information as to the nature of the Ca^{2+}-dependent conformational change in troponin A is required. Do other cations cause similar changes? Does the change occur in the presence of inhibitory factor? If the seemingly important carboxyl groups of troponin A are blocked, what conformation does the protein adopt? Do the tyrosine groups ionize similarly in the absence and in the presence of Ca^{2+}? What, if any, role does the one troponin A sulfhydryl play? These are a few of the points which require clarification. After labelling certain amino acid residues with reporter groups, perhaps the change in environment of various amino acids can be monitored. Of interest also is the question of whether or not ATP or any products of its hydrolysis affect the Ca^{2+}-induced conformational change. Since this protein is very acidic and probably contains quite high densities of negative charge, the arrangement of amino acids with respect to sequence may prove enlightening. Ultimately, crystallization of troponin A in the presence and absence of Ca^{2+} may lead to complete solution of questions as to the nature of the conformational change.

Methods for isolation and purification of the other proteins of the troponin system are becoming available. After characterization of these purified proteins, studying their interaction properties may lead eventually back to the native troponin complex, at which time determination of the exact role of troponin and its components may be more approachable.

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