INTERACTION BETWEEN TROPONIN I AND TROPONIN C OR CALMODULIN

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EDWARD EDMOND ZIESMANN

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INTERACTION BETWEEN TROPONIN I AND TROPONIN C OR CALMODULIN

BY

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"'T is a common proof,
That lowliness is young ambition's ladder,
Whereto the climber-upward turns his face;
But when he once attains the utmost round,
He then unto the ladder turns his back,
Looks into the clouds, scorning the base degrees
By which he did ascend."

William Shakespeare

ABBREVIATIONS

ATP	Adenosine Tri-Phosphate
BME	β-Mercaptoethanol
DEAE	Diethylaminoethyl
DMSO	Dimethylsulfoxide
DDT	Dithiothreitol
EGTA	Glycol Etherdiaminetetraacetic Acid
EMLC	Essential Myosin Light Chain
HPLC	High Pressure Liquid Chromatography
MBA	N, N' - Methylenebisacrylamide
PAGE	Polyacrylamide Gel Electrophoresis
PDE	Phosphodiesterase
PMSF	Phenylmethylsulfonyl Fluoride
RMLC	Regulatory Myosin Light Chain
SDS	Sodium Dodecyl Sulfate
TEMED	N,N,N',N', - Tetramethyl ethylenediamine
TM	Tropomyosin
TN	Troponin
TN-C	Troponin C
TN-I	Troponin I
TN-T	Troponin T
TRIS	Tris (hydroxy methyl) amino methane

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1. INTRODUCTION AND RATIONALE FOR THE STUDY

Calmodulin is a Ca^{++} binding protein which regulates a number of enzymes and biological systems. In this study we attempt to investigate the sites of interaction between calmodulin and the enzymes it modulates.

Calmodulin is homologous to troponin C and can replace troponin C in its Ca^{++}-dependent interaction with troponin I. Because troponin I is readily available, we decided to use it as an example of a calmodulin-binding protein.

The intent was to use chemical modification as a tool, to find out which residues in TN-I are essential for interaction with calmodulin. Assays would be used in which the effects of TN-I and modified TN-I, on the calmodulin activation of phosphodiesterase were compared. These competitive assays did not work. Therefore TN-I was not a good model for a calmodulin binding protein.

In the course of our studies we found that TN-I/TN-C could be crosslinked by nitration. This was followed up in the hope of being able to narrow down the site of interaction between TN-I and TN-C.

2. LITERATURE REVIEW

A. Calmodulin

a. Discovery

Calmodulin, the protein modulator of bovine brain cyclic nucleotide phosphodiesterase was discovered independently by Cheung (1970), in bovine brain and by Kakiuchi et al (1970), in rat brain, during attempts to purify phosphodiesterase. It had earlier been reported by Cheung (1969) that phosphodiesterase activity was gradually lost during the purification of the enzyme from beef brain and that phosphodiesterase was activated, upon incubation with brain extracts. Cheung (1970, 1971) demonstrated that the activator isolated free of phosphodiesterase activity, from the brain extract effectively reconstituted the activity of the purified enzyme.

Independently, Kakiuchi et al, (1970; 1970a; 1971) demonstrated the existence, in rat brain, of a calcium activatable cyclic nucleotide phosphodiesterase and a protein factor which could enhance Ca^{++} activation of the enzyme.

The phosphodiesterase activating factor was identified by Teo and Wang (1973) and was shown to be a calcium binding protein.

The calcium binding property of the protein and its subsequent physical and chemical properties led to its identification with other proteins isolated from other sources by other investigators. This protein became known as the calcium-dependent protein regulator. Subsequently Watterson et al (1976) referred to this protein as the Ca^{++}-dependent modulator protein. To avoid the confusion generated by the multiple nomenclatures, the protein was renamed calmodulin, by

general concensus of the researchers in the field.

Calmodulin has been purified to apparent homogeneity from many sources. These include several mammalian sources (Teo et al, 1973; Lin et al, 1974), lower vertebrates (Childers and Siegel, 1975), invertebrates (Waisman et al, 1975) and plants (Gomes et al, 1979; Anderson and Cormier, 1978).

The amino acid composition of different calmodulins has been found to be very similar. It is characterized by the absence of tryptophan and cysteine, a high threonine:serine ratio (12:4), a larger number of acidic residues and a single residue of histidine and trimethyllysine (Watterson et al, 1980; Jackson et al, 1977; Miyake and Kakiuchi, 1978).

Aside from similar amino acid compositions, sequences from widely different species have been found to be nearly identical. Todo et al (1981) have shown that there are only three sequence differences between calmodulin from bovine and scallop sources.

b. Mechanism of phosphodiesterase activation

Teo and Wang (1973) demonstrated using preparations of calmodulin deficient phosphodiesterase that activation of PDE by Ca^{++} and calmodulin is mutually dependent, i.e., the enzyme is not activated by Ca^{++} in the absence of calmodulin, nor is the enzyme activated by calmodulin at free Ca^{++} ion concentrations much below 1uM.

Activation could be the result of binding of both activators (Ca^{++} and calmodulin) to the enzyme, or secondly, calmodulin must bind Ca^{++} in order to activate the enzyme. Teo and Wang (1973) suggest that the second mechanism is operative in PDE activation on the basis

of equilibrium binding studies with $^{45}Ca^{++}$.

From gel filtration dissociation studies using EDTA, Lin et al (1975) suggested that the formation of the enzyme-modulator complex is Ca^{++} dependent. Wickson et al (1975) reported similar observations.

Based on these results a proposed mechanism for the activation of cyclic nucleotide PDE by the Ca^{++}-dependent modulator was proposed by several groups (Teshima and Kakiuchi, 1974; Liu et al, 1974; Wang et al, 1975). According to this model, the activation of PDE is a stepwise process initiated by the binding of Ca^{++} to the calmodulin molecule. Upon binding Ca^{++}, calmodulin is converted from an inactive to an active conformation. The active Ca^{++}-calmodulin complex then associates with PDE to form a ternary complex, simultaneously inducing a conformational change in the PDE through the protein-protein interaction. The overall result is a 6-10 fold enhancement of PDE activity. The mechamism of activation may be represented schematically as follows:

$$Ca^{++} + \text{Calmodulin} \rightleftharpoons [Ca^{++} - \text{Calmodulin}]$$

$$[Ca^{++} - \text{Calmodulin}] \rightleftharpoons [Ca^{++} - \text{Calmodulin}^*]$$

$$[Ca^{++} - \text{Calmodulin}^*] + \text{PDE} \rightleftharpoons [Ca^{++} - \text{Calmodulin}^* - \text{PDE}]$$

$$[Ca^{++} - \text{Calmodulin}^* - \text{PDE}] \rightleftharpoons [Ca^{++} - \text{Calmodulin}^* - \text{PDE}^*]$$

where *-denotes a different conformation.

Ca^{++}-binding studies have shown that each molecule of calmodulin has 4 Ca^{++}-binding sites, with dissociation constants from 4×10^{-6}M to 18×10^{-6}M (Liu et al, 1974; Lin et al, 1974).

In the proposed mechanism of PDE activation a conformational change takes place in calmodulin as it binds Ca^{++}. This results in the formation, within the calmodulin molecule, of a specific binding domain

which is involved in the interaction between calmodulin and PDE.

c. Family of calcium binding proteins

Calmodulin has been found to show sequence homology with a number of other calcium binding proteins such as troponin C (the Ca^{++}-dependent muscle regulatory protein), and parvalbumin (Kretsinger, 1975, 1976). These proteins have similar physical and chemical properties.

The proteins listed in table 1 make up a homologous family of calcium binding proteins. Based on the knowledge of the three dimensional structure of parvalbumin it can be concluded that each of these proteins contains one or more homologous calcium binding regions about 36-38 residues in length. Each binding region is made up of a helical segment, a calcium-binding loop and a second helical segment. Kretsinger (1971) has schematically represented the binding domain as a hand with extended thumb and forefinger (the two helical regions) and a clenched middle finger (the Ca^{++} binding loop) and has suggested that the so called "EF-hand" is the basic unit of evolution for this family of proteins.

As can be seen in table 1, some of the homologous proteins have more domains than Ca^{++} binding sites. In relation to the evolutionary aspects of these homologous Ca^{++}-binding proteins, it has been suggested that the ancestral protein from which these homologous proteins was derived contained a single calcium binding site with an "EF-hand" structure (Collins, 1976). This protein underwent two successive gene duplications and fusions to produce a protein four times the length of

Table 1: Family of homologous calcium binding proteins

Protein	MW	#Domains	#Ca^{++}-binding sites	Function
Parvalbumin	12,000	3	2	Ca^{++}-buffer
Troponin-C -				
cardiac	18,000	4	4	Regulation of Actomyosin ATPase
skeletal	18,000	4	4	Regulation of Actomyosin ATPase
Regulatory myosin Light Chain	19,000	4	1	Regulation of Actomyosin ATPase
Essential Myosin Light Chain	17,000-21,000	4	0	Essential for Myosin ATPase
Intestinal Ca^{++} Binding Protein	9,700	1	1	Ca^{++}-transport
Calmodulin	17,000	4	4	General Ca^{++} mediator
S-100	10,500	1	1	Role in Nervous System

the ancestral protein and contained 4 Ca^{++}-binding sites. This protein is then the common ancestor of TN-C, RMLC and EMLC, and parvalbumin. Of these proteins TN-C appears to be most closely related to this common ancestor, since it has the strongest internal sequence repeats. Parvalbumin apparently evolved by incomplete copying of the gene for the ancestral protein resulting in deletion of 1 domain and loss of Ca^{++} binding in a second domain. Internal homology in the myosin light chains is low. This results in structural changes which lead to distortions of the Ca^{++}-binding sites and consequent reduction in the affinity for Ca^{++} observed in both myosin light chains.

d. Comparison of calmodulin and troponin-C

Stevens et al (1976) showed that calmodulin resembles rabbit skeletal muscle TN-C in amino acid composition, molecular weight, isoelectric point, and U.V. absorption.

Watterson et al (1976) independently compared the physical and chemical properties of bovine brain calmodulin and TN-C. They observed both proteins to have blocked N-termini, similar and characteristic ultraviolet absorption spectra, similar Ca^{++} binding properties, similar amino acid compositions, and they co-migrate on SDS-polyacrylamide gels. Dedman et al (1977) also similarly concluded that calmodulin and TN-C are very similar and probably homologous, having evolved from a common ancestor.

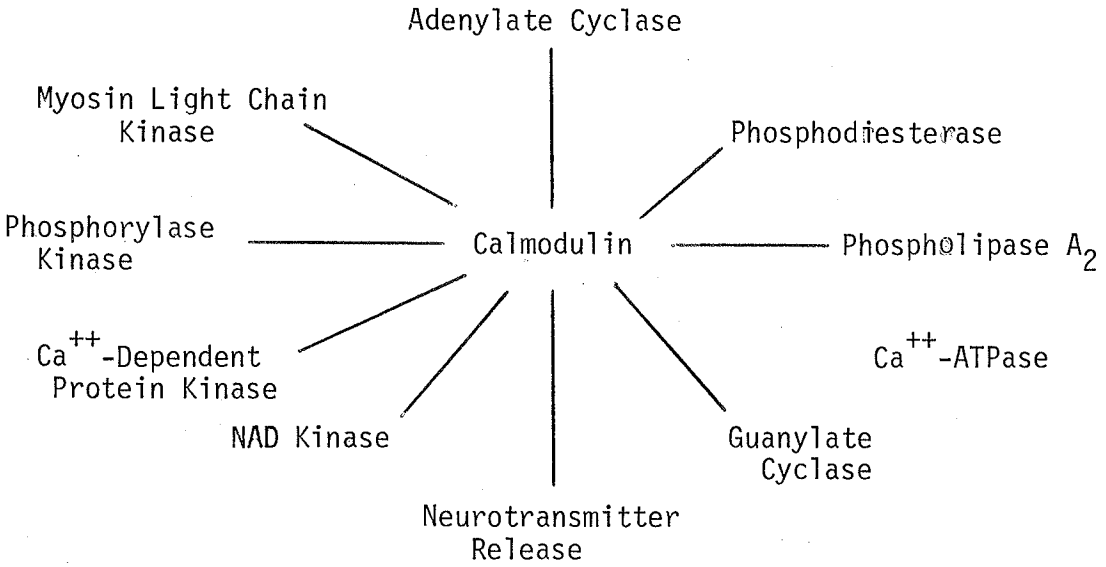
Appendix I compares the amino acid sequence of calmodulin to TN-C and unequivocally establishes that the primary structures of calmodulin and rabbit skeletal TN-C are homologous. The sequence homology between calmodulin and TN-C's is greater than that between

TN-C and myosin light chains and parvalbumins, indicating that calmodulin and TN-C form a family of structurally and functionally related proteins distinct from the more distantly related myosin light chains and parvalbumins. The total number of identical plus functionally conserved residues shared between bovine brain calmodulin and rabbit skeletal TN-C is 114 out of 148 positions.

TN-C can be divided into 4 homologous domains, each of which contains a potential Ca^{++}-binding site. Similarly, calmodulin also has 4 domains. The level of homology in domains of calmodulin and TN-C is greater when the first domain is aligned with the third domain and the second domain aligned with the forth.

e. Roles of Calmodulin

Calmodulin is involved as a modulator in a number of physiological systems. The following figure illustrates some of these systems.



Since calmodulin and TN-C are so remarkably similar in structure, they may be functionally interchangeable. Amphlett et al, (1976) demonstrated that calmodulin possesses a number of the biological properties of skeletal muscle TN-C. It can substitute for TN-C in a reconstituted Ca^{++}-sensitive actomyosin ATPase system. In the presence of TN-I and tropomyosin it is as effective in restoring Ca^{++}-sensitivity to desensitized actomyosin as is TN-C. Furthermore, calmodulin forms a Ca^{++}-dependent complex with TN-I as evidenced by PAGE in 15% slab gels. However, TN-C can not substitute for calmodulin as a modulator in systems calmodulin operates in. TN-C does not activate PDE (Wang et al, 1975; Klee, 1977), even at a 10^{-5}M concentration, while calmodulin gives a half maximal stimulation of the enzyme at $8x10^{-9}$M.

Calmodulin is a regulator for a number of enzymes, other than PDE including adenylate cyclase (Brostrom et al, 1975). It also is responsible for activation of the human erythocyte membrane (Ca^{++}-Mg^{++}- ATPase (Bond and Clough, 1973).

Calmodulin has also been implicated in mediating regulation of smooth muscle and non muscle contractile proteins. These are generally believed to be myosin-linked and regulated by a Ca^{++}-dependent, cAMP-independent myosin kinase (Klee et al, 1980).

B. Troponin in Muscle Contraction

a. General overview of muscle contraction

Muscle fibers are composed of four major proteins: myosin, actin, tropomyosin and troponin. The contraction of muscle is a function of their interaction, controlled by the presence of calcium ions. In the muscle cell, the four proteins are assembled into two multimolecular aggregates, the thick and thin filaments, which in turn are the basic components of the muscle fiber. The thick filaments are composed of myosin. A myosin molecule resembles a thin rod with two small globular heads. Within the thick filament, myosin molecules are arranged in a sheaf. A thick filament of skeletal muscle contains several hundred myosin molecules.

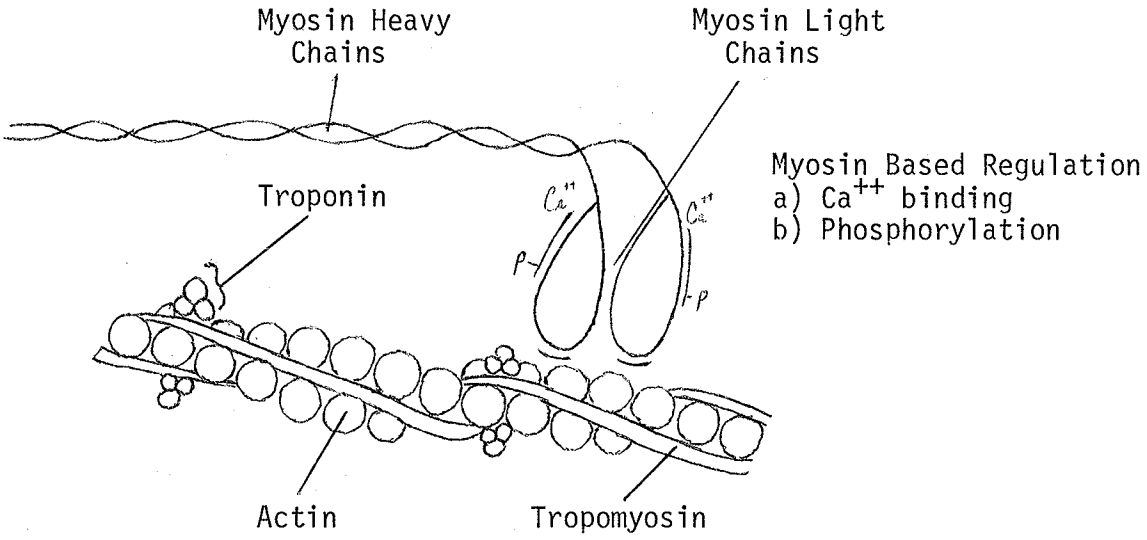
The other three major proteins are incorporated in the thin filaments. Of the three, actin is present in the largest amount. The actin molecules are arrayed to form a twisted double strand of beads.

Tropomyosins are long thin molecules that attach from end to end of the thin filaments forming a thin filament on the surface of an actin strand. Troponin, is more globular shaped and sits astride the tropomyosin molecule. In the thin filament one tropomyosin molecule extends over several actins, with one troponin molecule on each tropomyosin. The following figure illustrates (next page) this arrangement.

The two types of filaments are arranged in parallel arrays and overlap for part of their length. The spacing of the filaments is such that the two sets can slide past each other. It is this sliding motion that is responsible for the shortening during muscle contraction (Huxley, 1965).

The driving force for the sliding comes from the cross bridges on the thick filaments. They attach to the thin filaments at a certain angle and then presumably swivel to a different angle, pulling the thin filaments past the thick ones. The energy for this is provided by ATP.

In addition to the four proteins and ATP, calcium is required. Ebashi et al (1965) has shown that calcium control of muscle contraction resulted from the presence of troponin and tropomyosin on the actin filament. Calcium ions change the thin filament from an "off" to an "on" state, by binding to troponin (Murray, 1964).



Actin Based Regulation: Troponin - Tropomyosin

b. Discovery of troponin complex

The Ca^{++} requirement for actomyosin ATPase activity and super precipitation depends on the presence of tropomyosin and another protein factor, (Ebashi, 1963) termed troponin by Ebashi and Kodama (1965).

Troponin was originally described as a protein qualitatively resembling tropomyosin, but with a larger sedimentation constant and a higher viscosity (Ebashi and Kodama, 1965, 1966). Troponin alone could not sensitize the interaction between actin and myosin, but required the collaboration of tropomyosin (Ebashi and Endo, 1968; Ebashi et al, 1968). Further evidence indicated that troponin is the Ca^{++} receptive protein of the contractile system (Ebashi and Endo, 1968; Ebashi et al, 1967).

Hartshorne and Mueller (1968) separated troponin into two protein components which were termed troponin A and troponin B. The troponin A component had little effect on the Mg^{++}-ATPase activity, either in the presence or absence of tropomyosin, whereas troponin B caused a calcium insensitive inhibition of the Mg^{++} activated ATPase activity. Calcium sensitivity could be restored to the system by addition of the troponin A component (Hartshorne et al, 1969); the authors concluded that Ca^{++} sensitivity is associated with the troponin A component. This troponin A component was later found to be TN-C (Potter and Gergely, 1972). Troponin B was found to consist of Troponin I and troponin T (Hartshorne and Dreizen, 1972).

Many investigators have noted that troponin is a heterogeneous protein (Schaub and Perry, 1969; Greaser and Gergely, 1971; Drabikowski

et at, 1969). It is now accepted that troponin consists of three proteins, (Greaser and Gergely, 1971; Perry, 1971; Shagekawa and Tonomura, 1972). The protein component with molecular weight 23,000 was designated troponin I, the 18,000 molecular weight component was named troponin C and the third component with molecular weight 37,000 was named troponin T (Potter and Gergely, 1972).

c. Occurrence of troponin complex

Troponin is a complex of three components found in the thin filaments of skeletal muscle in a wide variety of species (Lehman et al, 1972). This protein complex in combination with tropomyosin regulates muscle contraction by conferring Ca^{++} sensitivity to the interaction of actin and myosin (Weber and Murray, 1973).

It has been isolated and purified to homogeneity from the entire spectrum of the evolutionary scale. Troponin has been found in insects (Lehman et al, 1972; Bullard et al, 1973), annelids (Lehman et al, 1972), fish and reptiles (Demaille and Dutruge, 1974), and crustaceans (Regenstein and Szent-Gyorgyi, 1975). Other sources include rabbit skeletal muscle (Greaser and Gergely, 1971; Shigekawa and Tonomura, 1972), rabbit cardiac muscle (Cole and Perry, 1975; Wilkinson and Grand, 1978), chicken skeletal muscle (Wilkinson, 1978) and bovine cardiac muscle (Burtnick et al, 1975).

d. Physical properties

Several groups of investigators have conducted physical and chemical characterization of the troponin complex, purified from

different sources. The different troponin's exhibit similar physical and chemical properties.

i) Molecular weights

Considerable discrepancies are apparent in the reported values of the molecular weights for the troponin subunits. The values obtained depend, on both the source of the material and the method used.

Troponin I molecular weights obtained, range from 20,500 to 27,000. Methods of determination used included gel filtration, sedimentation equilibrium, SDS-PAGE, and calculation from the amino acid sequence. As determined by sedimentation equilibrium TN-I from bovine cardiac muscle had an apparent molecular weight of 22,900 (Burtnick et al, 1975); SDS-PAGE determination indicated a molecular weight of 24,000 (Greaser and Gergely, 1971), 23,000 (Perry and Cole, 1974) and 22,000 (Wilkinson, 1974), for rabbit skeletal muscle and 27,000 for bovine cardiac muscle (Burtnick et al, 1971). Gel filtration studies found TN-I to have a molecular weight of 20,500 (Wilkinson, 1978), 23,000 (Schaub and Perry, 1971), and 23,000 by Wilkinson et al, (1971), for rabbit skeletal muscle. From the amino acid sequences of fast skeletal TN-I, (Wilkinson and Grand, 1975), a molecular weight of 20,721 was calculated and from slow skeletal TN-I (Grand and Wilkinson, 1977) a molecular weight of 21,123.

Troponin T has been purified by a number of investigators. Its molecular weight was found to be in the range of 30,500 to 44,000.

SDS-PAGE determination of TN-T from rabbit skeletal muscle indicated a molecular weight of 37,000 (Perry and Cole, 1974), 30,500 (Pearlstone et al, 1976), and 35,000 (Greaser and Gergely, 1971). Gel filtration for rabbit skeletal muscle gave a value of 44,000 (Perry and Cole, 1974), whereas for chicken skeletal muscle 33,500 (Wilkinson, 1977).

Troponin C was purified and its molecular weight was found to range between 18,000 and 21,000. SDS-PAGE gave a molecular weight of 21,000 (Greaser and Gergely, 1971) and 19,000 (Perry and Cole, 1974), for rabbit skeletal muscle. Gel filtration studies indicated a molecular weight of 18,000 (Wilkinson, 1978). Burtnick et al (1975), found an apparent molecular weight of 18,000 by sedimentation equilibrium. The molecular weight calculated from the sequence of TN-C (Collins et al, 1977) was 17,784.

e. Chemical structure of troponin

i) amino acid composition

Comparison of the amino acid compositions of the troponin subunits isolated from rabbit skeletal muscle (Greaser and Gergely, 1971; Wilkinson, 1974) rabbit cardiac muscle (Cole and Perry, 1975) and chicken skeletal muscle (Wilkinson and Grand, 1978) indicate that the troponin complex subunits from different sources exhibit a large degree of similarity. Appendixes II and III show comparisons of the amino acid compositions of TN-I and TN-C from various sources.

There is more variation in the composition of TN-I from the various sources, whereas TN-C values are virtually identical.

Troponin I has a large number of basic residues as compared to TN-C, which has a large percentage of acidic ones. This is

consistent with the observed isoelectric points for these proteins, with TN-I's being high and TN-C's being low.

ii) amino acid sequences

The amino acid sequences of the troponin components have been determined. Wilkinson and Grand, (1975) determined the sequence of rabbit fast muscle TN-I and Grand and Wilkinson, (1977) that for rabbit slow muscle TN-I. The TN-C sequence was done by Collins et al, (1977), and the TN-T sequence by Pearlstone et al, (1976).

Appendix IV illustrates the alligned amino acid sequences for TN-I from rabbit fast and slow skeletal muscle, along with that for rabbit cardiac TN-I. Appendix I compares the sequence of rabbit skeletal TN-C with calmodulin and bovine cardiac TN-C.

The different rabbit TN-I's show a great deal of homology. Overall there is approximately a 60% homology, between the sequences. If the proteins are divided in half one finds a 40% homology at the N-terminus and 75% homology at the C-terminus. There appears to be more conservation of residues in the latter half of the molecules.

Troponin C appears to be more conserved than TN-I. Comparing the homology between the three TN-C proteins, one finds about an 80% homology. There is a great deal more conservation in the TN-C proteins from different sources than in TN-I proteins from the same species.

f. Different forms of troponin

It has been established that TN-I, TN-T, and TN-C exist in polymorphic forms, characteristic for the type of muscle from which they were prepared (Perry, 1975; Cummins and Perry, 1978). In the case of cardiac muscle, which consists of one cell type, a single form of

each of the regulatory proteins is present (Swynghedauw et al, 1977).

Forms of troponin I found in skeletal muscle differ, depending on whether the muscle is fast or slow, and also differ from TN-I found in cardiac muscle (Greaser et al, 1972; Tsukui and Ebashi, 1973; Syska et al, 1974).

The amino acid sequences of the polymorphic forms of TN-I are different (Appendix IV). Wilkinson and Grand (1978) compared TN-I from rabbit fast skeletal, slow skeletal, and cardiac muscle. These proteins were found to differ in length and amino and carboxy terminal sequences. The cardiac TN-I has an extra 26 residues at the N-terminus as compared to both fast and slow skeletal TN-I. The sequence differences of these troponin I's may reflect differences in regulatory activity.

Different forms of TN-I were tested for antibody specificity (Dhoot et al, 1980). The antibodies produced against one type of TN-I do not cross react with other TN-I's. TN-I antibodies were found to be specific to the form of the protein, characteristic for the muscle type and not the species from which the muscle is derived.

Each form of TN-I possesses a net positive charge, being 8-9 for TN-I from fast skeletal muscle and 14-18 for TN-I from cardiac and slow skeletal muscle (Wilkinson and Grand, 1978). The slow muscle's higher positive charge is a consequence of an increased number of lysine and arginine residues and a decrease in aspartic and glutamic acid residues, as compared to fast skeletal TN-I.

The amino acid sequences of the three TN-I's are particularly variable towards the N-terminus, although residues 44-54 are more homologous than the surrounding areas. The proposed actin binding

sites (residues 125-145) (Syska et al, 1976) in the three molecules are very similar indeed and this lends support to the view that the actin molecules present in slow, fast and cardiac muscle are very similar.

g. Role in muscle contraction

Contractile activity in vertebrate skeletal muscle is regulated by the level of free Ca^{++} in the sarcoplasm by means of the molecular switch formed by the tropomyosin and interaction of the troponin subunits (Hartshorne and Muller, 1968).

Troponin, composed of three components, repeats itself every 385Å along the thin actin filaments (Ebashi et al, 1969; Weber and Murray, 1973). From this spacing Ebashi et al estimated that TN, TM, and actin are present in a molar ratio of 1:1:7. In agreement with this stoichiometry, Bremel and Weber (1972) have shown that one TN and one TM can block the combination of myosin with approximately seven actin molecules. Potter (1979) has shown that the ratio of troponin subunits, TN-T: TN-I: TN-C is 1:1:1.

TM is located within the two long pitch grooves of the actin double helix (Hanson et al, 1972). One strand of TM is in close association with one helical set of actin monomers and another TM strand with the other actin helix. In the relaxed state TM molecules are thought to sterically block the combination of myosin heads with actin. In the activated state, after the binding of Ca^{++} to TN, TM moves into a position closer to the groove and thus no longer blocks the interaction of myosin with action.

The three troponin subunits; TN-C, TN-T, and TN-I have been studied by many investigators and their specific role in this system outlined.

The target protein for Ca^{++} in the troponin complex is the calcium-binding protein, TN-C (Hartshorne and Mueller, 1968; Schaub and Perry, 1969). The changes in physical properties associated with the binding of Ca^{++}, reflect modifications in the conformation of the TN-C itself (Head and Perry, 1974). Murray and Kay, (1972) and Van Eerd and Kawasaki, (1972) have shown that Ca^{++} can induce conformational changes in isolated TN-C.

It was suggested that the primary function of the Ca^{++}-relaxing protein system is to regulate the sensitivity of the Mg^{++}-activated ATPase activity of actomyosin to the inhibitory effect of substrate Mg ATP (Ebashi and Ebashi, 1964). A structural effect on TN-C induced by Ca^{++}, is transmitted to actin through tropomyosin to affect the actin-myosin interaction (Tonomura et al, 1969).

Troponin I inhibits actomyosin ATPase (Ebashi et al, 1972; Perry et al, 1972), a property that is potentiated by the presence of TM, but is independent of Ca^{++} concentration. In the presence of Ca^{++} a complex between TN-I and TN-C neutralizes the inhibition of TN-I, allowing muscle contraction to be carried out (Head and Perry, 1974).

TN-T is the subunit of the troponin complex that binds to TM (Cohen et al, 1972). TN-T also binds to both TN-C and TN-I (Ebashi and Endo, 1968; Weber and Murray, 1973; Cohen et al, 1972). TN-T functions to increase the affinity of TN-C and TN-I from the TM containing actin filament; since it provides them with one or two additional binding

sites. TN-T may also function in increasing calcium "sensitivity" to the subunit complex (Ebashi and Endo, 1968).

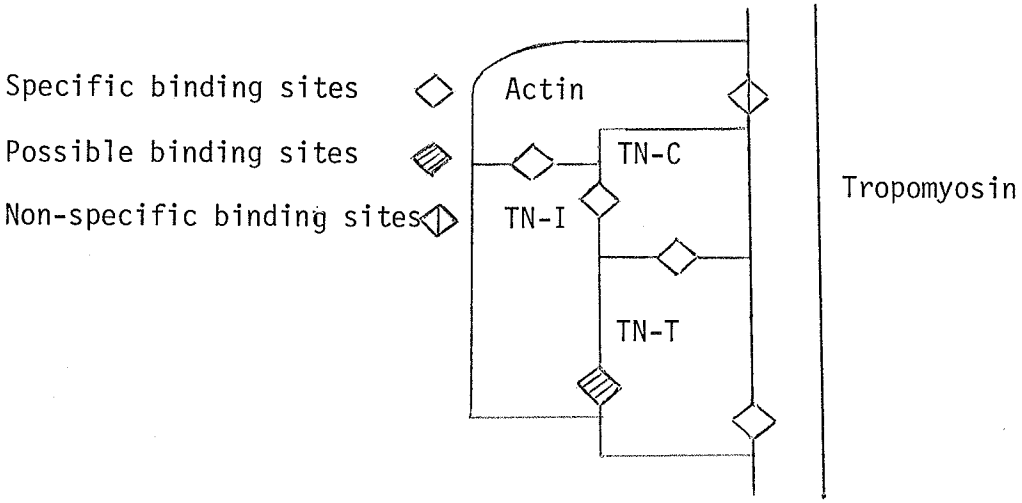
h. Sites of interaction

The active form of skeletal muscle troponin is a complex consisting of three components present in equimolar amounts, each responsible for different functions (Murray and Kay, 1971; Drabikowski et al, 1971).

TN-I inhibits the Mg^{++}-activated ATPase. This inhibition is removed in the presence of Ca^{++}, by TN-C, which binds Ca^{++} strongly (Fuchs, 1971). After binding Ca^{++}, TN-C undergoes a conformational change which enhances the formation of complex between TN-C and Tn-I (McCubbin et al, 1974).

The TN-C subunit binds to both TN-T (Cohen, 1971) and TN-I (Hartshorne and Dreizen, 1972). The following diagram illustrates some of the binding sites of the troponin subunits with each other and the other thin filament proteins.

Schematic representation of presumed interaction sites between troponin subunits and between troponin, tropomyosin, and actin (Ebashi et al, 1969)



Many of the actual sites of interaction have been located.

Syska et al (1976) have localized the areas of interaction of fast skeletal TN-I with TN-C and actin. The actin binding site is located near the C-terminus of the TN-I molecule, comprising residues 125-145 (Syska et al, 1976). Grand et al (1976) have found analogous regions of cardiac and slow skeletal TN-I to bind actin.

The TN-C binding site, at the N-terminus of the TN-I molecule, is less well defined, as compared to the actin binding site. Syska et al (1976) have presumed that the TN-C binding region is located near residues 44-54.

Troponin C is composed of four Ca^{++}-binding sites. Weeks and Perry (1978) and Leavis et al (1978) have shown at least one and possibly two sites of interaction exist for TN-C with TN-I. Using fluorescence titration and observing complex formation on polyacrylamide gels Leavis et al (1978) have shown that the third Ca^{++}-binding site, consisting of residues 92-105, interacts strongly with TN-I and that site two, representing residues 53-65 interacts weakly with TN-I. Potter et al (1976) has shown that the third Ca^{++} binding site is one which has high affinity for Ca^{++}.

Wilkinson and Grand (1975) have shown that threonine 11 and serine 117 of TN-I are blocked from phosphorylation when TN-I is in complex with TN-C. It may be that these residues are involved in the binding between these two subunits.

Troponin C also forms an interaction with TN-T. The binding region on TN-C for the TN-T molecule was found to be between residues 120 to 159 (Leavis et al, 1978).

3. GENERAL EXPERIMENTAL PROCEDURES

A. Assay of Calmodulin Activity

Calmodulin activity was assayed by measuring the extent of stimulation of a fixed amount of calmodulin deficient PDE (0.4-0.5 units) under standard conditions. The reaction mixture, in a volume of 0.9 ml, contained in addition to the calmodulin and a standard amount of PDE (prepared as described in 3.E), 1.2 mM cAMP, and 0.25 units of 5' nucleotidase in 40 mM Tris, 40 mM imidazole, 3 mM magnesium acetate, 0.11 mM $CaCl_2$, pH 7.5. The reaction mixture was incubated at 30°C for 30 min, at which time the reaction was terminated by the addition of 0.1 ml of 55% (w/v) TCA. To the mixture is added 1 ml of 55% (w/v) ammonium molybdate in 1.1 N sulfuric acid followed by 0.1 ml reducing agent (1.15 M sodium bisulfite, 0.05 sodium sulfite, 0.01 M 1-amino-1-napthol-4-sulfonic acid). Colour development was allowed to proceed for at least 30 min and the absorbance at 660 nm was measured against distilled water in a Beckman model 25 spectrophotometer.

Varying amounts of calmodulin samples were assayed in order to construct a standard curve relating stimulation of PDE activity to amount of calmodulin (fig. 1 as an example). With saturating concentrations of calmodulin, phosphodiesterase is stimulated 6-10 fold. One unit of calmodulin is defined as the amount required to give ½ maximal stimulation of a standard amount of PDE.

B. Assay of Cyclic Nucleotide Phosphodiesterase

PDE activity was measured by the method of Butcher and Sutherland (1962), as modified by Wang et al (1972). The assay involves measuring the inorganic phosphate obtained from 5'AMP, (the product of the action

of PDE on cAMP) by the action of 5' nucleotidase. The reaction mixture (0.9 ml) contained in addition to PDE and calmodulin, 1.2 mM cAMP, 0.25 units of 5' nucleotidase in 40 mM Tris, 40 mM imidazole, 3 mM magnesium acetate, 0.11 mM $CaCl_2$, pH 7.5. The reaction mixture was incubated for 30 minutes at 30°C, at which time the reaction was terminated by the addition of 0.1 ml of 55% (w/v) TCA. To the mixture was added 1 ml of ammonium molybdate (55% w/v) in 1.1 N sulfuric acid, followed by 0.1 ml reducing agent (1.15 M sodium bisulfite, 0.05 M sodium sulfite, 0.01 M 1-amino-2-napthol-4-sulfonic acid). Colour development was allowed to proceed for at least 30 minutes and absorbance measured at 660 nm against distilled water, in a Beckman model 25 spectrophotometer. A typical standard curve relating amount of PDE to A_{660}nm is illustrated in fig. 2.

C. Electrophoretic Procedures

a. 15% PAGE

Gel electrophoresis was performed on 15% polyacrylamide slab gels employing the discontinuous buffer system of Davis (1964). The following stock solutions were used.

Solution	Composition
A	22.2 g acrylamide 0.6 g MBA H_2O ---- 100 ml
B	0.6 M Tris-HCl, pH 8.9
C	0.6 M Tris-phosphate, pH 6.9
D (separating gel buffer)	40 g sucrose 40 ml solution B H_2O ---- 100 ml

Solution	Composition
E (sample buffer)	70 g sucrose 20 ml solution C 2.5 mg bromophenol blue H_2O ---- 100 ml
F (running buffer)	4.32 g glycine 0.9 g Tris, pH 8.3 H_2O ---- 1500 ml
G (spacer gel buffer)	40 g sucrose 40 ml solution C H_2O ---- 100 ml

The following solutions were made prior to use:

Ammonium persulfate: 5 mg/ml in H_2O

Running Buffer: 60 ml solution F plus 2940 ml H_2O

The separating gel (15% acrylamide, 0.4% MBA) was prepared by mixing the following solutions:

Solution A	20.3 ml
Solution D	7.5 ml
H_2O	0.7 ml
Ammonium persulfate	1.5 ml
Temed	0.02 ml

The spacer gel (5% acrylamide, 0.13% MBA) was prepared by mixing the following solutions:

Solution A	3.375 ml
Solution G	3.75 ml
H_2O	7.125 ml
Ammonium persulfate	0.75 ml
Temed	0.02 ml

Protein samples were dissolved, normally at a concentration of 1 mg/ml in solution E (sample buffer), diluted 1:1 with water.

Electrophoresis was performed at 100 volts until the sample focused in the separating gel, and then at 300 volts until the dye front neared the bottom of the gel. Gels were stained overnight with 0.25% Coomassie Brilliant Blue G-250 in 7.5% acetic acid and destained electrophoretically with 7.5% acetic acid.

b. SDS-PAGE

SDS-PAGE was performed in slab gels employing a discontinuous buffer system essentially as described by Swank and Munkres (1971). The following solutions were used:

Solution	Composition
A	30 g acrylamide 0.8 g MBA H_2O ---- 100 ml
B	1.5 M Tris-HCl, pH 6.8
C	10% SDS
D	0.5 M Tris-HCl, pH 6.8
E (running buffer)	3 g Tris 14.4 g glycine 1 g SDS H_2O ---- 1000 ml
F (sample buffer)	0.05 M Tris 1% SDS 0.01% Bromophenol Blue 30% glycerol

The separating gel (12% acrylamide, 0.32% MBA) was prepared by mixing the following solutions:

Solution A	12 ml
Solution B	7.5 ml
H_2O	8.4 ml

Solution C	0.6 ml
Ammonium persulfate 4 mg/ml	1.5 ml
Temed	0.02 ml

The spacer gel (5% acrylamide, 0.13% MBA) was prepared by mixing the following solutions:

Solution A	5 ml
Solution B	7.5 ml
H_2O	15.7 ml
Solution C	0.3 ml
Ammonium persulfate	1.5 ml
Temed	0.02 ml.

Protein samples were dissolved normally at a concentration of 1 mg/ml in solution F (sample buffer) and immersed in a boiling water bath for 10 minutes. Electrophoresis was performed at 25 mamps, until the dye front neared the bottom of the gel. Gels were stained overnight with 0.25% Coomassie Brilliant Blue G-250 in methanol:acetic acid:H_2O (5:1:5) and subsequently destained electrophoretically with 7.5% acetic acid.

D. Preparation of Calmodulin

Bovine brain calmodulin was isolated and purified by a modification of the procedure described by Teo et al, (1973) for the purification of the bovine heart modulator. The modifications were necessary to remove protein contaminants, notably the S100 protein (Watterson et al, 1976), not found in bovine heart. The following steps were performed at 4°C unless otherwise stated.

Homogenization

1 kg of beef brain (stored frozen at -20°C) was thawed, chopped into small pieces and homogenized in 2.5 volumes of 0.1 M Tris, 2 mM EDTA, pH 7.5 in a Waring blender at high speed for 20-30 seconds. The homogenate was centrifuged at 11,000 g for 45 minutes.

Ammonium sulfate fractionation

The homogenate supernatant was adjusted to pH 8.8 with 5 N NaOH and brought to 40% saturation with ammonium sulfate, stirred well and left standing for 30 minutes. The mixture was then centrifuged at 11,000 g for 30 minutes. This step of the procedure removes the modulator dependent phosphodiesterase, which is in the pellet, from the calmodulin, located in the supernatant.

Heat treatment

To the supernatant from the previous step were added 100 ml of 1 M magnesium acetate and additional ammonium sulfate to give a final saturation of 60%. The pH was adjusted to 4.2 with HCl and the resulting mixture was left overnight in the coldroom. The mixture was centrifuged at 11,000 g for 30 minutes. The supernatant was discarded and the pellet resuspended in 300 ml of .08 M Tris, 1 mM magnesium acetate, pH 8.0. The pH was adjusted to 8.0 with 5 N NaOH. Calcium chloride (45 mM) was added to a final concentration of 20 μM. The slurry was immersed in a boiling water bath with constant stirring until a precipitate formed. Heating was continued for an additional minute, the sample cooled immediately on ice and centrifuged at 23,000 g for 20 minutes. The supernatant was dialyzed against 0.02 M imidazole, 1 mM magnesium acetate, pH 6.5.

DEAE - Cellulose column chromatography

The dialyzed supernatant was applied to a DEAE-Cellulose column (2.5 x 90 cm) previously equilibrated with buffer A (0.02 M imidazole, 1 mM magnesium acetate, 0.2 M NaCl, 20 μM $CaCl_2$, pH 6.5). The column was washed with buffer A and elution achieved with a linear salt gradient consisting of 1 litre each of buffer A and buffer B (0.02 M imidazole, 1 mM magnesium acetate, 0.65 M NaCl, 20 μM $CaCl_2$, pH 6.5). The flow rate was 65 ml/h and 6.5 ml fractions were collected. The elution profile is shown in fig. 3. Selected fractions were subjected to assays for calmodulin activity, see Section 3A (fig. 3) and electrophoresis on a 15% polyacrylamide slab gel (fig. 4); appropriate fractions enriched with calmodulin were pooled, dialyzed against water and lyophilyzed.

Sephadex G-100 gel filtration

The lyophilyzed material was dissolved in 5 ml of 0.02 M Tris, 1 mM imidazole, 1 mM magnesium acetate, 0.01 mM $CaCl_2$, 0.5 M NaCl, pH 7.5 and applied to a Sephadex G-100 column (2.5 x 90 cm) equilibrated with the same buffer. Fractions (3.2 ml) were collected at a rate of 25 ml/h (fig. 5). The fractions were monitored as described above (fig. 5, fig. 6) and the appropriate fractions were pooled, dialyzed against water and lyophilyzed.

A summary of the purification of bovine brain calmodulin is shown in table I. The average yield of purified calmodulin by this method is 50 - 70 mg/Kg of bovine brain.

Criteria of homogeneity

The purity of the final product was assessed by gel electro-

phoresis (fig. 6) and also by amino acid composition (table II). Table II shows results of typical amino acid analysis of calmodulin. The theoretical composition was calculated from the amino acid sequence (Vanaman et al, 1979) and is shown for comparison.

E. Preparation of Calmodulin Deficient Phosphodiesterase

Calmodulin free phosphodiesterase (PDE) was prepared from bovine brain as described by Wang et al, (1980). All steps were performed at 4°C unless otherwise stated.

Homogenization

2 Kg of bovine brain (stored frozen at -20°C) were thawed, chopped into small pieces and homogenized with 3 litres of buffer A (100 mM Tris, 2 mM EDTA, pH 7.5) in a Waring blender at top speed for 20 seconds. The homogenate was centrifuged at 11,000 g for 30 minutes. The pellet was reextracted with 3 litres of buffer A and centrifugation repeated. The supernatants were combined and EGTA was added to give a final concentration of 0.1 mM. Mercaptoethanol was also added to give a final concentration of 10 mM.

DEAE-Cellulose column chromatography

The combined supernatants were applied to a DEAE - Cellulose column (6 x 30 cm) previously equilibrated with buffer B (20 mM Tris, 1 mM magnesium acetate, 1 mM imidazole, 0.1 mM EGTA, 10 mM mercaptoethanol, pH 7.0) and washed with buffer B containing 0.05 M NaCl for 1-2 bed volumes. Elution was achieved with a linear salt gradient consisting of 2 litres each of buffer B containing 0.05 M NaCl and buffer B containing 0.4 M NaCl. The flow rate was 200 ml/h and 20 ml fractions were collected. The elution profile is shown in fig. 7.

Selected fractions were subjected to assays for phosphodiesterase activity in the presence and absence of Ca^{++}, conductivity measurements, and the absorbance was read at 280 nm. PDE-I (calmodulin dependent PDE) is identified as the peak of PDE activity which is inhibited by EGTA. PDE-II is unaffected by EGTA and represents calmodulin independent PDE. The fractions corresponding to PDE-I were pooled.

Affi-gel blue column chromatography

The pooled material was applied to an affi-gel blue column (6 x 15 cm) previously equilibrated with buffer B. The column was washed with 4 column volumes of buffer B containing 0.15 M NaCl. Elution of PDE was achieved with a linear salt gradient consisting of 1.5 litres each of buffer B plus 0.15 M NaCl and buffer B plus 1 M NaCl. The flow rate was 200 ml/h and 15 ml fractions were collected. The elution profile is shown in fig. 8. The fractions indicated by the bar were pooled and stored frozen in 1 ml aliquots.

F. Preparation of Troponin

The troponin complex (consisting of troponin I, troponin C, and troponin T) was isolated and purified by a modification of the method of Greaser and Gergeley, (1971). The modifications were necessary to produce a single type of troponin I, since troponin I from fast and slow muscle are now known to have different amino acid sequences. Reagents were also added to limit protein degradation during the preparation. The purification was monitored using SDS - polyacrylamide gel electrophoresis on 12% slab gels. The steps were performed at 4°C unless otherwise stated.

Washing

1 Kg of back muscle was removed from New Zealand White rabbits, cleaned of fat and connective tissue and ground. The ground muscle was stirred for 5 minutes in 2 litres of a solution containing 50 mM KCl, 5 mM Tris, 0.1 mM $CaCl_2$, pH 7.5 with 350 µl of a solution of 50 µg PMSF/ml DMSO added per litre of wash solution to inactivate proteolytic enzymes. The suspension was filtered through cheesecloth and the ground muscle washing was repeated 4 more times.

2 litres of 95% ethanol was then added to the ground muscle and the solution was filtered through cheesecloth after 10 minutes. The ethanol wash was repeated 2 additional times.

The muscle was then washed three times, each time with 2 litres of diethyl ether for 10 minutes and then was filtered through cheesecloth and allowed to dry at room temperature for 2-3 hours.

Extraction

The dried powder was extracted overnight at room temperature with 2 litres of a solution containing 1 M KCl, 25 mM Tris, 0.2 mM $CaCl_2$, 0.1 mM DTT, pH 8.0, with 400 µg of Pepstatin A added per litre of extraction solution to prevent proteolytic breakdown. The solution was filtered through cheesecloth and the residue was reextracted with 1 litre of 1 M KCl solution.

Ammonium sulfate fractionation

The combined extracts were cooled to 4°C. Solid ammonium sulfate was added to produce approximately 40% saturation. The solution was centrifuged at 11,000 g for 25 minutes. Ammonium sulfate was added to the supernatant to give a final 60% saturation. This mixture was

centrifuged at 11,000 g for 25 minutes and the supernatant discarded. The precipitate was dissolved in 500 ml of a solution containing 5 mM Tris, 0.1 mM $CaCl_2$, 0.1 mM DTT, pH 7.5 and dialyzed against 15 litres of the same solution for 6 h and against 15 litres of fresh solution overnight. Solid KCl was added to give a final concentration of 1 M and 1 M KCl solution was added to bring the volume to 1 litre. The pH was adjusted to 4.6 by addition of HCl and tropomyosin was removed by centrifugation at 23,000 g for 20 minutes. The supernatant was adjusted to pH 7.0 with 5 N KOH and ammonium sulfate added to 70% saturation. The solution was centrifuged at 11,000 g for 30 minutes and the precipitate dissolved in 400 ml of a solution containing 5 mM Tris, 0.1 mM $CaCl_2$, 0.1 mM DTT, pH 7.5 and dialyzed against 15 litres of the same solution overnight. Solid KCl was added to bring the final concentration to 1 M and the pH adjusted to 4.6 with HCl. The precipitate was removed by centrifugation at 23,000 g for 20 minutes. The supernatant was adjusted to pH 7.0 with 5N KOH and dialyzed against 2 mM Tris, pH 7.5 overnight. This solution contains the crude troponin complex as seen in fig. 9.

DEAE-Sephadex A-50 column chromatography

The dialyzed supernatant was applied to a column (6 x 30 cm) of DEAE Sephadex A-50 previously equilibrated with buffer A (7 M urea, 50 mM Tris, 1 mM DTT, 2 mM EGTA, pH 7.5). Excess protein was washed off with buffer A and elution achieved with a linear salt gradient consisting of 800 ml buffer A and 800 ml buffer A containing .6 M KCl. The flow rate was 125 ml/h and 6.2 ml fractions were collected. The elution profile is shown in fig. 10. Selected fractions were subjected

to electrophoresis on 12% SDS-PAGE slab gels (fig. 11, fig. 12). The appropriate fractions of each subunit of the troponin complex were pooled and dialyzed against several changes of a solution containing 0.3 M KCl, 10 mM Tris, 2 mM βME, pH 7.5. After all the urea had been removed, βME was omitted in the final dialysis.

a. Troponin C purification

The Troponin C, obtained from DEAE-Sephadex A-50 chromatography was judged to be homogeneous by amino acid composition (Table III), and SDS-PAGE (fig. 12, slots 13, 14).

b. Troponin I purification

The troponin I fraction obtained from DEAE-Sephadex A-50 chromatography was further purified by passage through a Sepharose 4B-calmodulin affinity column. Coupling of calmodulin to Sepharose 4B was as described by Watterson and Vanaman (1976).

The TN-I sample was applied to a Sepharose 4B - calmodulin affinity column (1.5 x 30 cm) previously equilibrated with 50 mM Tris, 2 mM $CaCl_2$, 0.2 M KCl, pH 7.5. Excess protein was washed off and TN-I was eluted with a solution of 50 mM Tris, 0.2 M KCl, 5 mM EGTA, pH 7.5. The flow rate was 30 ml/h and 2.5 ml fractions were collected. The elution profile is shown in fig. 13, and electrophoresis of the crude and purified TN-I is seen in fig. 14.

The purity of the final product was assessed by amino acid composition (Table IV) and SDS-PAGE (fig. 14).

G. Acid Hydrolysis and Amino Acid Analysis

Samples containing 0.02 to 0.1 μmoles of protein were hydrolyzed with 6 N HCl, containing 50 μl of 55% (w/v) aqueous phenol per ml to protect tyrosine residues against destruction (Howard and Pierce, 1969), at 110°C in sealed evacuated tubes for 22 hours. Samples were dried in a vacuum desicator prior to amino acid analysis which was carried out on a Spinco 120/139 amino acid analyzer as outlined in the Spinco manual.

H. Peptide Purification by PAGE

Samples of digested protein were subjected to preparative gel electrophoresis employing 15% polyacrylamide slab gels (see 3.C.a), in batches of 1 mg of protein. The entire gels were stained for location of specific peptide bands. To elute the protein from the gel the gel slices containing bands of interest were cut out, cut into 1 mm cubes and homogenized in a minimum volume of 0.1 Tris-HCl, 0.1 M KCl, pH 8.1. The homogenate was stirred overnight and then centrifuged at 27,000 g for 30 minutes. The supernatant was decanted into a lyopholization flask and the pellet was resuspended in a minimum volume of 0.1 M Tris-HCl, 0.1 M KCl, pH 8.1 and centrifuged as before. The resultant supernatant was combined with the first supernatant and the mixture lyophilized. The residue was dissolved in 3.5 ml of water and applied to a Sephadex G-25 column (2.5 x 90 cm) previously equilibrated with 0.1 M Tris-HCl, 0.1 KCl, pH 8.1 and elution achieved with the same buffer. Fractions (5 ml) were collected at a flow rate of 80 ml/h. The absorbance of each fraction was measured at 280 nm in a Beckman Model 25 spectrophotometer. The peptide samples eluted between

fractions 38-48, which coincided with the void volume as previously determined using blue dextran. These fractions were pooled and lyophilized.

The residue, shown to be homogeneous by 15% PAGE, was dissolved in 5 ml of 0.1 M KCl. Aliquots were lyophilized in hydrolysis tubes and amino acid hydrolysis done in the usual manner.

I. Peptide Purification by HPLC

Samples containing 10-20 μg (1 mg/ml) of protein after enzymatic digestion were applied to a Beckman model 322 gradient liquid chromatograph system with an Altex Ultrasphere ODS reverse phase column (4.6 x 25 mm). Solvent A was 0.1% H_3PO_4 and solvent B, acetonitrile. Gradients were run from 0-70% B over a 40 minute time span. The method used was a modification of the method of Fullmer and Wasserman (1981). The effluent was monitored at 210 nm with a Hitachi 100-40 spectrophotometer with a flow cell. Appropriate samples were collected, pooled, lyophilized and rerun, to assure homogeneity. Aliquots were lyophilized in hydrolysis tubes and amino acid hydrolysis done in the usual manner.

4. EXPERIMENTAL

A. Complex Formation Between TN-I and TN-C or Calmodulin

a. Introduction

It has been reported that TN-I and TN-C can form a stable Ca^{++} dependent complex. In the presence of Ca^{++} the complex did not dissociate even in the presence of 8 M urea (Head and Perry, 1974). As well, Vanaman et al, (1975), reported that calmodulin can also form a stable complex with TN-I. Troponin C and calmodulin are rich in acidic residues and have a higher electrophoretic mobility at pH 8.3 than TN-I, whose basic nature tends to keep it near the origin at this pH value. The complex formed between TN-I and either TN-C or calmodulin is readily visible on PAGE in the presence of Ca^{++}. It would be of interest to look at the stoichiometry of the complexes formed; to look at the ratio of TN-I to either calcium binding protein.

b. Experimental procedure

The first experiments included qualitative descriptions of the complexes formed between TN-I and either calmodulin or TN-C. Samples containing 20 µg of TN-I were mixed with 40 µg of either calcium binding protein, in the presence and absence of Ca^{++}, and the mixture run on 15% PAGE slab gels (fig. 15).

Subsequent experiments included titration gels. 20 µg of TN-C or calmodulin were applied to each slot of a 15% PAGE and increasing amounts of TN-I were added to the same slots (figs. 16 and 17) in order to determine the amount of TN-I needed to fully complex TN-C or calmodulin.

c. Results and discussion

In agreement with the previous studies of Head and Perry (1974) we were able to show that the complex formed between TN-I and either TN-C or calmodulin is calcium dependent. One can see from fig. 15 (slots 5, 9) that TN-I forms a complex with TN-C or calmodulin in the presence of Ca^{++}, that on PAGE runs inbetween TN-I and the calcium binding protein it is complexed with. Fig. 15, (slots 6, 10) illustrate that this complex does not form, when EGTA is present. EGTA is a calcium chelator which removes the free calcium, thus preventing calcium binding with either calcium binding protein.

The complexes formed ran as two bands on PAGE. A major band and a minor, slower moving band. Head and Perry (1974) found that with higher ratios of TN-C to TN-I a slower moving band was observed which they postulated was of different stoicheiometry.

Results of the titration gel (fig. 16) show that as the ratio of TN-I to TN-C is increased, beyond two to one, all of the TN-C is bound up in complex. This is in complete agreement with the findings of Burtnick et al, (1975). These results do not necessarily represent a physiological ratio between these two proteins.

Fig. 17, the titration gel of calmodulin with TN-I, shows similar results as that of TN-I : TN-C, but a slightly higher ratio of TN-I to calmodulin is needed to bind up all of the calmodulin.

B. Isolation of the TN-I: Calmodulin Complex

a. Introduction

An attempt was made to isolate the TN-I: calmodulin complex. The isolated complex would be subjected to chemical modification,

separated back into its components and the modifications examined by amino acid analysis. A TN-I:TN-C complex has been isolated by gel filtration on Sephadex G-200 chromatography (Head and Perry, 1974).

b. Experimental procedure

Troponin I (25 mg) and calmodulin (50 mg) were dissolved in 5 ml of 0.2 M KCl, 50 mM ammonium bicarbonate, 2 mM $CaCl_2$, pH 8.0. This sample was applied to a Sephadex G-200 column (1.7 x 90 cm) previously equilibrated with the same buffer. 5 ml fractions were collected at a flow rate of 6 ml/h (fig. 18) and selected fractions were subjected to 15% PAGE (fig. 19). Absorbance of fractions was monitored by reading absorbance at 280 nm on a Beckman model 25 spectrophotometer.

Ion exchange chromatography was also used to try and separate out the TN-I:calmodulin complex. Troponin I (20 mg) and calmodulin (40 mg) were dissolved in 10 ml of 50 mM ammonium bicarbonate, 0.10 M KCl, 2 mM $CaCl_2$, pH 8.0 and dialyzed against the same buffer overnight. This sample was applied to a DEAE-Cellulose column (2.5 x 45 cm) previously equilibrated with the same buffer. Elution was achieved with a step wise salt gradient consisting of washing with 200 ml of buffer at each step. The first step after washing with initial buffer consisted of 50 mM ammonium bicarbonate, 0.15 M KCl, 2 mM$CaCl_2$, pH 8.0. The second step was 0.2 M KCl, 50 mM ammonium bicarbonate, 2 mM $CaCl_2$, pH 8.0. The flow rate was 30 ml/h and 1.5 ml fractions were collected. The elution profile is shown in (fig. 20). Selected fractions were subjected to electrophoresis on 15% PAGE slab gel (fig 21) and the appropriate fractions enriched with complex were pooled, dialyzed, and

stored. A sample of complex material was subjected to amino acid analysis.

c. Results and discussion

It was not possible to separate the complex of TN-I and calmodulin from excess calmodulin by gel filtration on Sephadex G-200 chromatography. In theory the complex should come off ahead of the native calmodulin, but in fact that was not the case. It has been reported that calmodulin runs anomously on gel filtration with an apparent molecular weight between 30,000 and 40,000 (Lin et al, 1974; Brooks and Siegel, 1972; Wolff and Siegel, 1972), approximately the same value as the theoretical 1:1 complex would. Thus, it would appear that the complex of TN-I and calmodulin can not easily be separated from excess calmodulin by gel filtration. Isolation of the complex was then tried by means of ion-exchange chromatography.

It was possible to isolate a pure sample of TN-I - calmodulin complex by ion exchange chromatography on DEAE - Cellulose, by the method described. The complex material eluted at a salt concentration between 0.15 M and 0.20 M KCl. Amino acid analysis was performed on the sample (Table V) and the complex appears to be a 1:1 stoichiometric conformation, when compared to a theoretical 1:1 complex using the values of TN-I from Wilkinson and Grand (1975) and for calmodulin from Vanaman et al (1979). However, when this sample was later thawed for chemical modification, it had broken down into its constituent of TN-I and calmodulin, and could not be used for modification studies.

C. Effect of TN-I on the Calmodulin Stimulation of Phosphodiesterase

a. Introduction

Calmodulin is the protein activator for a number of enzymes, including phosphodiesterase. This activation requires the presence of Ca^{++} and can result in a 6-10 fold stimulation in PDE activity. Calmodulin also binds TN-I. If the TN-I and PDE binding sites on calmodulin were the same, one would expect that, with limiting amounts of calmodulin present, TN-I would interfere with the calmodulin activation of PDE. This would show up as a decrease in PDE activity.

Any modification in TN-I which would result in a decrease in its affinity for calmodulin, could then be detected by a reduction in its ability to interfere with the calmodulin activation of PDE. This assay system could then be used to monitor the effect of chemical modification, of specific amino acid residues in TN-I, on its ability to bind calmodulin.

b. Experimental procedure

To a constant amount of native calmodulin (sufficient to give 50% maximal stimulation of phosphodiesterase) were added increasing amounts of TN-I, and the effects on the stimulation of PDE activity were observed by assaying modulator activity as described in 3.D. The results are shown in (fig. 22). If the troponin I binding site is the same as the binding site for phosphodiesterase, one would expect it to compete for the native modulator. Such competition would result in a decrease in A_{660}nm, with increasing TN-I: calmodulin ratios. On the other hand; if TN-I binds calmodulin at a site other than PDE binds,

one would expect to see no competitive inhibition and no effect on A_{660}nm, with increasing TN-I : calmodulin ratios. The effect observed (fig. 22) is a combination of the above.

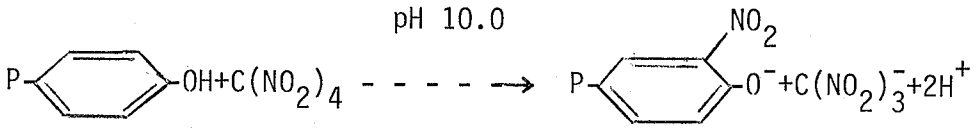
c. Results and discussion

A decrease in A_{660} was seen with increasing troponin I concentration, but this decrease reached a plateau at only a 10% loss in absorbance. Even with a molar excess of 250:1 (TN-I : calmodulin) only a slight decrease in A_{660} was observed. This indicates that the TN-I and PDE binding sites on calmodulin are probably different. Walsh and Stevens (1978) have reached a similar conclusion. It may be that the TN-I : calmodulin complex has slightly poorer affinity for PDE and it is this that causes the 10% loss in activity.

D. Tyrosine Modification

a. Introduction

Tyrosine residues in proteins can be chemically modified by treatment with tetranitromethane, which converts reactive tyrosine residues to 3-nitrotyrosine.



Tyrosine residue | tetranitromethane | 3-nitrotyrosine | nitroformate anion

The degree of nitration of tyrosine residues may be determined by measurement of the absorbance at 428 nm using a value of 4100 $M^{-1}cm^{-1}$ for the molar extinction coefficient of 3- nitrotyrosine (Riordan et al, 1967), and also by amino acid analysis following acid hydrolysis since 3-nitrotyrosine is an acid stable derivative. 3-Nitrotyrosine elutes from the long column of the amino acid analyzer as a discrete peak after phenylalanine.

Nitration with tetranitromethane affects not only tyrosyl groups but also cysteinyl residues. However, the nitration of tyrosyl residues does not proceed at pH 6 where cysteinyl residues continue to be oxidized. The variance on pH dependence of oxidation vs nitration can be employed to differentiate them (Sokolovsky et al, 1966).

Several instances have been reported of inter and intra molecular cross linking occurring as a side reaction during nitration with tetranitromethane (Means and Feeney, 1971); and these cross-linkages appear to be between tyrosine residues. It is recommended always to check for the possibility of this side reaction during nitration. This is achieved by polyacrylamide gel electrophoresis in the presence of SDS.

b. Experimental procedure

i) nitration of troponin I

Nitration of tyrosine residues was achieved by the method of Sokolovsky et al (1966). Troponin I (final concentration 1 mg/ml) was incubated at 23°C in 0.05 M Tris, 1M NaCl, 1 mM $CaCl_2$, pH 10.0 in the reference and sample cuvettes of a Beckman model 25 double beam spectrophotometer. Nitration was initiated by the addition of a

concentrated solution of tetranitromethane (60 mM in absolute ethanol) to the sample to give a final reagent concentration of 0.6 mM. An equal volume of absolute ethanol was added to the reference cell and the reaction was monitored by recording the absorbance at 428 nm. The final concentration of ethanol in the sample and reference cuvettes was less than 1%. The degree of tyrosine residue nitration was calculated from the absorbance at 428 nm using a value of 4100 $M^{-1}cm^{-1}$ for the molar extinction coefficient of 3-nitrotyrosine (Riordan et al, 1967). After completion of the reaction, the reaction mixture was dialyzed extensively against 0.3 M KCl to remove excess reagent, lyopholized, and acid hydrolyzed prior to amino acid analysis.

A sample of TN-I was incubated with tetranitromethane in the presence of Ca^{++} for 5h, as described above, at which time the reaction was complete. A control sample of TN-I was treated in identical fashion with the exception that tetranitromethane was omitted from the reaction mixture. The reaction was terminated by extensive dialysis against 0.5 M KCl to remove excess reagent. Aliquots were withdrawn from the sample and control before initiation and after completion of the 5 h incubation for: (1) acid hydrolysis and amino acid analysis and (2) PAGE to see if complex formation with either calmodulin or TN-C could be achieved.

ii) nitration of the TN-I: calmodulin complex

In an attempt to determine if one or both of the tyrosine residues are directly involved in the calmodulin binding site of TN-I, nitration of the TN-I: calmodulin complex was performed. One would expect that if the complex is stable under the reaction conditions used, residues involved in complex formation would be shielded from the

reagent in the complex and therefore not modified. If, however, neither tyrosine is involved in the binding site, both tyrosines should be modified. To ensure that all of the TN-I would be in complex with calmodulin a 5 fold molar excess of calmodulin was used.

A sample of TN-I (5 mg) and calmodulin (20 mg) in the presence of Ca^{++} was incubated with tetranitromethane for 5 h, as described earlier. The reaction mixture was dialyzed overnight against 0.15 M KCl, 50 mM Tris, 2 mM $CaCl_2$, pH 7.5, to remove excess reagent. The following day the sample was dialyzed against 0.15 M KCl, 50 mM Tris, 5 mM EGTA, pH 7.5 (to dissociate the complex) and applied to a DEAE-Cellulose (0.5 x 15 cm) column equilibrated with the same buffer. The column was washed with a column volume of initial buffer. Elution was achieved with a linear salt gradient consisting of 100 ml of initial buffer and 100 ml initial containing 0.3 M KCl. The flow rate was 30 ml/h and 3 ml fractions were collected. Fractions were monitored by reading absorbance at 280 nm on a Beckman model 25 spectrophotometer.

iii) nitration of the TN-I:TN-C complex

The previous experiment was repeated with the exception that TN-C was substituted for calmodulin. Again a 5 fold molar excess of TN-C to TN-I was used, to ensure that all of the TN-I would be in the complex form during modification with tetranitromethane.

A sample of TN-I (5 mg) and TN-C (20 mg) in the presence of Ca^{++} was incubated for 5 h at room temperature with tetranitromethane as described above. After 5 h the reaction was terminated by dialysis against 0.2 M KCl, 50 mM Tris, 2 mM $CaCl_2$, pH 7.5 to remove excess reagent. The mixture was then dialyzed against 0.15 M KCl, 50 mM Tris, 5 mM EGTA, pH 7.5 to dissociate the complex. This sample was applied

to a DEAE-Cellulose column (0.5 x 15 cm) equilibrated with the same buffer. The column was washed with a column volume of initial buffer and eluted with a linear salt gradient consisting of 100 ml each of initial buffer and initial buffer containing 0.3 M KCl. The flow rate was 30 ml/h and 3 ml fractions were collected. Fractions were monitored by reading absorbance at 280 nm. Selected fractions were subjected to electrophoresis on 15% PAGE slab gel. Each peak was pooled, dialyzed and examined by electrophoresis on 15% PAGE.

c. Results and discussion

Troponin I contains two tyrosine residues per molecule. The kinetics of nitration of these two residues by tetranitromethane are shown in (fig. 23). Modification of both tyrosine residues (positions 43 and 79 according to the sequence as determined by Wilkinson and Grand, 1975) has been achieved as determined by amino acid analysis (Table VI). As judged by PAGE (fig. 24) modification of TN-I with tetranitromethane results in concomitant loss of most of TN-I's ability to bind either troponin C or calmodulin. This suggests that one or both of TN-I's tyrosine residues may play a role in the binding site with these Ca^{++} binding proteins.

DEAE-Cellulose chromatography on the tetranitromethane treated sample of TN-I and calmodulin resulted in 2 peaks (fig. 25). By gel electrophoresis (fig. 26) peak I was identified as TN-I and peak II as calmodulin. Amino acid analysis of the troponin I from peak I revealed that both tyrosines 43 and 79 were nitrated (Table VII). Thus the

presence of calmodulin offers no protection to TN-I's tyrosine residues. The nitrated troponin I recovered after nitration no longer forms a complex with calmodulin (fig 26, slot 4).

DEAE-Cellulose chromatography of the tetranitromethane treated TN-I: TN-C mixture yielded 3 peaks (fig 27). From PAGE (figs 28,29) it is clear that peak I is TN-I, peak III is TN-C and peak II appears to behave as a TN-I : TN-C complex, even in the presence of EGTA. From amino acid analysis (Table VIII) it is apparent that both tyrosine residues of TN-I have been modified and that the presence of TN-C during nitration offers no protection to either tyrosine residue. The material in peak II represents a cross-linked 1:1 molar TN-I : TN-C complex as judged by SDS PAGE (fig 30) and amino acid analysis (Table IX). From SDS - PAGE gel, the complex was found to run between the molecular weight markers 23,000 (TN-I) and 67,000 (BSA). A theoretical TN-I : TN-C complex would have a molecular weight of 40,000, assuming the molar ratio of each component was 1.

As mentioned earlier there have been several previous reports of intermolecular cross-linking as a side reaction to nitration with tetranitromethane (Means and Feeney, 1971). This complex of TN-I and TN-C would appear to be crosslinked. If cross-linking does occur between tyrosine residues as suggested by Means and Feeney, a tyrosine residue from TN-I must come in close proximity to a tyrosine residue of TN-C in the TN-I: TN-C complex and the binding sites between the two proteins are also in the neighbourhood of those tyrosine residues.

E. Tryptic Cleavage of TN-I, TN-C, TN-I:TN-C Complexes

a. Introduction

In an attempt to determine the site of cross-linking, tryptic digests of native and cross-linked TN-I: TN-C complexes were compared by PAGE and HPLC chromatography. If, as expected, the molecules are cross-linked between two tyrosine residues, the tryptic digest of cross-linked material as compared to native material should be missing two peptides and contain a new cross-linked peptide. This peptide should show up as a new band on PAGE or as a new peak on HPLC chromatography.

a. Experimental procedure

Tryptic digests of TN-I, TN-C, TN-I: TN-C complex, and cross-linked TN-I: TN-C were performed at 37°C in 1% ammonium bicarbonate, pH 8.0 at a protein concentration of 1 mg/ml and an enzyme/substrate ratio of 1:20. The samples were incubated for 5, 10, 15, 30, 60, 90, and 120 minutes. Digestion was terminated by boiling the samples for 3 minutes in a boiling water bath.

The digests were examined by PAGE (figs 31, 32, 33, 34). The tryptic peptides of interest were purified by PAGE as described by Drabikowski et al, (1977).

Digested protein samples were also examined by HPLC (figs 35, a, b, c), by a modification of the method of Fulmer and Wasserman, (1979). Protein digest (20 µl of 2 mg/ml solution) was applied to an ODS column (4.6x25cm) and the peaks indicated by the arrows collected. Elution was achieved by a linear gradient from 0-70% acetonitrile over 40 minutes.

Selected fractions from several runs were pooled, lyopholyzed and hydrolyzed for amino acid analysis. The fractions collected were monitored by reading OD at 210 nm, with a Hitachi 100-40 spectrophotometer with a flow through cell.

c. Results and discussion

By the method of preparative PAGE a new band was seen, as indicated by the arrow (fig. 34) and the band indicated by the arrow (fig. 32) was absent. The band of interest was purified by PAGE. Its amino acid composition is shown in Table X. Within experimental error the amino acid composition could account for the sum of amino acid compositions in known tryptic peptides of TN-I (residues 41-65) and TN-C (residues 105-120). These tryptic peptides each contain a tyrosine residue; tyrosine 43 of TN-I and tyrosine 109 of TN-C.

A comparison of the HPLC runs of the different tryptic digests (fig. 35, a, b, c) shows that a peptide from nitrated TN-I (19.68 min.) and TN-C (18.67 min.) is absent in the cross-linked TN-I: TN-C run. One also sees that a new band runs at 26.6 minutes in the cross-linked TN-I: TN-C sample. The peptide isolated from the TN-I: TN-C mixture (26.6 min.) was collected and its amino acid composition is seen in Table XI. Within experimental error, these values account for the sum of TN-I peptide (residues 41-65) and TN-C (residues 105-120). Each of these peptides has a tyrosine residue in them; tyrosine 43 of TN-I and tyrosine 109 of TN-C.

The composition of peptides obtained from nitrated TN-I run (19.68 min.) and nitrated TN-C (18.67 min.) run are shown in Tables XII, XIII. Within experimental error, the TN-I peptide (19.68 min.) accounts

for residues 41-65 of the TN-I sequence and the TN-C peptide (18.67 min.) accounts for residues 105-120 of the TN-C sequence.

Together, both methods of isolation of the cross-linked peptide, give similar results and within experimental error point to the cross-linking being between tyrosine 43 of TN-I and tyrosine 109 of TN-C.

5. CONCLUDING REMARKS

From the experiments performed a number of conclusions can be drawn. First of all, the initial experiments show that 2 different TN-I: TN-C complexes can be formed. The major complex being of 1:1 stoichiometry and the other of unknown molar ratio. These results were seen when TN-I formed a complex with either calmodulin or TN-C.

From PDE assay experiments in which TN-I was to act as a competitive inhibitor for calmodulin, one can conclude that the PDE site and TN-I site on calmodulin are not the same. TN-I does not act as a competitive inhibitor, competing with PDE for the calmodulin molecule. Work by Walsh and Stevens (1978) support this conclusion. They found that calmodulin which had been chemically modified could still form a complex with TN-I, but had lost its ability to activate phosphodiesterase. From this they inferred that the binding sites for PDE and TN-I on the calmodulin molecule are different.

Modification of the two tyrosines (positions 43 and 79) of TN-I resulted in loss of the ability of the protein to form a Ca^{++}-dependent complex with either TN-C or calmodulin. Neither tyrosine residue was protected from modification when the reaction was carried out in the presence of TN-C or calmodulin under conditions favorable for complex formation.

It is surprising on the one hand that both tyrosine residues are still available to the reagent in the complexes, indicating that they are not part of the binding site as such, whereas, on the other hand their modification prevents the formation of complex, indicating that they play some role in it.

The crosslinking studies show that the crosslinked complex contains TN-I and TN-C in a one to one molar ratio. The crosslinked peptide isolated from tryptic digests of the crosslinked complex appears to be composed of TN-I region 41-65 and TN-C region 105-120. Each of these regions contain one tyrosine residue, tyrosine 43 of TN-I and tyrosine 109 of TN-C. These results are in agreement with the results of Weeks and Perry (1978) who showed that a CNBr peptide representing residues 83-134 of TN-C possesses many of the properties of the entire molecule and contains the TN-I binding region. Leavis et al (1977) has shown that the TN-C binding site for TN-I is between residues 88-119. The homologous region was shown to be involved in calmodulin (Walsh and Stevens, 1978).

Syska et al (1976) have shown that regions representing residues 1-47 and residues 97-117 of TN-I can interact with TN-C. They also found that when TN-I is in complex with TN-C, threonine (position 37) is blocked from phosphorylation. This suggests that this residue is within the binding site of TN-I or very close to it, making it inaccessible for phosphorylation.

These studies by other workers coincide with the results from this study and lend further support to our conclusion that tyrosine 43 of TN-I and tyrosine 109 of TN-C must be in close proximity in the TN-I: TN-C complex, and probably very close to the binding regions of these two proteins.

Calmodulin and TN-C are homologous proteins (Watterson et al, 1980) and in calmodulin the equivalent position to tyrosine 109 of TN-C is also occupied by a tyrosine residue (tyrosine 99). It is therefore

unexpected that a crosslinked complex after nitration did not occur in the TN-I: calmodulin complex. The reason for this is not clear, but it may be the result of a slightly different topology in the vicinity of the TN-I binding site in calmodulin as compared to TN-C.

Finally, the experiments provide further evidence for the evolutionary homology between both calcium binding proteins, TN-C and calmodulin. With both proteins, the majority of complex formed with TN-I is in a 1:1 ratio. A second minor complex of different stoichiometry is seen with both these proteins. With both proteins one can see that nitrated TN-I no longer forms a complex with and as well nitration of the TN-I: calmoculin or TN-I: TN-C complexes does not block TN-I tyrosine nitration.

APPENDIX I

																		10	
A.	Ac	met	ASP	asp	ile	tyr	lys	ala	ALA	val	glu	GLN	LEU	THR	GLU	GLU	GLN	lys	asn	GLU
B.									Ac	(ala | asx)	GLN	LEU	THR	GLU	GLU	GLN	ILE	ALA	GLU
C.		Ac	ASP	thr	gln	gln	ala	glu	ALA	arg	ser	tyr	LEU	SER	GLU	GLU	met	ILE	ALA	GLU
								20											30	
PHE	LYS	ALA	ALA	PHE	ASP	ILE	PHE	val	leu	gly	ALA	GLU	asp	gly	cys	ILE	SER	THR	LYS	GLU
PHE	LYS	glu	ALA	PHE	SER	LEU	PHE	ASP	lys	ASP	GLY	ASX	GLY	THR		ILE	THR	THR	LYS	GLU
PHE	LYS	ALA	ALA	PHE	ASP	MET	PHE	ASP	ala	ASP	GLY	gly	GLY	ASP		ILE	SER	val	LYS	GLU
								40										50		
LEU	GLY	lys	VAL	MET	ARG	MET	LEU	GLY	GLN	ASN	PRO	THR	pro	GLU	GLU	LEU	GLN	GLU	MET	ILE
LEU	GLY	THR	VAL	MET	ARG	ser	LEU	GLY	GLN	ASN	PRO	THR	glu	ala	GLU	LEU	GLX	ASX	MET	ILE
LEU	GLY	THR	VAL	MET	ARG	MET	LEU	GLY	GLN	THR	PRO	THR	lys	GLU	GLU	LEU	ASP	ala	ILE	ILE
							60										70			
ASP	GLU	VAL	ASP	GLU	ASP	GLY	SER	GLY	THR	VAL	ASP	PHE	ASP	GLU	PHE	LEU	VAL	MET	MET	VAL
ASN	GLU	VAL	ASP	ala	ASP	GLY	ASX	GLY	THR	ILE	ASP	PHE	pro	GLU	PHE	LEU	thr	MET	MET	ALA
GLU	GLU	VAL	ASP	GLU	ASP	GLY	SER	GLY	THR	ILE	ASP	PHE	GLU	GLU	PHE	LEU	VAL	MET	MET	VAL
								80											90	
ARG	cys	MET	LYS	ASP	ASP	SER	LYS	GLY	LYS	SER	GLU	GLU	GLU	LEU	SER	ASP	leu	PHE	ARG	MET
ARG	lys	MET	LYS	ASP	thr	asp				SER	GLU	GLU	GLU	ILE	arg	GLU	ala	PHE	ARG	VAL
ARG	gln	MET	LYS	GLU	ASP	ALA	LYS	GLY	LYS	SER	GLU	GLU	GLU	LEU	ALA	GLU	cys	PHE	ARG	ILE
								100										110		
PHE	ASP	LYS	ASN	ALA	ASP	GLY	TYR	ILE	ASP	leu	GLU	GLU	LEU	LYS	ile	MET	LEU	gln	ALA	THR
PHE	ASP	LYS	ASP	GLY	ASN	GLY	TYR	ILE	SER	ALA	ala	GLU	LEU	ARG	his	VAL	MET	thr	asx	leu
PHE	ASP	ARG	ASN	ALA	ASP	GLY	TYR	ILE	ASP	ALA	GLU	GLU	LEU	ala	glu	ILE	PHE	arg	ALA	SER
							120										130			
GLY	GLU	thr	ILE	THR	GLU	ASP	ASP	ILE	GLU	GLU	LEU	MET	LYS	ASP	GLY	ASP	LYS	ASN	ASN	ASP
GLY	GLU	tml	LEU	THR	ASP	GLU	GLU	VAL	ASP	GLU	MET	ILE	ARG	GLU	ALA	ASN	ile	ASP	gly	ASP
GLY	GLU	his	VAL	THR	ASP	GLU	GLU	ILE	GLU	ser	LEU	MET	LYS	ASP	GLY	ASP	LYS	ASN	ASN	ASP
						140														
GLY	ARG	ILE	ASP	TYR	ASP	GLU	PHE	LEU	GLU	PHE	MET	lys	GLY	VAL	GLU	COOH				
GLY	glx	VAL	ASX	TYR	GLX	GLX	PHE	VAL	GLN	MET	MET	thr	ALA	lys	COOH					
GLY	ARG	ILE	ASP	PHE	ASP	GLU	PHE	LEU	lys	MET	MET	glu	GLY	VAL	GLN	COOH				

Amino acid sequences of calmodulin from bovine brain and TN-C's. A.-Bovine cardiac TN-C taken from vanEerd and Takahashi (1976). B.-Calmodulin taken from Vanaman et al, (1977). C.-Rabbit skeletal TN-C taken from Collins (1976). Residues in capital letters are identical or functionally conservative replacements.

APPENDIX II

Comparison of amino acid composition of TN-I's

Amino Acid	Rabbit Skeletal[1]	Rabbit Cardiac[2]	Chicken Skeletal[3]
Lys	28	24	23
His	4	3	5
Arg	16	25	12
Asp	19	18	16
Thr	4	10	8
Ser	10	9	8
Glu	36	32	27
Pro	7	6	8
Gly	9	11	10
Ala	17	25	17
Cys	3	2	1
Val	8	9	10
Met	8	3	5
Ile	5	7	5
Leu	20	23	18
Tyr	2	3	3
Phe	3	4	4
Trp	1	1	1

All values are rounded to nearest integer.

1. From Wilkinson, 1974.

2. From Wilkinson and Grand, 1977.

3. From Wilkinson, 1978.

APPENDIX III

Comparison of amino acid composition of TN-C's

Amino Acid	Rabbit Skeletal[1]	Bovine Cardiac[2]	Chicken Skeletal[3]
Lys	10	12	10
His	1	2	1
Arg	7	6	6
Asp	24	24	25
Thr	6	6	7
Ser	7	5	6
Glu	35	36	28
Pro	2	7	1
Gly	13	12	14
Ala	13	10	14
Cys	1	1	1
Val	7	9	6
Met	9	8	11
Ile	9	7	11
Leu	9	12	11
Tyr	2	3	2
Phe	9	8	10
Trp	0	0	0

All values rounded to nearest integer.

1. From Greaser and Gergely, 1971.

2. From Burtnick et al, 1975.

3. From Wilkinson, 1978.

APPENDIX IV

A.
B.
C.

	x-ala	asp	glu	ser	arg	asp	ala	ala	gly	glu	ala	arg	pro	ala	pro	ala	val	arg	arg
10									30										40
							Ac-gly	asp	GLU	glu	LYS	ARG	asn	ARG	ALA	ILE	THR	ALA		ARG
								NH_2-PRO	GLU	VAL	glu	ARG	LYS	ser	LYS	ILE	THR	ALA	SER	ARG
ser	asp	arg	ala	tyr	ala	thr	glu	PRO	his	ALA	LYS	ser	LYS	LYS	LYS	ILE	SER	ALA	SER	ARG
									50										60
ARG	gln	his	LEU	LYS	SER	VAL	MET	LEU	GLN	ILE	ALA	ALA	THR	GLU	LEU	GLU	LYS	GLU	GLU	GLY
LYS	LEU		LEU	LYS	SER	LEU	MET	LEU	ala	LYS	ALA	LYS	GLU	cys	gln	GLN	glu	his	GLU	ALA
LYS	LEU	gln	LEU	LYS	THR	LEU	MET	LEU	GLN	ILE	ALA	LYS	GLN	GLU	LEU	GLN	ARG	GLU	ala	glu
								70										80		
arg	ARG	GLU	ALA	GLU	LYS	gln	asn	TYR	LEU	ALA	GLU	his	CYS	PRO	PRO	LEU	ser	LEU	PRO	GLY
	ARG	GLU	ALA	GLU	LYS	VAL	ARG	TYR	LEU	ALA	GLU	ARG	ile	PRO	ALA	LEU	GLN	thr	arg	GLY
glu	ARG	arg	GLY	GLU	LYS	GLY	ARG	ala	LEU	ser	thr	ARG	CYS	gln	PRO	LEU	GLU	LEU	ALA	GLY
									90										100	
	SER	MET	ALA	GLU	VAL	GLN	GLN	LEU	CYS	LYS	GLN	LEU	HIS	ALA	LYS	ILE	ASP	ALA		
LEU	SER	LEU	ser	ala	LEU	GLN	ASP	LEU	CYS	ARG	GLN	LEU	HIS	ALA	LYS	VAL	GLU	VAL		
LEU	gly	phe	ALA	GLU	LEU	GLN	ASP	LEU	CYS	ARG	GLN	LEU	HIS	ALA	ARG	VAL	ASP	lys		
						110										120				
ALA	GLU	GLU	GLU	LYS	TYR	ASP	MET	GLU	ile	LYS	VAL	GLN	LYS	SER	SER	LYS	GLU	LEU	glu	ASP
VAL	ASP	GLU	GLU	ARG	TYR	ASP	ILE	GLU	ALA	LYS	cys	leu	his	ASN	THR	ARG	GLU	ILE	lys	ASP
VAL	ASP	GLU	GLU	ARG	TYR	ASP	VAL	GLU	ALA	LYS	VAL	THR	LYS	ASN	ile	thr	GLU	ILE	ala	ASP
							130										140			
MET	asn	GLN	LYS	LEU	PHE	ASP	LEU	ARG	GLY	LYS	PHE	LYS	ARG	PRO	PRO	LEU	ARG		ARG	VAL
LEU	lys	leu	LYS	VAL	LEU	ASP	LEU	ARG	GLY	LYS	PHE	LYS	ARG	PRO	PRO	LEU	ARG		ARG	VAL
LEU	thr	GLN	LYS	ILE	PHE	ASP	LEU	ARG	GLY	LYS	PHE	LYS	ARG	PRO	thr	LEU	ARG	leu	ARG	VAL
						150										160				
ARG	MET	SER	ALA	ASP	ALA	MET	LEU	LYS	ALA	LEU	LEU	GLY	SER	LYS	HIS	LYS	VAL	cys	MET	ASP
ARG	VAL	SER	ALA	ASP	ALA	MET	LEU	ARG	ALA	LEU	LEU	GLY	SER	LYS	HIS	LYS	VAL	SER	MET	ASP
ARG	ILE	SER	ALA	ASP	ALA	MET	MET	gln	ALA	LEU	LEU	GLY	THR	ARG	ala	LYS	glu	THR	LEU	ASP

APPENDIX IV (continued)

					170									180					
LEU	ARG	ALA	ASN	LEU	LYS	GLN	VAL	LYS	LYS	GLU	ASP	THR	GLU	LYS	GLU			ARG	ASP
LEU	ARG	ALA	ASN	LEU	LYS	ser	VAL	LYS	LYS	GLU	ASP	THR	GLU	LYS	GLU	arg	pro	val		GLU
LEU	ARG	ALA	his	LEU	LYS	GLN	VAL	LYS	LYS	GLU	ASP	THR	GLU	LYS	GLU			asn	ARG	GLU
				190										200					
VAL	GLY	ASP	TRP	ARG	LYS	ASN	ILE	GLU	glu	LYS	SER	GLY	MET	GLU	GLY	ARG	LYS	LYS	MET	PHE
VAL	GLY	ASP	TRP	ARG	LYS	ASP	VAL	GLU	ala	MET	SER	GLY	MET	GLU	GLY	ARG	LYS	LYS	MET	PHE
VAL	GLY	ASP	TRP	ARG	LYS	ASN	ILE	ASP	leu	LEU	SER	GLY	MET	GLU	GLY	ARG	LYS	LYS	lys	PHE
			210																
GLU	ser		glu	SER-COOH															
ASP	ALA	ala	lys	SER	pro	thr	ser	gln-COOH											
GLU	GLY-COOH																		

Amino acid sequences of TN-I. A.-Rabbit fast skeletal taken from Wilkinson and Grand (1975). B.-Rabbit slow skeletal taken from Grand and Wilkinson (1977). C.-Rabbit cardiac taken from Grand and Wilkinson (1976).

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TABLE I: Purification of Calmodulin

	Fraction	Vol(ml)	[1]Protein(mg)	[2]Total Units	Specific Activity units/mg Protein	Purification factor	Yield
I	Homogenate	1680	34,250	11.0×10^6	320	1	100
II	40% ammonium sulfate supernatant	1740	20,180	10.7×10^6	530	1.66	97
III	Heat treatment supernatant	325	1,015	9.1×10^6	9,230	28.8	85
IV	DEAE-Cellulose pool	125	107	6.8×10^6	63,400	198.1	62
V	Sephadex G-100 pool	65	80	6.4×10^6	80,250	250.8	58

1. Protein concentration was measured by method of Lowry, 1951.

2. 1 unit of modulator activity is defined as the amount which gives 50% maximal activation of a standard amount (0.4-0.5 units of modulator defecient PDE, Teo et al, 1973).

TABLE II: Purification of Calmodulin: Amino Acid Composition of Calmodulin Prepared as Described in Text

Amino Acid	Amino Acid Composition[1] 1	2	3	Average	Integer	Theoretical[2]
Lysine	8.8	8.7	8.6	8.7	9	9[3]
Histidine	1.1	.9	1.2	1.1	1	1
Arginine	6.9	6.7	6.7	6.8	7	6
Aspartic Acid	23.8	23.6	23.5	23.6	24	23
Threonine	11.3	11.2	11.4	11.3	11	12
Serine	4.1	4.1	4.0	4.1	4	4
Glutamic Acid	27.0	27.5	27.3	27.3	27	27
Proline	2.9	2.8	3.0	2.9	3	2
Glycine	12.0	12.0	11.9	12.0	12	11
Alanine	12.8	12.7	12.5	12.7	13	11
Half Cystine	0	0	0	0	0	0
Valine	7.5	7.4	7.4	7.4	7	7
Methionine	8.6	8.7	8.6	8.6	9	9
Isoleucine	8.2	8.2	8.1	8.2	8	8
Leucine	10.6	10.6	10.5	10.6	11	9
Tyrosine	1.9	2.0	1.9	1.9	2	2
Phenylalanine	8.2	8.1	8.1	8.1	8	8

1. The results represent amino acid analysis of 3 independent hydrolyses.

2. Values taken from the amino acid sequence as determined by Vanaman et al, (1979).

3. This value includes 1 residue of trimethyl lysine.

TABLE III: Amino Acid Composition of Troponin C
Prepared as Described in Text

Amino Acid	Amino Acid Composition[1] 1	2	3	Average	Integer	Theoretical[2]
Lysine	9.9	9.2	9.3	9.4	9	9
Histidine	1.3	1.2	1.4	1.3	1	1
Arginine	7.9	7.3	7.2	7.4	7	7
Aspartic Acid	23.8	22.1	22.0	22.6	23	22
Threonine	5.8	5.5	5.7	5.7	6	6
Serine	7.9	7.4	7.2	7.5	7	7
Glutamic Acid	35.5	33.0	31.9	33.4	33	31
Proline	1.6	1.5	2.3	1.8	2	1
Glycine	14.1	13.1	13.7	13.6	14	13
Alanine	13.3	12.4	13.6	13.1	13	13
Half Cystine	--	--	--	--	--	1
Valine	7.7	7.1	6.9	7.2	7	7
Methionine	10.9	10.1	8.1	9.7	10	10
Isoleucine	9.7	9.1	8.2	9.0	9	10
Leucine	11.1	10.3	9.6	10.3	10	9
Tyrosine	1.9	2.1	1.8	1.9	2	2
Phenylalanine	10.9	10.2	9.7	10.3	10	10

1. The results represent amino acid analysis of 3 independent samples from different preparations.

2. Values taken from the amino acid sequence as determined by Collins et al (1977).

TABLE IV: Amino Acid Composition of Troponin I
Prepared as Described in Text

Amino Acid	Amino Acid Composition[1] 1	2	3	Average	Integer	Theoretical[2]
Lysine	20.3	22.6	20.4	21.1	21	24
Histidine	3.4	4.5	3.6	3.8	4	4
Arginine	12.1	14.2	12.8	13.0	13	15
Aspartic Acid	15.0	14.6	14.6	14.7	15	15
Threonine	3.7	3.1	3.6	3.5	4	3
Serine	6.9	7.8	7.9	7.5	8	10
Glutamic Acid	27.5	29.2	26.5	27.7	28	33
Proline	6.1	5.9	6.3	6.1	6	5
Glycine	8.9	8.9	8.7	8.8	9	8
Alanine	13.9	13.8	14.1	13.9	14	14
Half Cystine	--	--	--	--	--	3
Valine	7.7	6.3	7.5	7.1	7	7
Methionine	6.4	6.5	6.2	6.4	6	9
Isoleucine	5.4	4.7	5.2	5.1	5	5
Leucine	15.2	17.3	16.1	16.2	16	17
Tyrosine	2.1	2.2	1.9	2.1	2	2
Phenylalanine	3.3	2.8	3.2	3.1	3	3

1. The results represent amino acid analysis of 3 independent samples, from different preparations.

2. Values taken from the amino acid sequence as determined by Wilkenson and Grand (1975).

TABLE V: Isolation of the TN-I: Calmodulin Complex: Amino Acid Composition of the Complex Isolated by Ion Exchange Chromatography

Amino Acid	Amino Acid Composition	Integer	Theoretical[1]
Lysine	29.9	30	33[2]
Histidine	7.3	7	5
Arginine	19.6	20	21
Aspartic Acid	36.8	37	38
Threonine	13.2	13	15
Serine	15.6	16	14
Glutamic Acid	60.5	61	60
Proline	7.4	7	7
Glycine	18.8	19	19
Alanine	26.9	27	25
Half Cystine	--	--	3
Valine	13.4	14	14
Methionine	17.8	18	18
Isoleucine	11.8	12	13
Leucine	24.9	25	26
Tyrosine	4.2	4	4
Phenylalanine	12.2	12	11

1. Theoretical values are based on a 1:1 molar ratio complex using the values of TN-I from sequence work of Wilkinson and Grand, (1975) and values for calmodulin from the sequence determined by Vanaman et al (1979).

2. Includes 1 residue trimethyl lysine from Calmodulin.

TABLE VI: Tyrosine Modification: Amino Acid Analysis of Troponin I - nitration with tetranitromethane

Amino Acid	[2]Native	Nitrated
Lysine	21	20
Histidine	4	4
Arginine	13	13
Aspartic Acid	15	15
Threonine	4	4
Serine	8	8
Glutamic Acid	28	29
Proline	6	6
Glycine	9	9
Alanine	14	14
Half Cystine	8	8
Valine	7	7
Methionine	6	6
Isoleucine	5	5
Leucine	16	16
Tyrosine	2	0
Phenylalanine	3	3

1. The values are in moles/mole and rounded to the nearest integer.

2. Native values are the average of 3 separate analysis of TN-I prior to addition of TNM.

TABLE VII: Tyrosine Modification : Nitration of the TN-I: Calmodulin Complex. Amino Acid Composition of Peak I (TN-I) recovered from DEAE-Cellulose Column Chromatorgraphy (fig. 25)

Amino Acid	[2]Native	Recovered from Calmodulin Complex (Peak I)
Lysine	21	21
Histidine	4	4
Arginine	13	13
Aspartic Acid	15	15
Threonine	4	5
Serine	8	11
Glutamic Acid	28	26
Proline	6	7
Glycine	9	9
Alanine	14	14
Half Cystine	--	--
Valine	7	8
Methionine	6	6
Isoleucine	5	6
Leucine	16	16
Tyrosine	2	0
Phenylalanine	3	3

1. The values are in moles/mole and rounded to the nearest integer.

2. Native values are the average of 3 separate analysis of TN-I prior to complex formation.

TABLE VIII: Tyrosine Modification: Nitration of the TN-I:TN-C Complex. Amino Acid Composition of Peak I (TN-I) obtained from DEAE-Cellulose Chromatography (fig. 27)

Amino Acid	[2]Native TN-I	TN-I from Complex with TN-C (PEAK I)
Lysine	21	20
Histidine	4	4
Arginine	13	12
Aspartic Acid	15	15
Threonine	4	4
Serine	8	8
Glutamic Acid	28	26
Proline	6	7
Glycine	9	9
Alanine	14	14
Half Cystine	--	--
Valine	7	7
Methionine	6	6
Isoleucine	5	5
Leucine	16	16
Tyrosine	2	0
Phenylalanine	3	3

1. The values are in moles/mole and rounded to the nearest integer.

2. Native values are the average of 3 separate analysis of TN-I prior to complex formation.

TABLE IX: Tyrosine Modification: Nitration of the TN-I:TN-C Complex Amino Acid Composition of Peak II from DEAE-Cellulose Chromatography (fig. 27)

Amino Acid	Peak II	Theoretical TN-I:TN-C[1] 1:1 complex
Lysine	30	33
Histidine	6	5
Arginine	21	22
Aspartic Acid	36	37
Threonine	10	9
Serine	18	17
Glutamic Acid	54	64
Proline	8	6
Glycine	25	21
Alanine	26	27
Half Cystine	--	4
Valine	14	14
Methionine	18	19
Isoleucine	15	15
Leucine	27	26
Tyrosine	0	4
Phenylalanine	12	13

1. Based on an assumed 1:1 molar complex using amino acid composition data of Wilkinson and Grand, (1975) for TN-I and of Collins et al, (1977) for TN-C.

TABLE X: Amino Acid Composition : Isolated Tryptic Fragments by Preparative PAGE as Described in Text

Amino Acid	Peptide	Theoretical Peptide[1]
Lysine	1	1
Histidine	1	1
Arginine	1	1
Aspartic Acid	3	4
Threonine	1	0
Serine	3	2
Glutamic Acid	6	8
Proline	3	3
Glycine	5[2]	2
Alanine	3	5
Half Cystine	0	0
Valine	1	1
Methionine	0	1
Isoleucine	2	2
Leucine	4	5
Tyrosine	0[3]	2
Phenylalanine	1	1

1. The values are obtained by adding residues of tryptic peptide 41-65 of TN-I (Wilkinson and Grand, 1975) and peptide 105-120 of TN-C (Collins et al, 1977).

2. Glycine values are probably high because the peptides were isolated by electrophoresis in presence of Tris/glycine buffer.

3. No tyrosine residues were seen, as tyrosines are modified by tetranitromethone.

TABLE XI: Amino Acid Composition : Sample Collected From Cross-Linked TN-I:TN-C Tryptic Digest Collected By HPLC at 26.6 Minutes

Amino Acid	Peptide	Theoretical Peptide[1]
Lysine	2	1
Histidine	1	1
Arginine	2	1
Aspartic Acid	6	4
Threonine	0	0
Serine	3	2
Glutamic Acid	9	8
Proline	3	3
Glycine	4[2]	2
Alanine	4	5
Half Cystine	0	0
Valine	1	1
Methionine	1	1
Isoleucine	2	2
Leucine	5	5
Tyrosine	0[2]	2
Phenylalanine	2	1

1. The values are obtained by adding of tryptic peptides 41-65 of TN-I (Wilkinson and Grand, 1975) and peptide 105-120 of TN-C (Collins et al, 1977).

2. No tyrosine residues were seen as tyrosines are modified by tetranitromethane.

TABLE XII: Amino Acid Composition : Isolated Tryptic Fragment From HPLC of Nitrated TN-I at 19.68 minutes

Amino Acid	Peptide	Theoretical Peptide[1]
Lysine	3	1
Histidine	1	1
Arginine	0	0
Aspartic Acid	2	1
Threonine	0	0
Serine	2	2
Glutamic Acid	5	5
Proline	3	3
Glycine	2	1
Alanine	2	2
Half Cystine	0	0
Valine	1	1
Methionine	1	1
Isoleucine	0	0
Leucine	4	4
Tyrosine	0[2]	1
Phenylalanine	1	1

1. Theoretical values are from residues 41-65 of TN-I sequence (Wilkinson and Grand, 1975).

2. No tyrosine residue is found as tyrosine is modified by tetranitromethane.

TABLE XIII: Amino Acid Composition : Isolated Tryptic Fragment From HPLC of Nitrated TN-C at 18.67 minutes

Amino Acid	Peptide	Theoretical Peptide[1]
Lysine	1	0
Histidine	0	0
Arginine	2	1
Aspartic Acid	3	3
Threonine	0	0
Serine	1	0
Glutamic Acid	4	3
Proline	0	0
Glycine	2	1
Alanine	2	3
Half Cystine	0	0
Valine	0	0
Methionine	0	0
Isoleucine	2	2
Leucine	1	1
Tyrosine	0[2]	1
Phenylalanine	1	0

1. The values are taken from region 105-120 of TN-C sequence as determined by Collins et al, 1977.

2. No tyrosine residue is seen as tyrosine residues are modified by tetranitromethane.

Fig. 1:

Standard curve of calmodulin activity. Increasing amounts of calmodulin were assayed for their capacity to stimulate a fixed amount of calmodulin deficient PDE, as described in 3.A.

Fig. 1

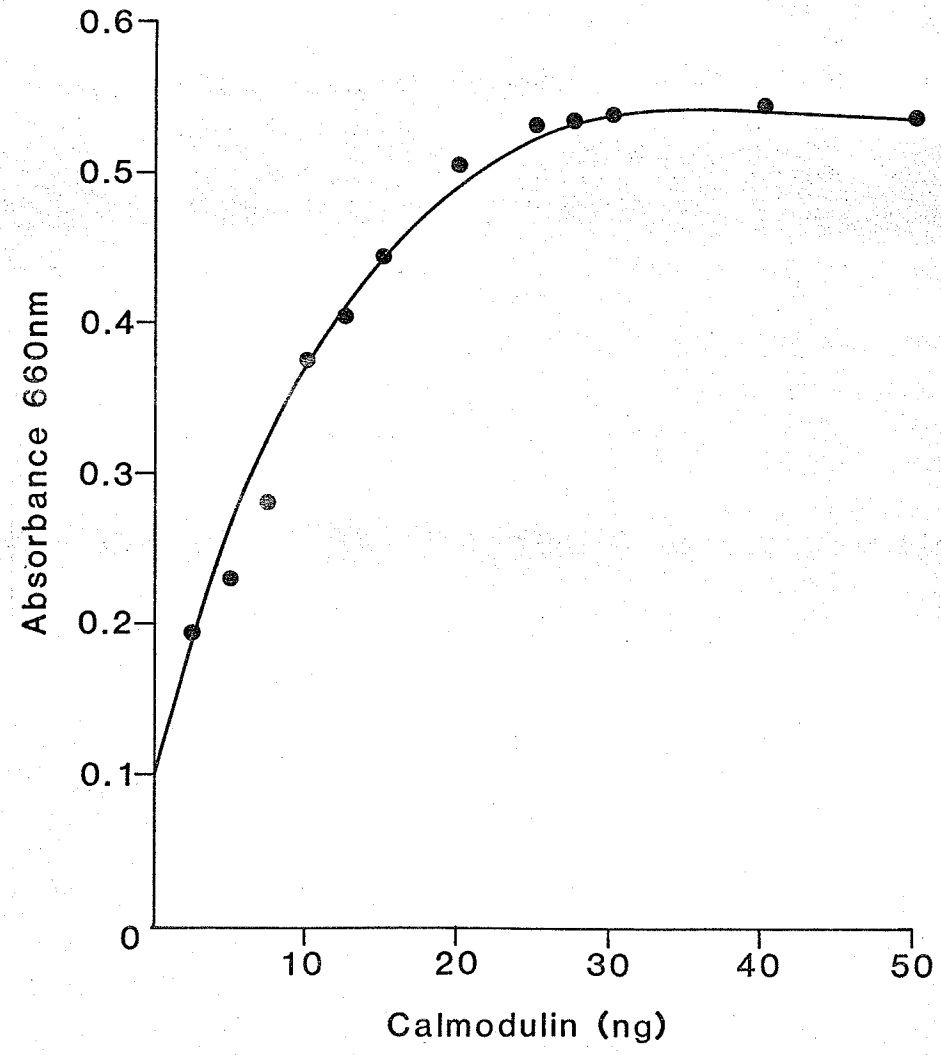

0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance 660nm
10
20
30
40
50
Calmodulin (ng)

Fig. 2:

Standard curve of phosphodiesterase activity. Increasing amounts of PDE were assayed for activity, with a constant amount of calmodulin, as described in 3.B.

Fig. 2

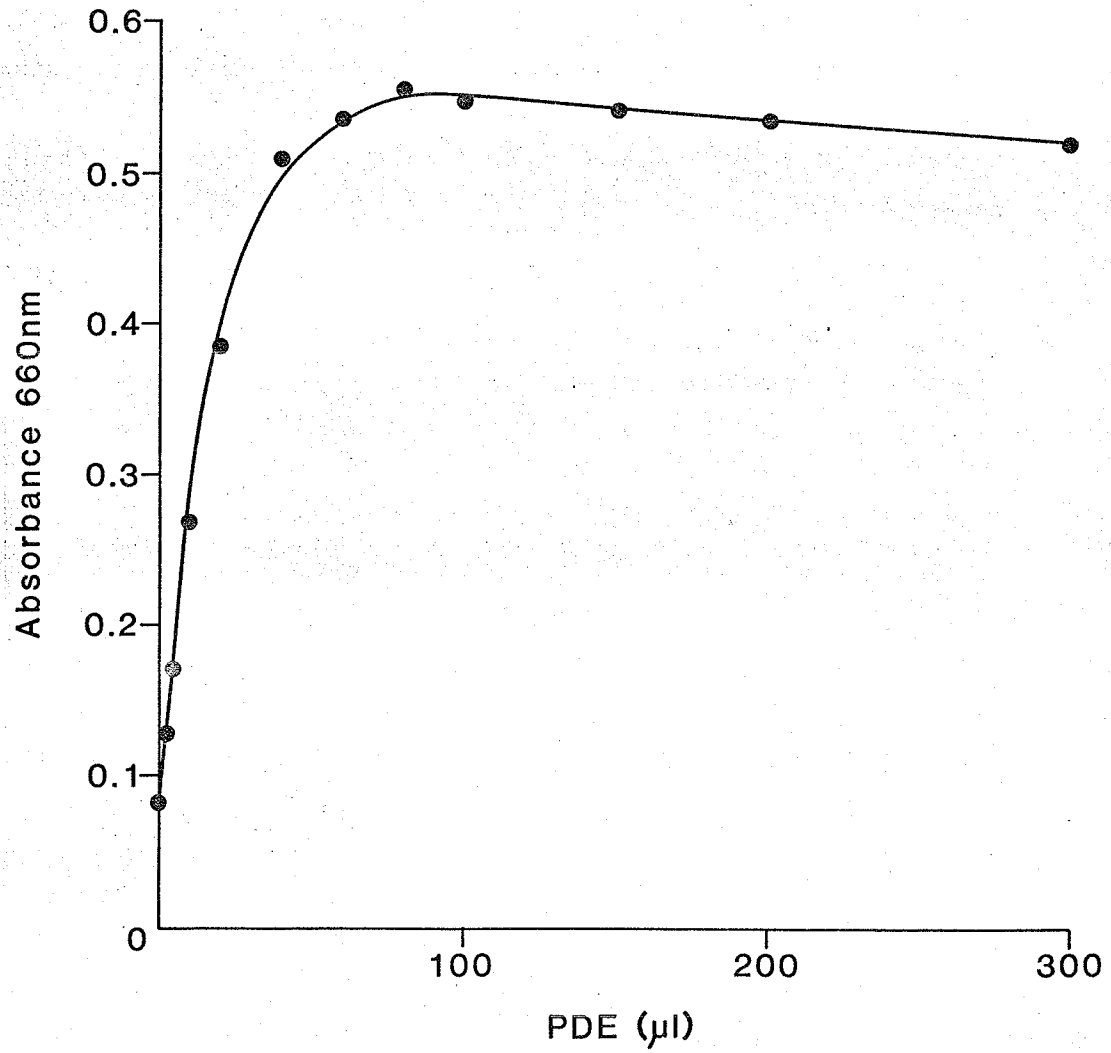


Fig. 3:

Purification of calmodulin: DEAE-Cellulose column chromatography. The separation shown was performed and monitored as described in the text. Fractions (6.5 ml) were collected and those fractions indicated by the bar were pooled.

Fig. 3

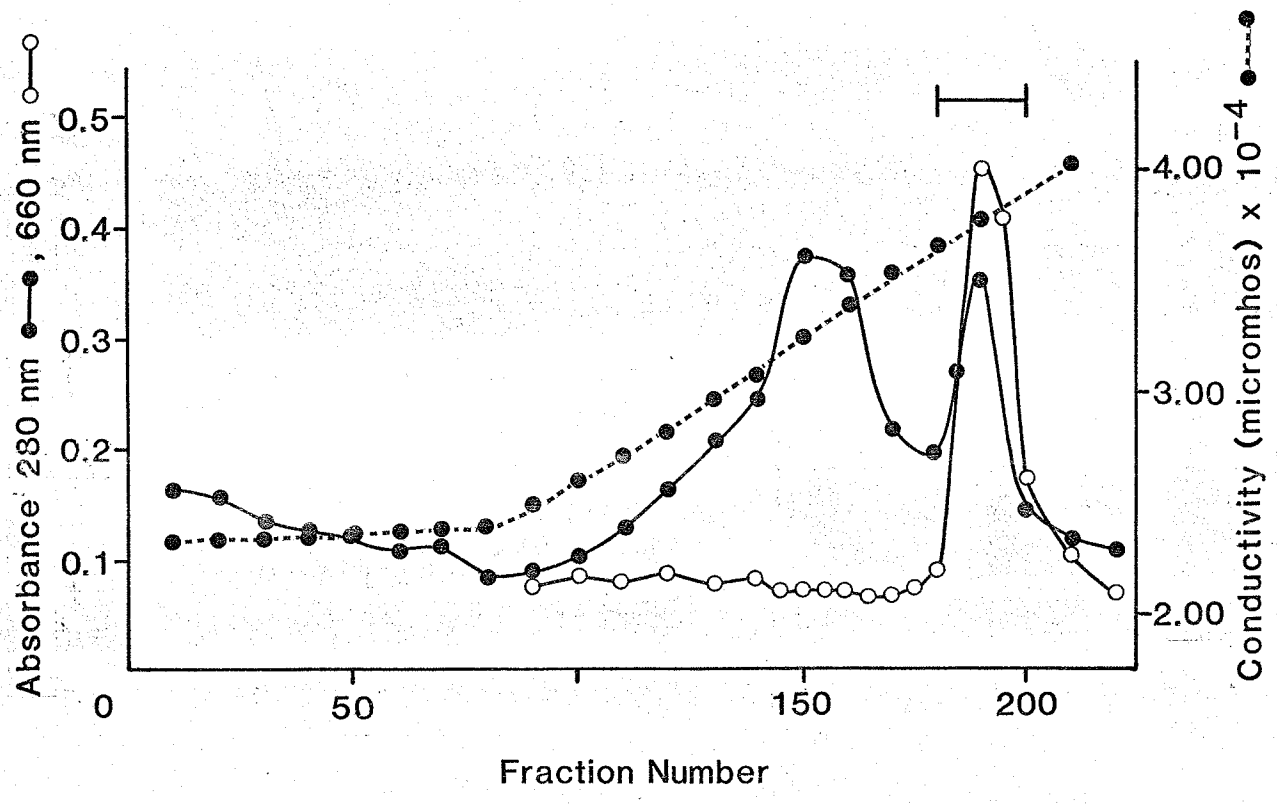

Absorbance 280 nm ●—●, 660 nm ○—○
0.5
0.4
0.3
0.2
0.1
0
50
150
200
Fraction Number
4.00
3.00
2.00
Conductivity (micromhos) x 10^{-4} ●---●

Fig. 4:

Purification of calmodulin: PAGE electrophoresis on 15% slabs of the fractions obtained by DEAE - Cellulose chromatography (Fig. 3). From left to right (slots 1 to 13), the fractions represented on the gel are: 120, 130, 140, 150, 160, 170, 180, 185, 190, 195, 200, 210.

Fig. 4

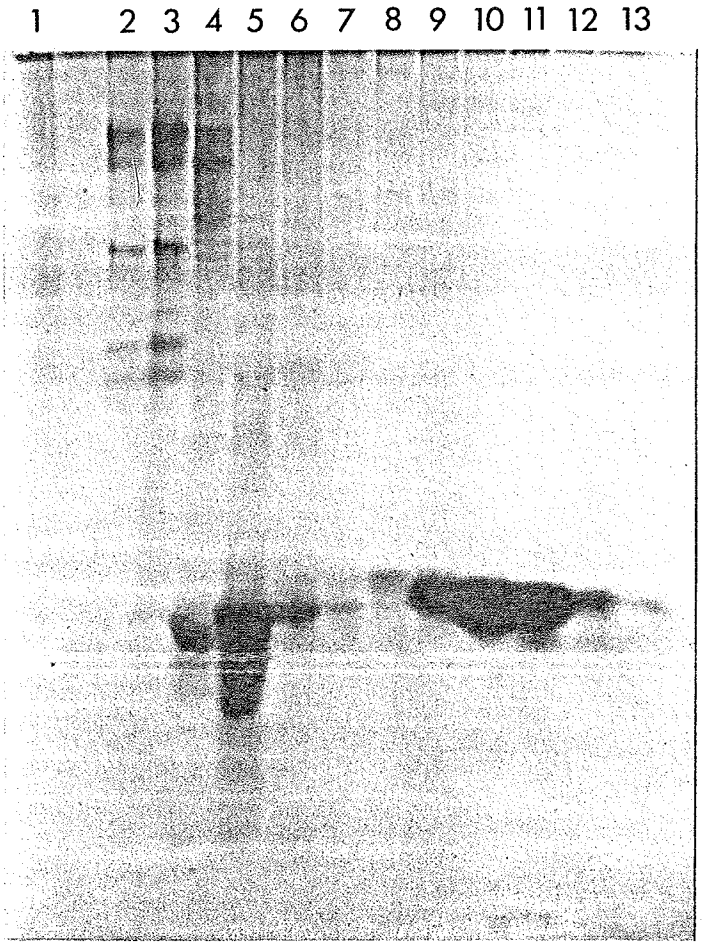


Fig. 5:

Purification of calmodulin: Gel filtration on Sephadex G-100, of the peak obtained by DEAE - Cellulose column (Fig. 3). The separation shown was performed and monitored as described in text. Fractions (3.2 ml) were collected and pooled, as indicated by the bar.

Fig. 5

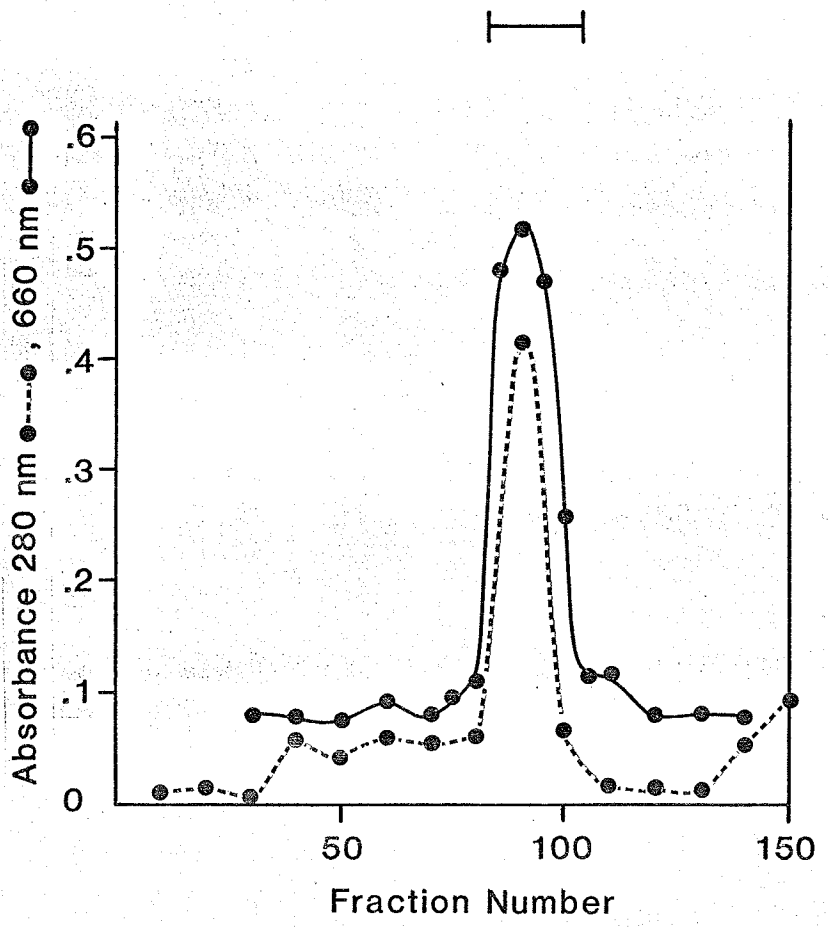


Fig. 6:

Purification of calmodulin: PAGE electrophoresis on 15% slabs of the fraction obtained from the Sephadex G-100 column (fig. 5). Reading left to right (slots 1-13), the fractions represented on the gel are 40, 50, 60, 70, 75, 80, 85, 90, 95, 100, 105, 110, 120.

Fig. 6

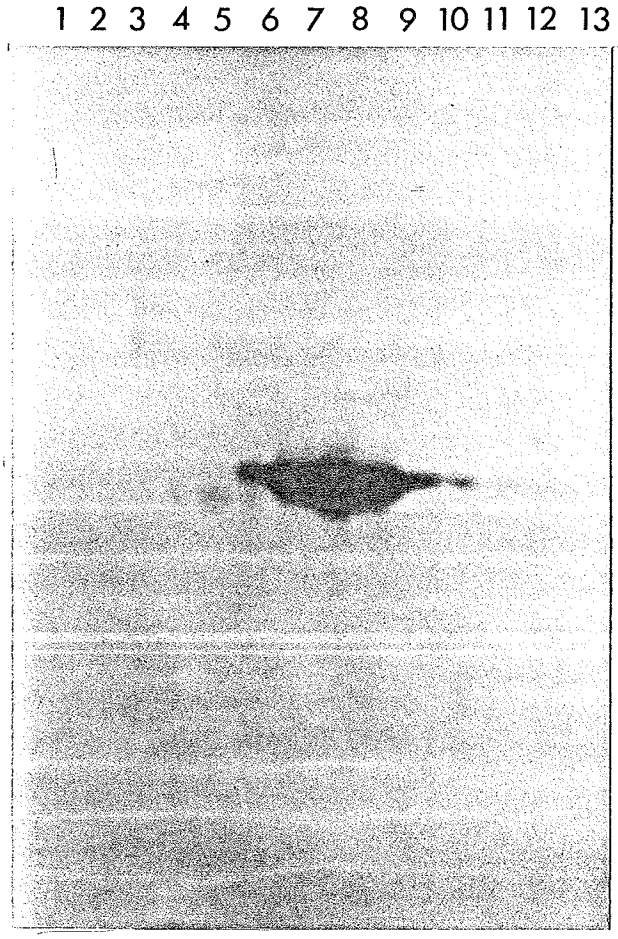


Fig. 7:

Preparation of phosphodiesterase: DEAE - Cellulose column chromatography. The separation shown was performed and monitored as described in the text. Fractions (20 ml) were collected and those fractions indicated by the bar were pooled.

Fig. 7

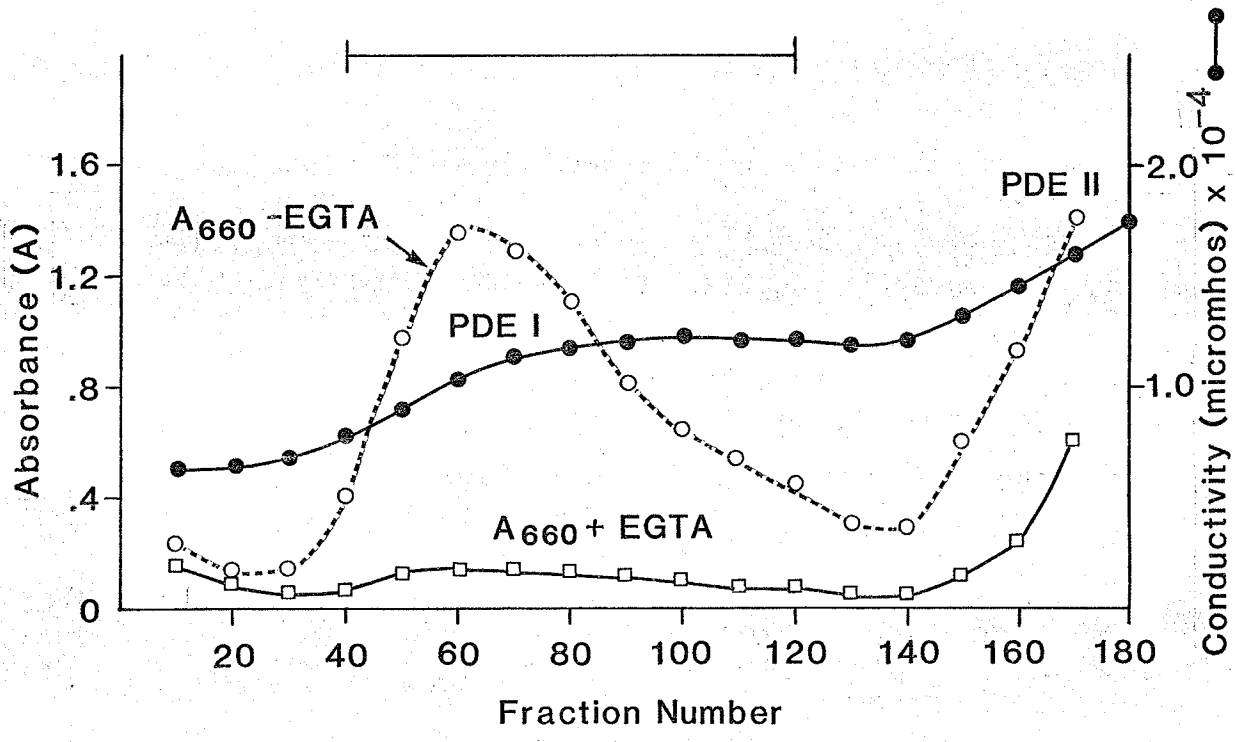

Absorbance (A)
1.6
1.2
.8
.4
0
A_{660} -EGTA
PDE I
PDE II
A_{660} + EGTA
20
40
60
80
100
120
140
160
180
Fraction Number
2.0
1.0
Conductivity (micromhos) x 10^{-4}

Fig. 8:

Preparation of phosphodiesterase: Affi-gel Blue Affinity chromatography, of the peak obtained by DEAE - Cellulose column (Fig. 7). The separation shown was performed and monitored as described in text. Fractions (15 ml) were collected and those fractions indicated by the bar pooled.

Fig. 8

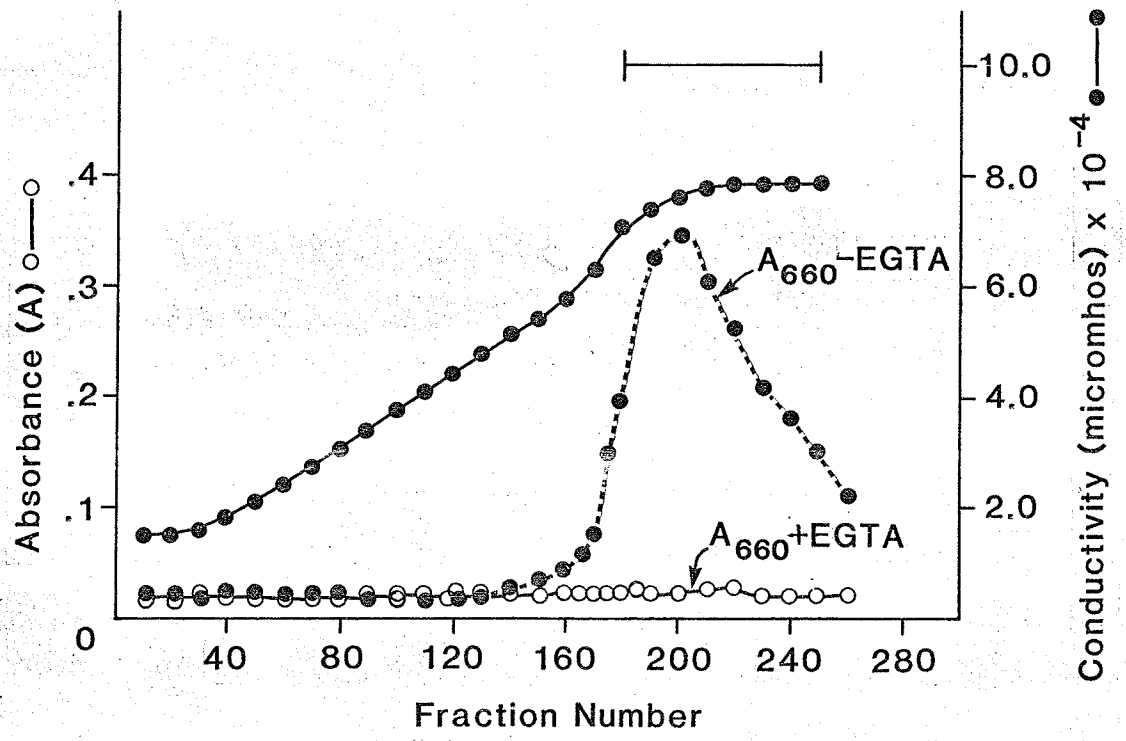

Absorbance (A) ○—○
.4
.3
.2
.1
0
40
80
120
160
200
240
280
Fraction Number
A_{660}-EGTA
A_{660}+EGTA
10.0
8.0
6.0
4.0
2.0
Conductivity (micromhos) x 10^{-4} ●—●

Fig. 9:

Preparation of troponin: Crude troponin prepared as described in text was run on 12% SDS polyacrylamide slabs. Reading from left to right slots 1 and 2 contain 5 μg of sample; 4 and 5, 15 μg; 7 and 8, 25 μg; slots 10 and 11, 35μg.

Fig. 9

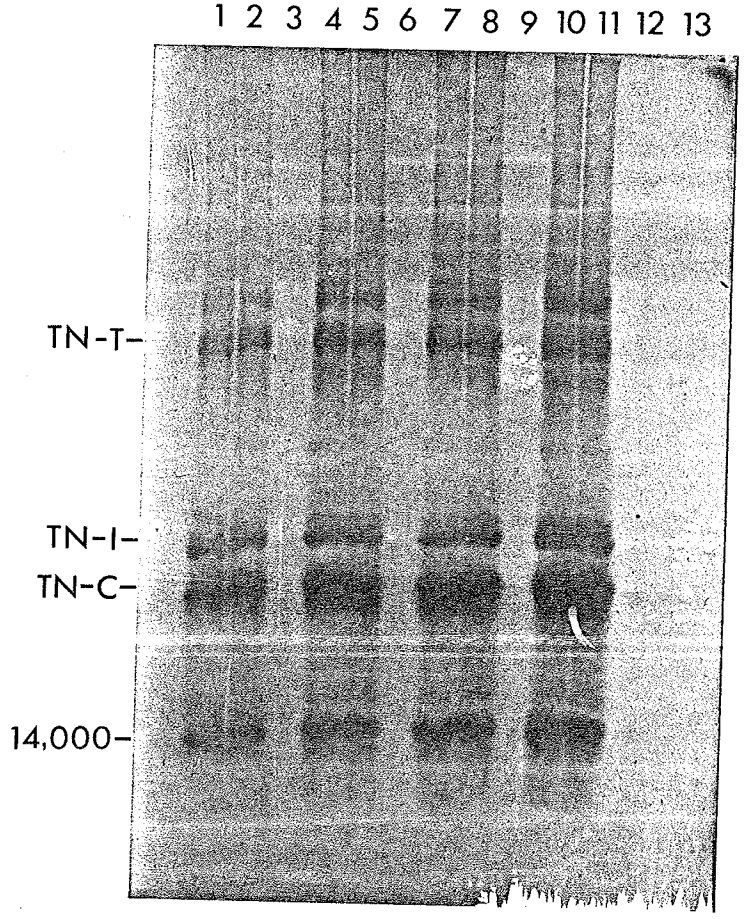


Fig. 10:

Preparation of troponin: DEAE - Sephadex A-50 column chromatography. The separation shown was performed and monitored as described in the text. Fractions (2 ml) were collected and those fractions indicated by the bar were pooled.

Fig. 10

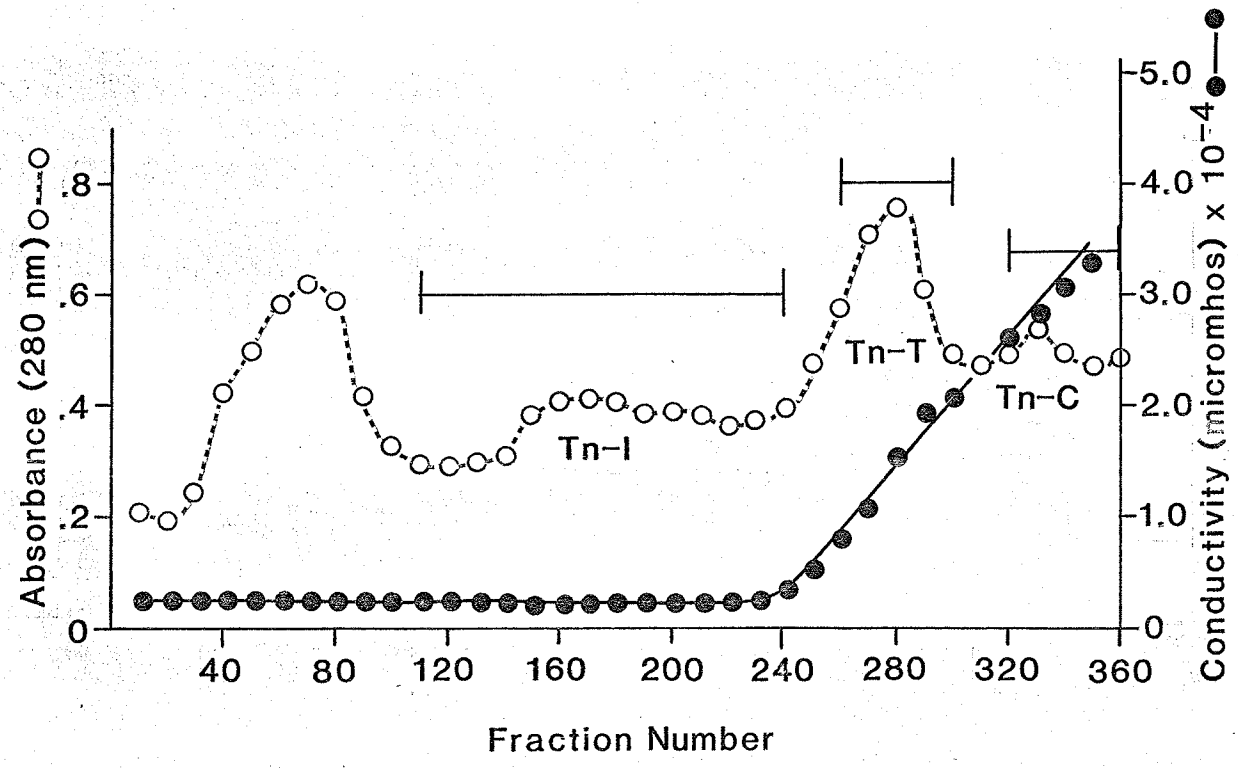

Absorbance (280 nm)
0
.2
.4
.6
.8
Tn-I
Tn-T
Tn-C
40
80
120
160
200
240
280
320
360
Fraction Number
Conductivity (micromhos) x 10⁻⁴
0
1.0
2.0
3.0
4.0
5.0

Fig. 11:

Preparation of troponin: SDS-PAGE electrophoresis on 12% slabs of selected fractions obtained by chromatography on DEAE - Sephadex A-50 (Fig. 10). From left to right (slots 1-18) the fractions represented on the gel are: 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210.

Fig. 11

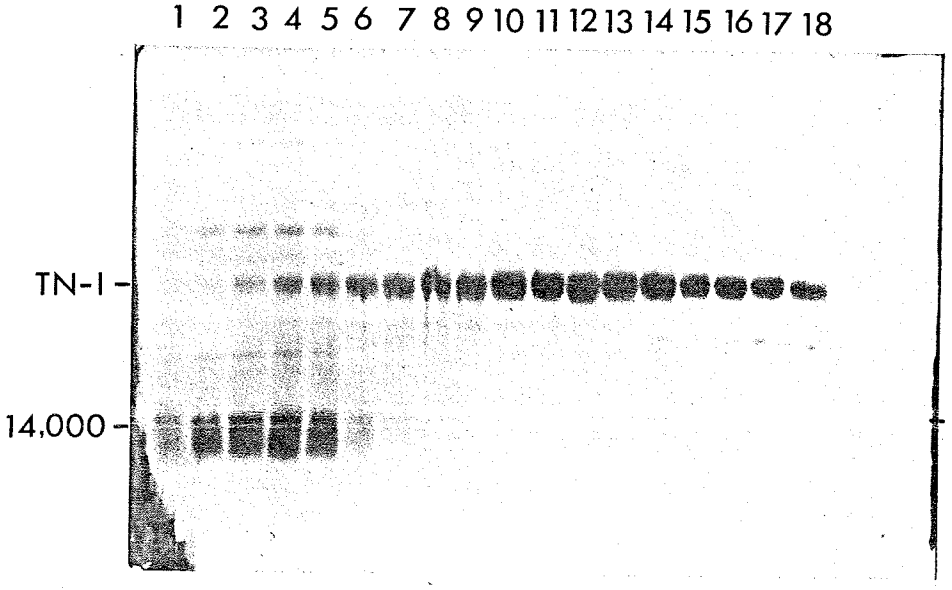


Fig. 12:

Preparation of troponin: SDS-PAGE electrophoresis on 12% slabs of selected fractions obtained by chromatography on DEAE - Sephadex A-50 (Fig 10). From left to right (slots 2-18) the fractions represented on the gel are: 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380.

Fig. 12

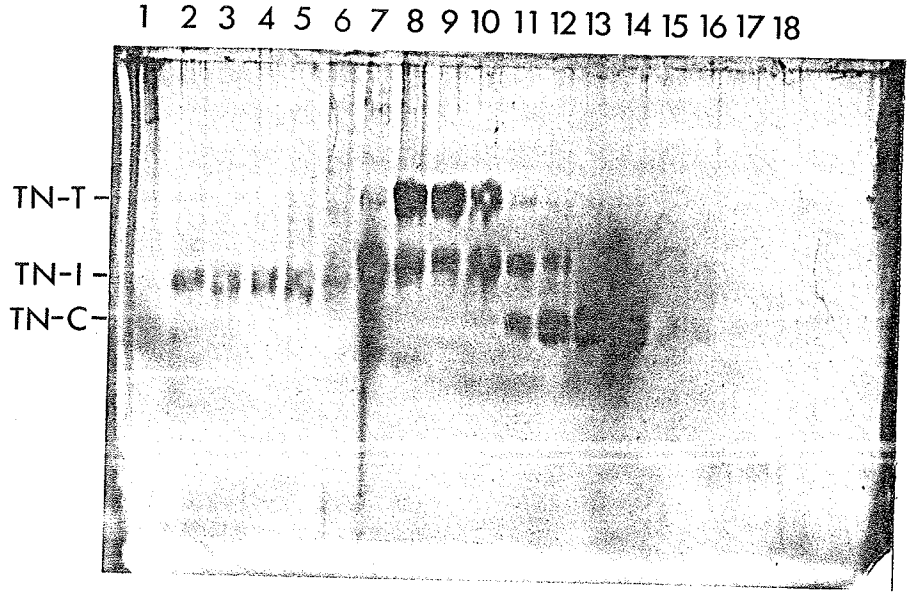


Fig. 13:

Purification of troponin I: Sepharose 4B - Calmodulin Affinity column chromatography. The separation shown was performed and monitored as described in text. Fractions (2.5 ml) were collected and those fractions indicated by the bar were pooled.

Fig. 13

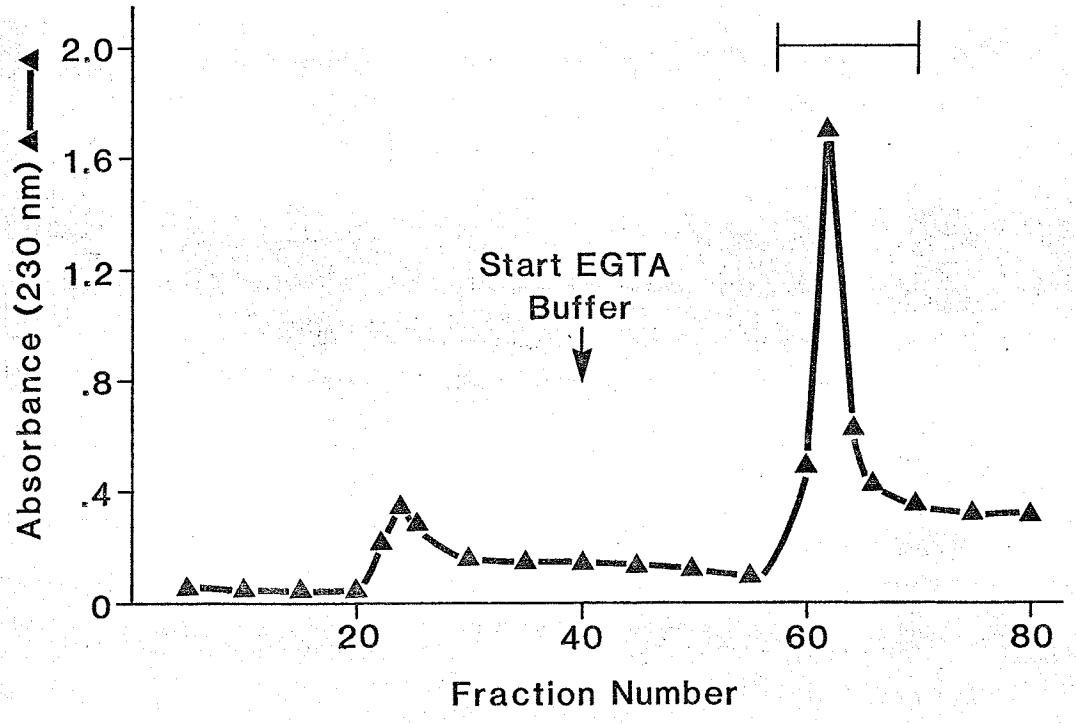

Absorbance (230 nm)
2.0
1.6
1.2
.8
.4
0
Start EGTA
Buffer
20
40
60
80
Fraction Number

Fig. 14:

Purification of troponin I: SDS-PAGE on 12% slabs of crude TN-I and fractions from Sepharose 4B - Calmodulin Affinity chromatography (Fig. 13). Reading from left to right slots 3 and 4 contain crude TN-I (15 and 20 μg respectively) and slots 8, 9, 10, represent fractions 62, 65 and 70.

Fig. 14

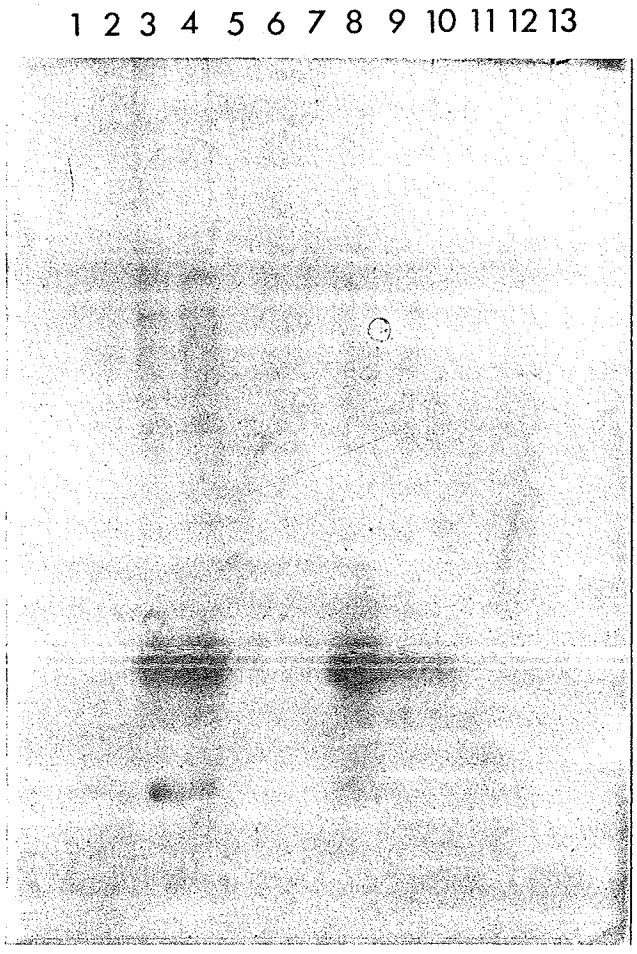


Fig. 15:

Complex formation between TN-I and TN-C or calmodulin: PAGE electrophoresis on 15% slabs. Reading from left to right the samples on the gel are: slot 1 - TN-I + Ca^{++}; slot 3 - TN-I + EGTA; slot 5 - TN-I, calmodulin + Ca^{++}; slot 6 - TN-I, calmodulin + EGTA; slot 7 - calmodulin + Ca^{++}; slot 9 - TN-I, TN-C + Ca^{++}; slot 10 - TN-I, TN-C + EGTA; slot 11 - TN-C + Ca^{++}.

Fig. 15

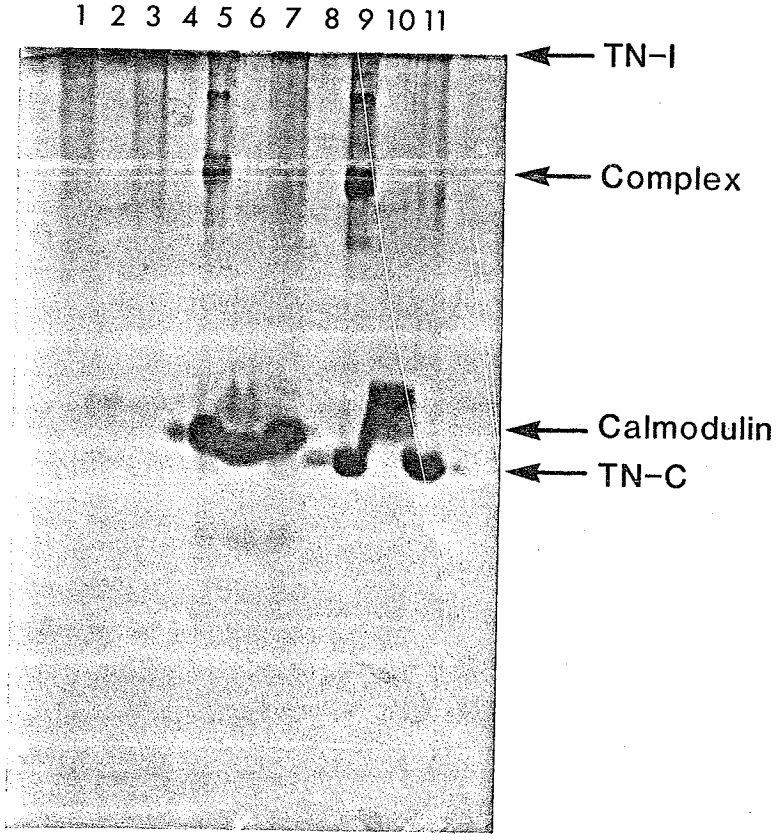


Fig. 16:

Complex formation between TN-I and TN-C: PAGE electrophoresis on 15% slabs comparing the ratio of TN-I to TN-C needed for total TN-C binding. Reading from left to right (slots 1-7) the fractions represented on the gel are in the following molar ratios (TN-I:TN-C): 0:1; .5:1; 1:1; 1.5:1; 2:1; 2.5:1; 3:1.

Fig. 16

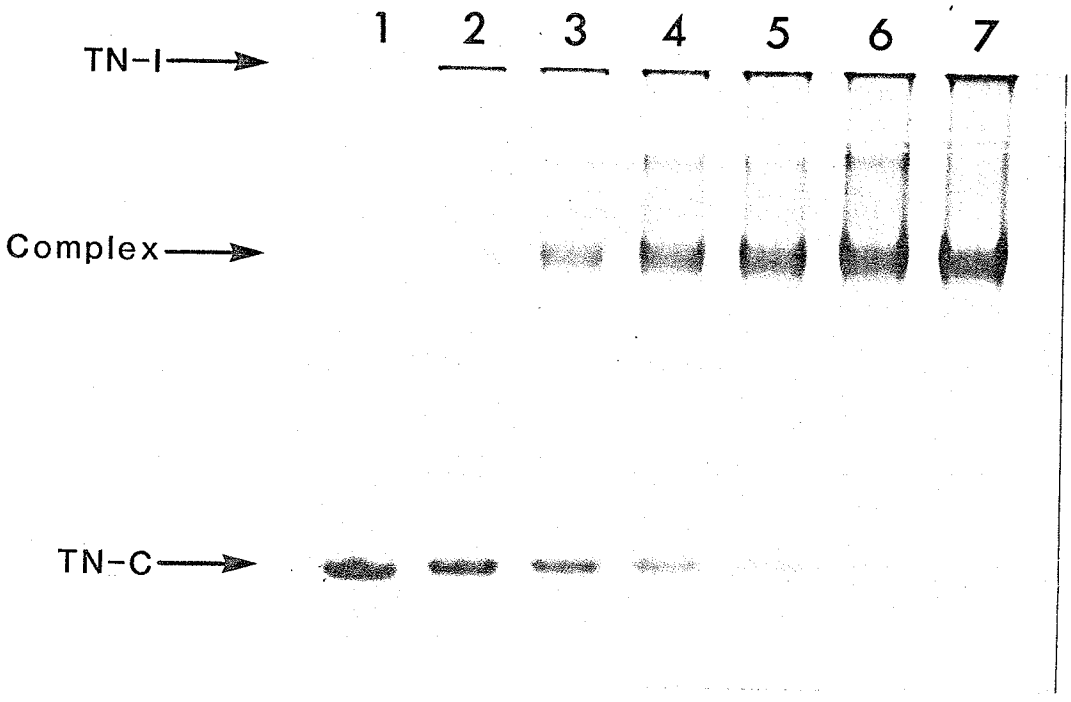


Fig. 17:

Complex formation between TN-I and calmodulin: PAGE electrophoresis on 15% slabs comparing the ratio of TN-I to calmodulin needed for total calmodulin binding. Reading from left to right (slots 1-7) the fractions represented on the gel are in the following molar ratios of (TN-I:calmodulin) 0:1, .5:1, 1:1, 1.5:1, 2:1, 2.5:1, 3:1.

Fig. 17

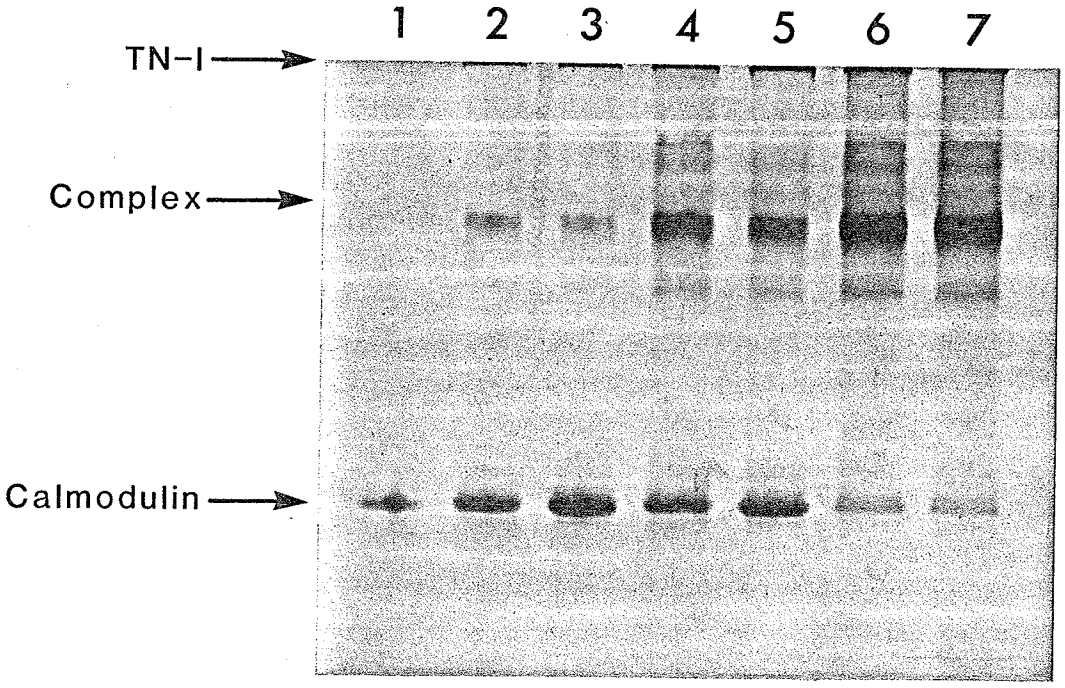


Fig. 18:

Isolation of troponin I : calmodulin complex: Sephdex G-200 column chromatography. The separation shown was performed and monitored as described in text. Fractions (5.0 ml) were collected.

Fig. 18

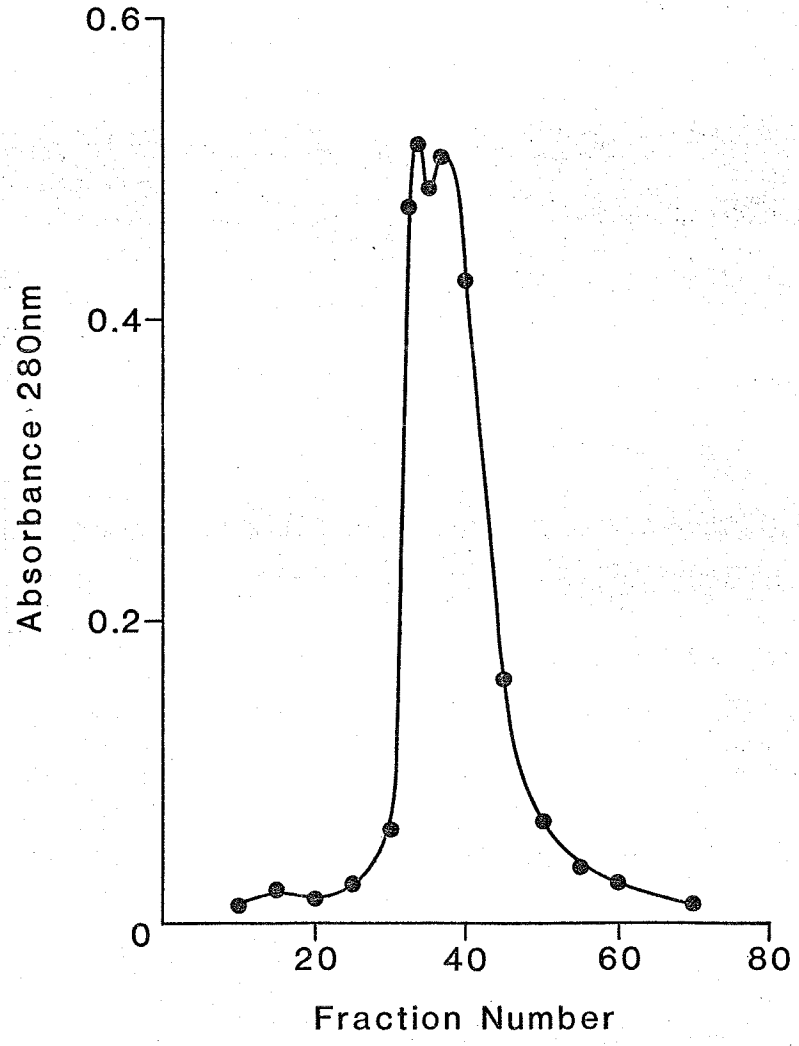


Fig. 19:

Isolation of the TN-I:calmodulin complex: PAGE electrophoresis on 15% slabs of the fractions from Sephadex G-200 (Fig. 18). From left to right (slots 1-13) the fractions represented on the gel are tubes 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40.

Fig. 19

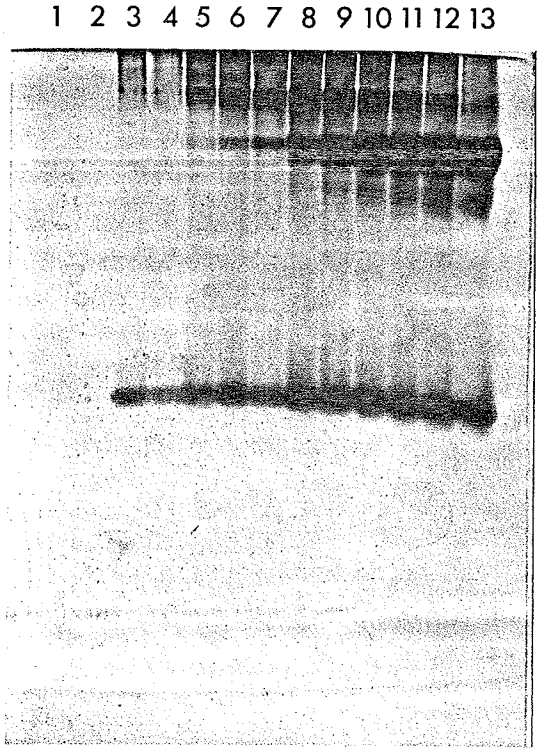


Fig. 20:

Isolation of troponin I : calmodulin complex: DEAE - Cellulose column chromatography. The separation shown was performed as described in text. Fractions (1.5 ml) were collected and these fractions indicated by the bar were pooled.

Fig. 20

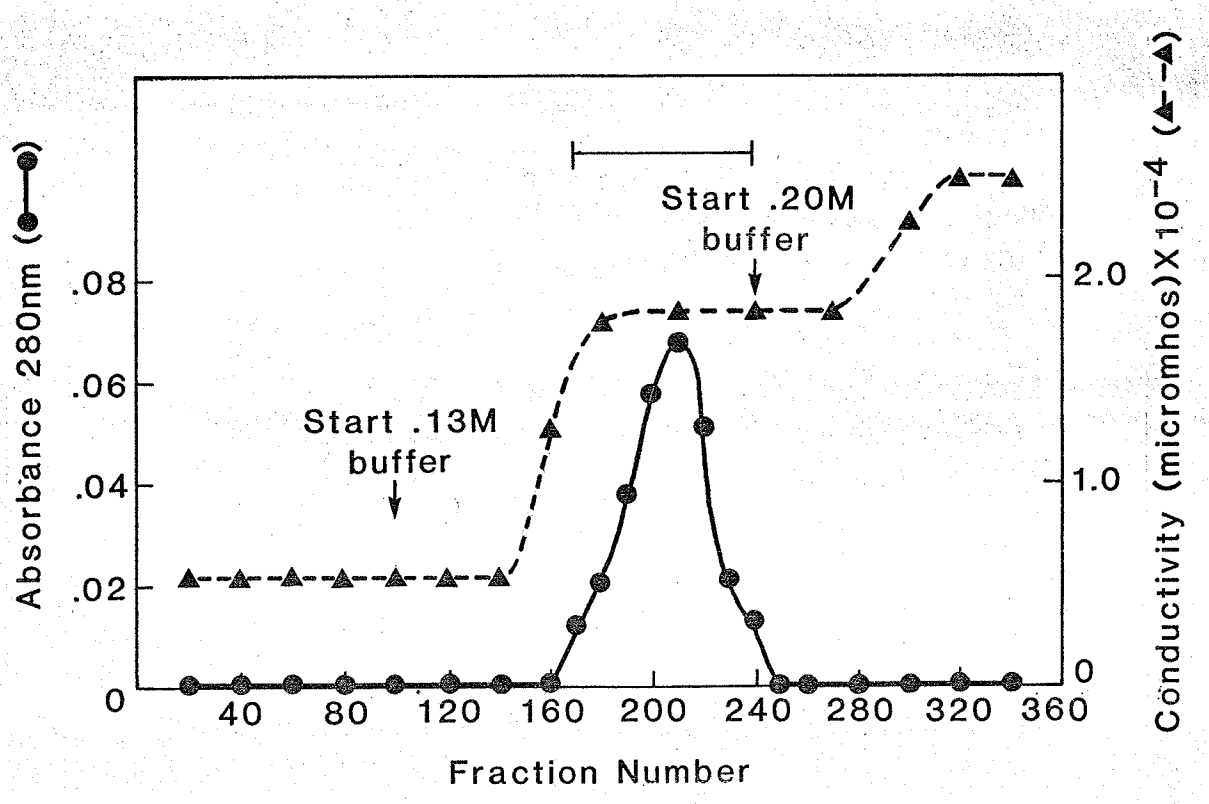

Absorbance 280nm (●—●)
.08
.06
.04
.02
0
Start .13M
buffer
Start .20M
buffer
2.0
1.0
0
Conductivity (micromhos)X10⁻⁴ (▲--▲)
40
80
120
160
200
240
280
320
360
Fraction Number

Fig. 21:

Isolation of the TN-I:calmodulin complex. PAGE on 15% slabs of selected fractions from DEAE - Cellulose column (Fig. 20). From left to right (slots 1-13) the samples represent: calmodulin, TN-I + calmodulin, 130, 140, 150, 160, 170, 180, 200, 210, 220, 240, 250.

Fig. 21

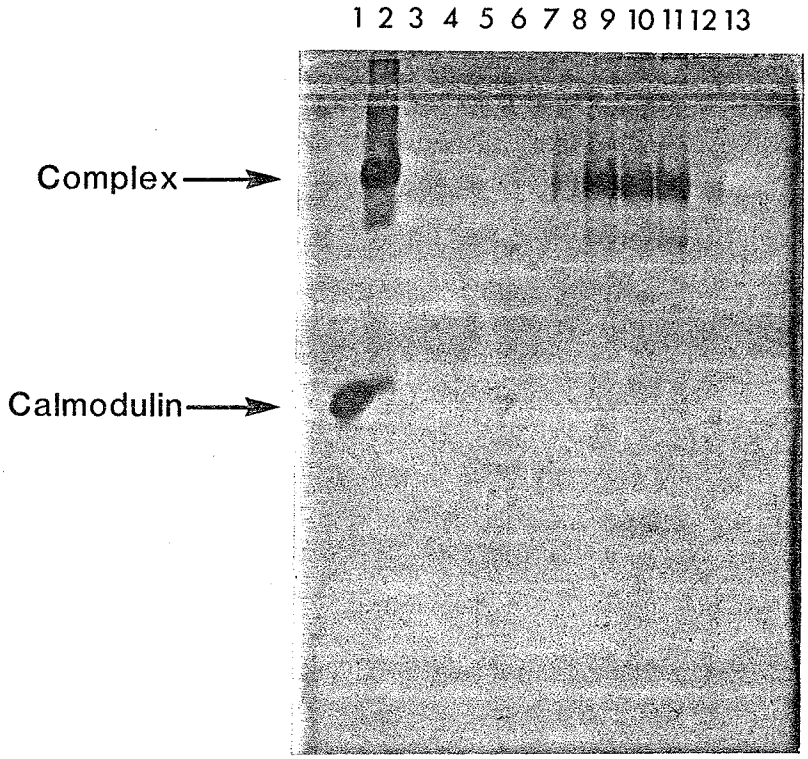


Fig. 22:

Effect of TN-I on the calmodulin stimulation of PDE: Increasing amounts of TN-I were added to a standard assay mixture of PDE and calmodulin. The results are expressed in terms of PDE activity (as measured by O.D. 660 nm) as a function of TN-I:calmodulin ratio.

Fig. 22

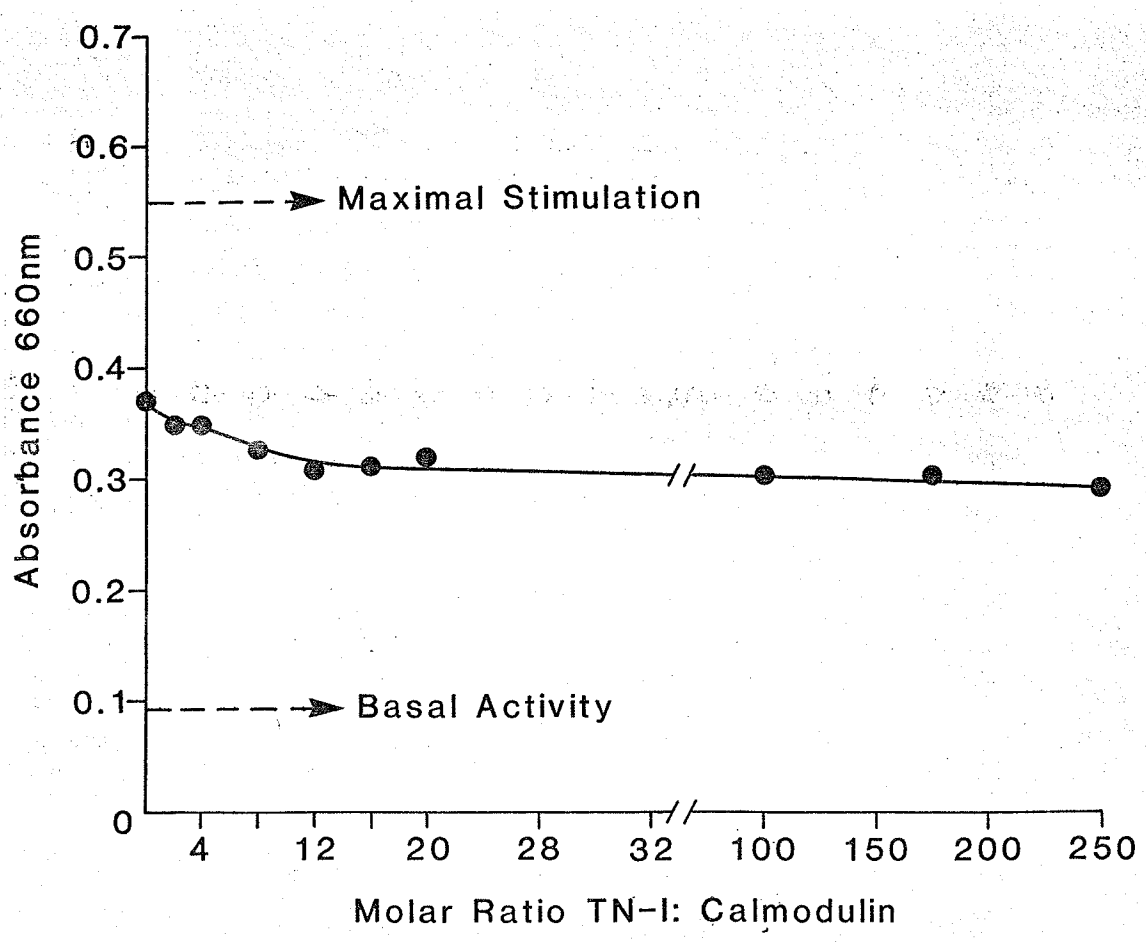

0.7
0.6
Maximal Stimulation
0.5
0.4
0.3
0.2
0.1
Basal Activity
0
Absorbance 660nm
4
12
20
28
32
100
150
200
250
Molar Ratio TN-I: Calmodulin

Fig. 23:

Tyrosine modification: Kinetics of nitration of troponin I. Troponin I (1 mg/ml) was incubated at 23°C, pH 11.0 with tetranitromethane (0.6 nm). The nitrotyrosine content was estimated from the absorbance at 428 nm using a molar extinction coefficient of 4100 M^{-1} cm^{-1}.

Fig. 23

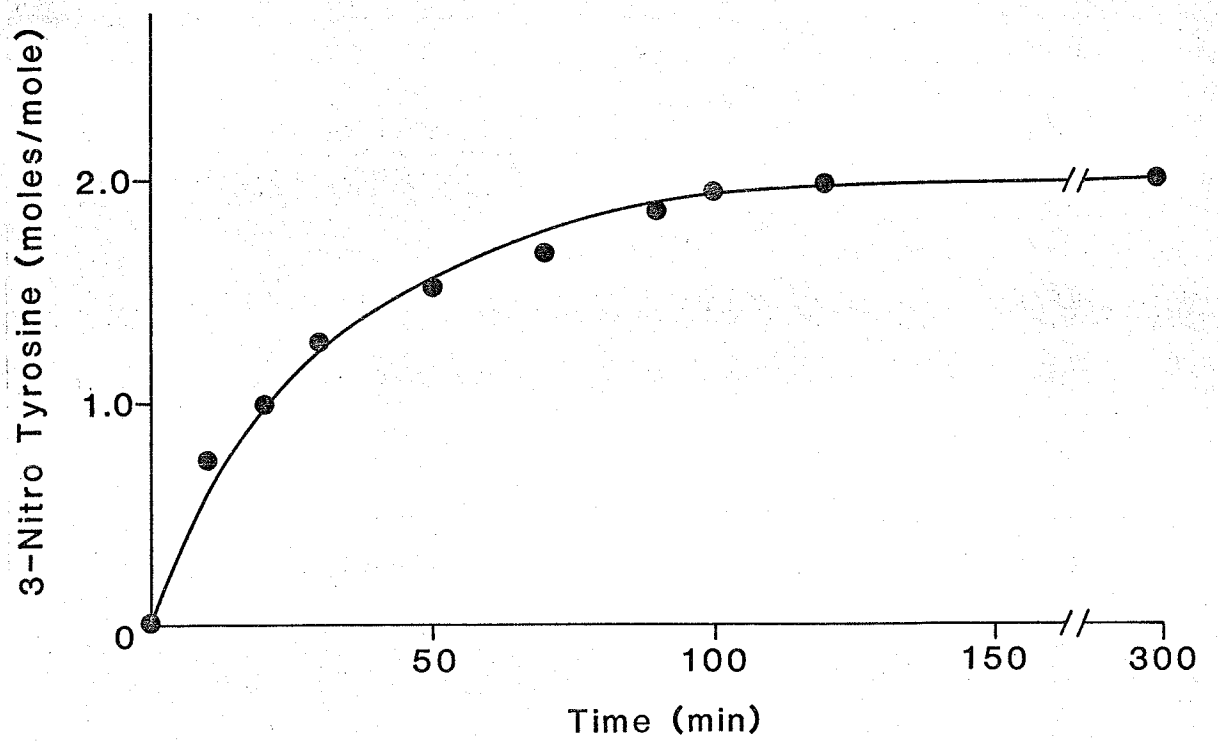


Fig. 24:

Tyrosine modification: PAGE electrophoresis on 15% slabs. Reading from left to right (slots 1 to 4), the following samples are represented: 20 μg TN-I + 20 μg calmodulin; 20 μg TNM treated TN-I + 20 μg calmodulin; 20 μg TN-I + 20 μg TN-C; 20 μg TNM treated TN-I + 20 μg TN-C. All slots contain Ca^{++}.

Fig. 24

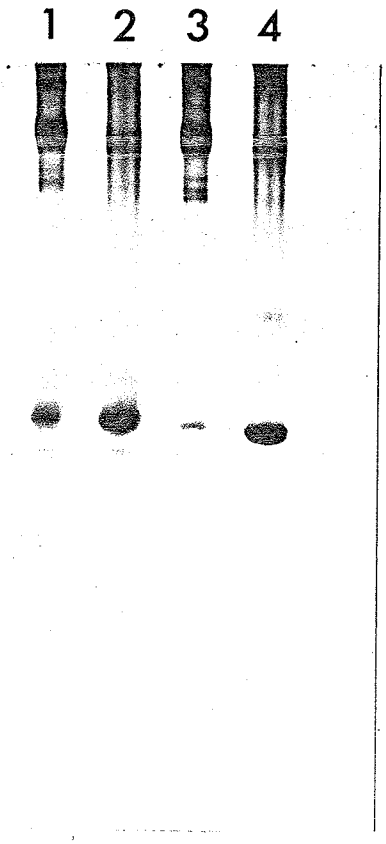


Fig. 25:

Tyrosine modification: Nitration of the TN-I:calmodulin complex. DEAE-Cellulose column chromatography of the nitrated complex. The separation shown was performed and monitored as described in the text. Fractions (3.0 ml) were collected and those fractions indicated by the bar were pooled.

Fig. 25

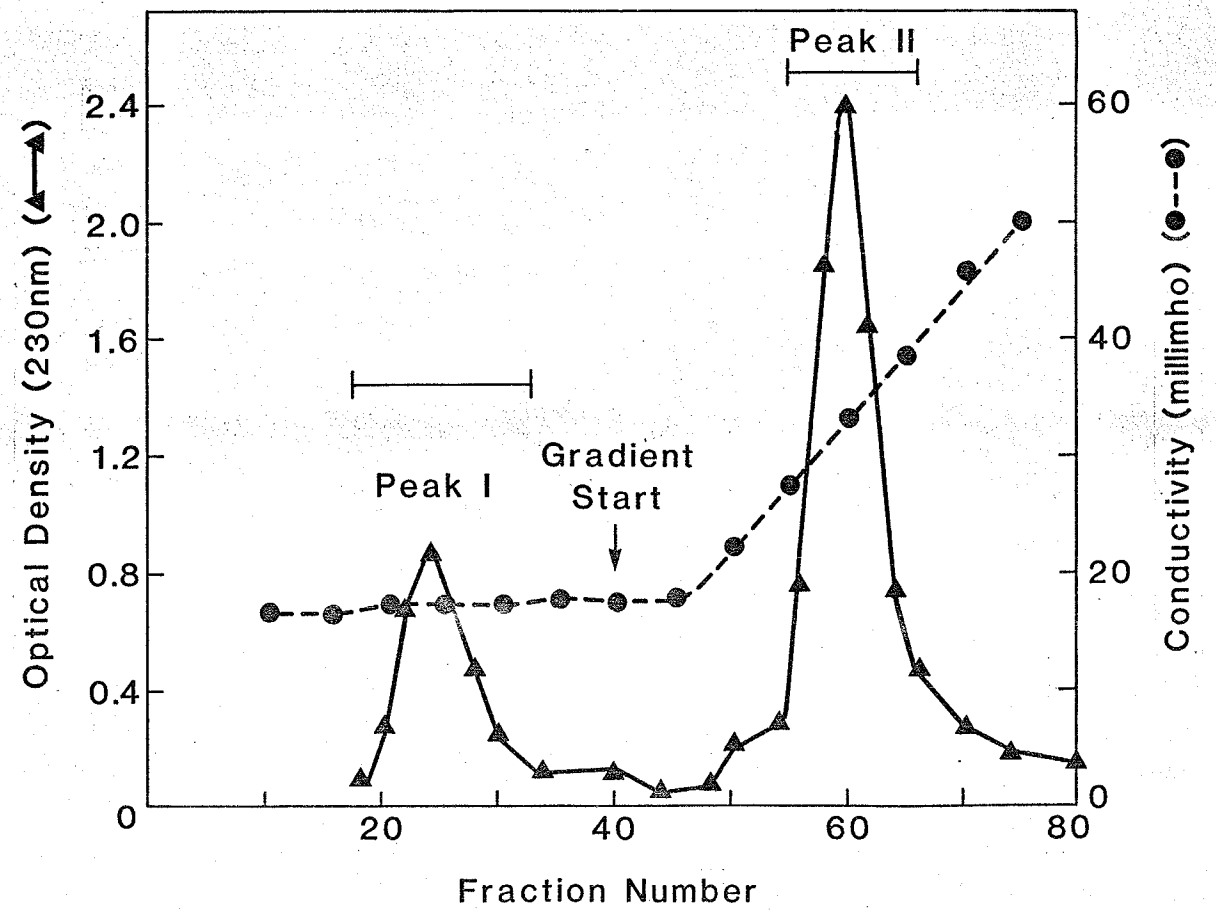

Peak II
Peak I
Gradient
Start
Optical Density (230nm)
Conductivity (millimho)
Fraction Number
0
0.4
0.8
1.2
1.6
2.0
2.4
0
20
40
60
20
40
60
80

Fig. 26:

Tyrosine modification: Nitration of the TN-I:calmodulin complex. PAGE electrophoresis on 15% slabs of the following samples: 20 μg TN-I; Peak I (Fig. 25); Peak II (Fig. 25); Peak II and Peak I + Ca^{++}; TN-I and Peak II + Ca^{++}.

Fig. 26

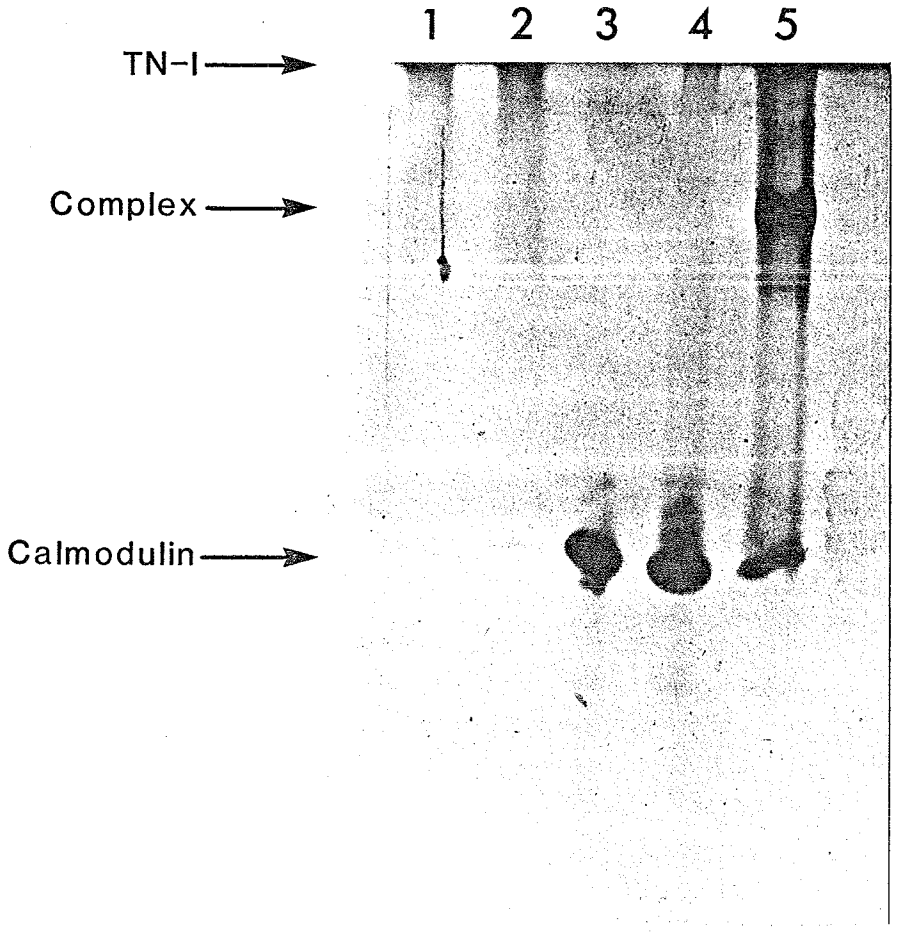


Fig. 27:

Tyrosine modification: Nitration of the TN-I:TN-C complex: DEAE - Cellulose column chromatography. The separation shown was performed and monitored as described in text. Fractions (3.0 ml) were collected and those fractions indicated by the bar were pooled.

Fig. 27

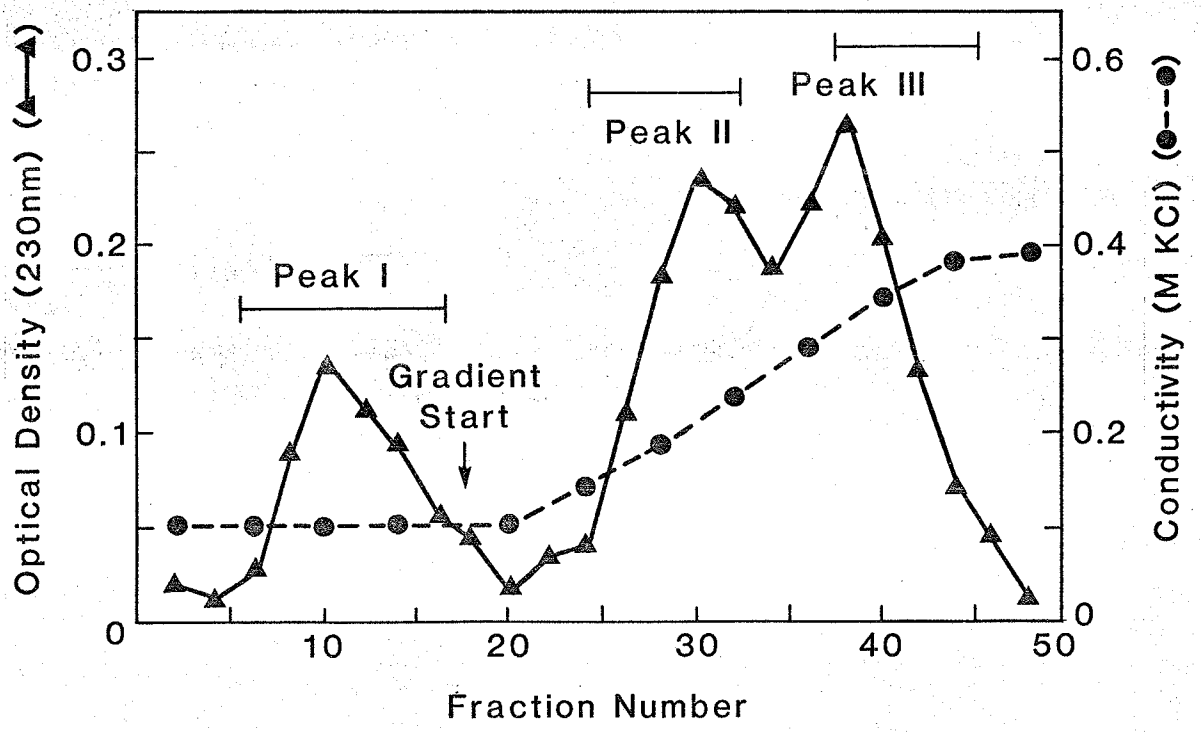

Optical Density (230nm)
Conductivity (M KCl)
Fraction Number
Peak I
Peak II
Peak III
Gradient Start
0.3
0.2
0.1
0
0.6
0.4
0.2
0
10
20
30
40
50

Fig. 28:

Tyrosine modification: Nitration of the TN-I:TN-C complex. PAGE electrophoresis on 15% slabs of fractions obtained by DEAE-Cellulose chromatography (Fig. 27). From left to right (slots 1-13) the fractions represented on the gel are tubes 10, 20, 24, 27, 30, 33, 37, 39, 40, 45, 48, 50, 55.

Fig. 28

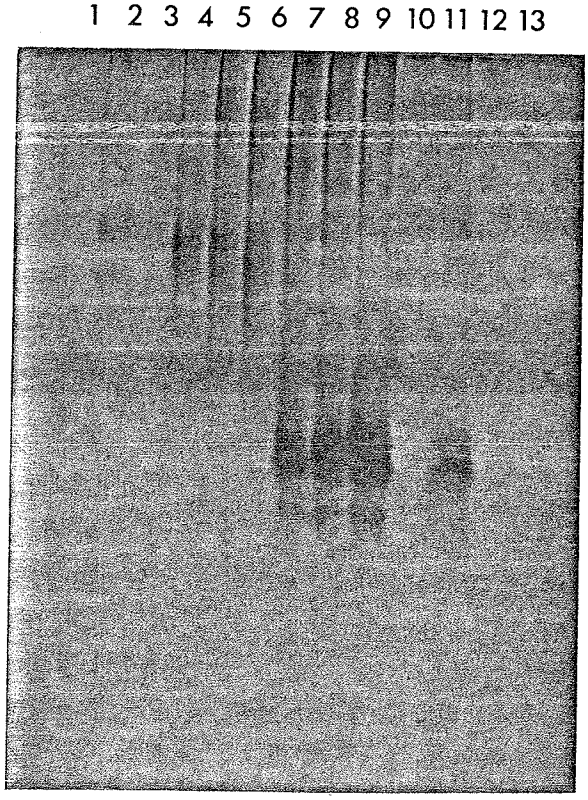


Fig. 29:

Tyrosine modification: Nitration of the TN-I:TN-C complex. PAGE electrophoresis on 15% slabs of peaks obtained from DEAE-Cellulose chromatography (Fig. 27). Reading from left to right (slots 1-4) the samples represented on the gel are: Peak I - tubes 5-16, Peak II - tubes 22-33, and tubes 34-38, Peak III - tubes 39-45.

Fig. 29

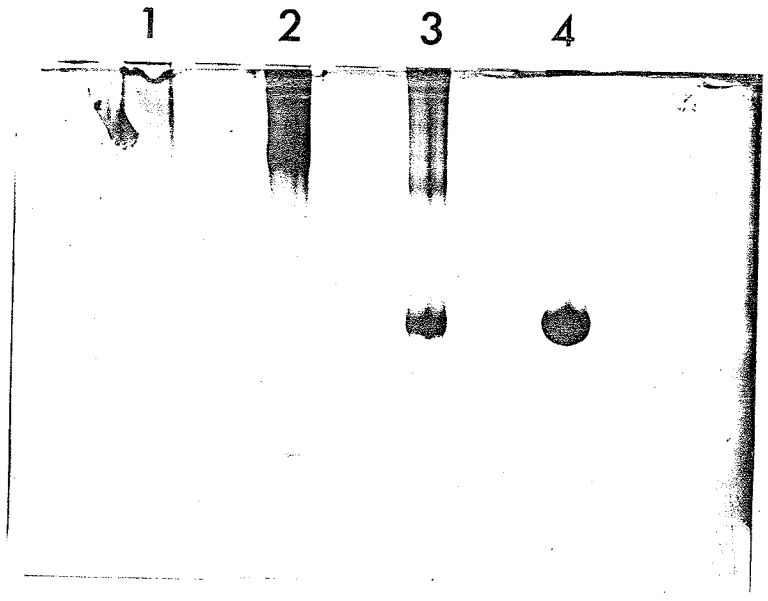


Fig. 30:

Tyrosine modification: Nitration of the TN-I:TN-C complex. SDS-PAGE on 12% slabs of Peak II obtained from DEAE-Cellulose chromatography (Fig. 27) and molecular weight standards. Reading from left to right (slots 1-6) the samples represented on the gel are: 7.5 μg Peak II; 10 μg Peak II; 15 μg Peak II; cytochrome-C - 12,500 and TN-I - 22,000; calmodulin - 17,500; BSA - 67,000.

Fig. 30

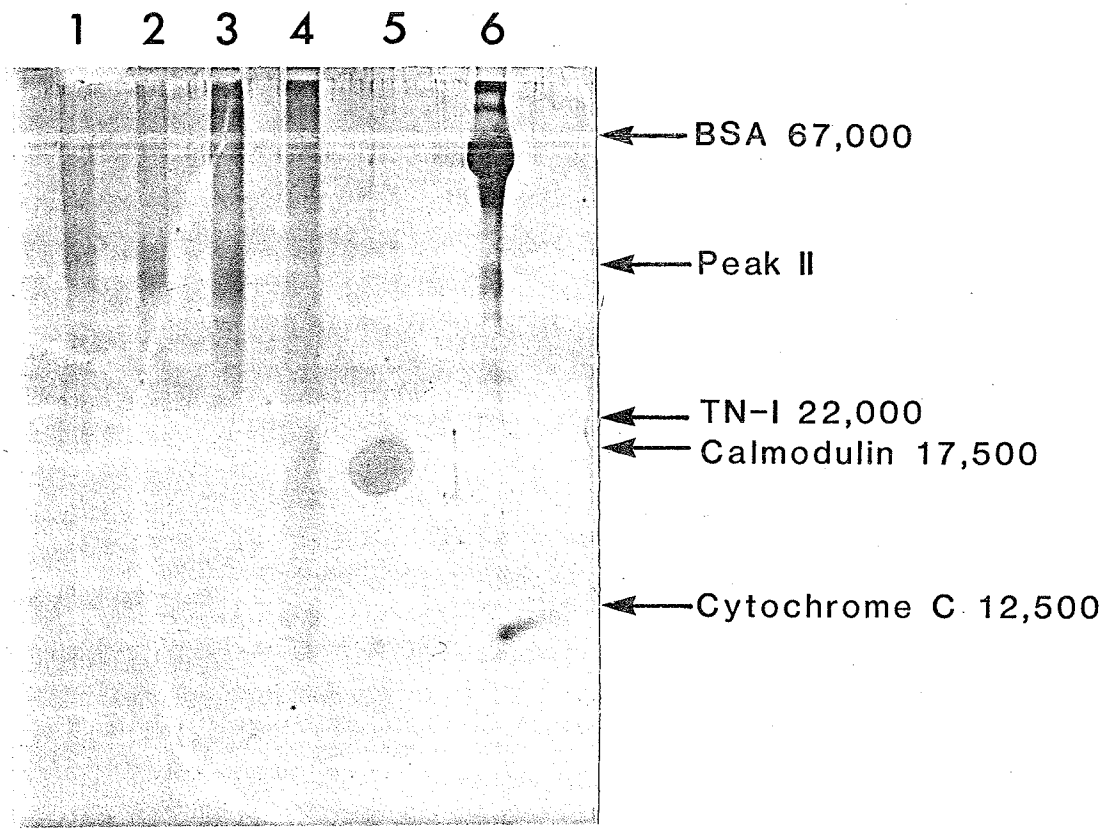

1 2 3 4 5 6
BSA 67,000
Peak II
TN-I 22,000
Calmodulin 17,500
Cytochrome C 12,500

Fig. 31:

Tryptic cleavage of troponin. Time course tryptic digestion of nitrated TN-I as monitored by PAGE electrophoresis on 15% slabs. TN-I (1.0 mg) was digested at 37°C with an enzyme/substrate ratio of 1:20. Reading from left to right (slots 1-7) each sample represents 100 μg of protein at the following incubation times: 0, 5 min, 10 min, 15 min, 30 min, 60 min, 90 min.

Fig. 31

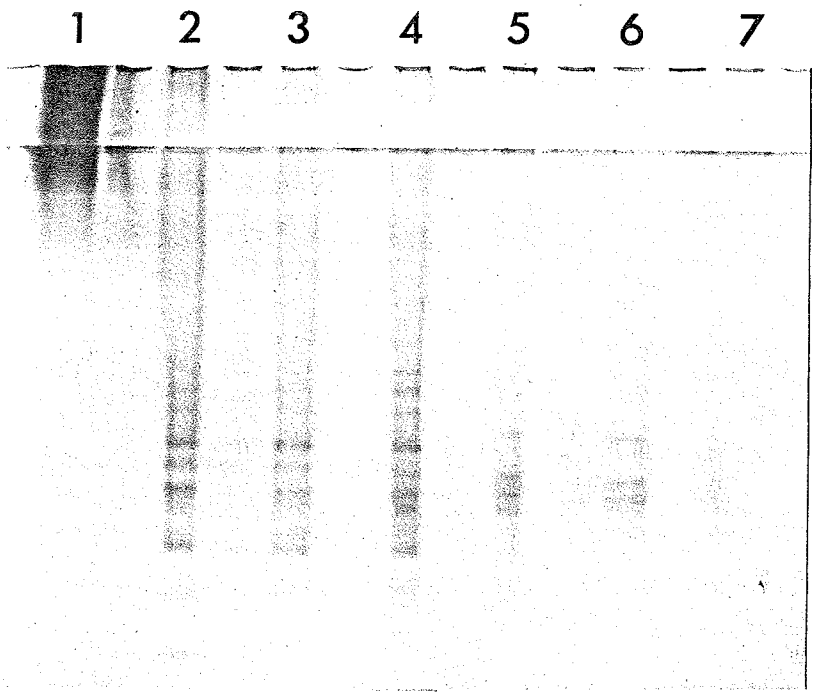


Fig. 32:

Tryptic cleavage of troponin: Time course tryptic digestion of nitrated TN-C as monitored by PAGE electrophoresis on 15% slabs. TN-C (1.0 mg) was digested at 37°C with an enzyme/substrate rate of 1:20. Reading from left to right (slots 1-8) each sample represents 100 μg of protein at the following incubation times: 0, 5 min, 10 min, 15 min, 30 min, 60 min, 90 min, 0 min.

Fig. 32

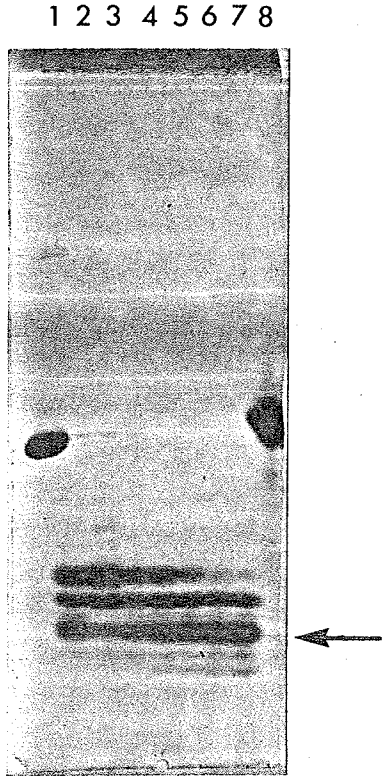


Fig. 33:

Tryptic cleavage of troponin: Time course tryptic digestion of unmodified TN-I:TN-C complex as monitored by PAGE electrophoresis on 15% slabs. TN-I:TN-C complex (1.0 mg) was digested at 37°C with an enzyme/substrate ratio of 1:20. Reading from left to right (slots 1-8) each sample represents 100 μg of protein at the following incubation times: 0, 2 min, 5 min, 10 min, 15 min, 30 min, 60 min, 90 min.

Fig. 33

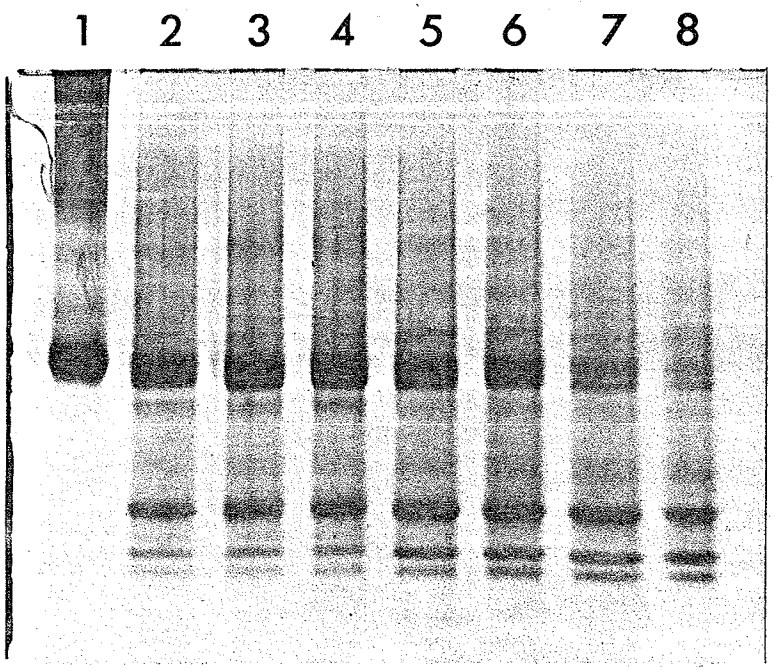


Fig. 34:

Tryptic cleavage of troponin: Time course tryptic digestion of the cross-linked TN-I:TN-C complex as monitored by PAGE electrophoresis on 15% slabs. Cross-linked TN-I:TN-C (1.0 mg) was digested at 37°C with an enzyme/substrate ratio of 1:20. Reading from left to right (slots 1-3) each sample represents 100 μg of protein at the following incubation times 0, 60 min, 90 min.

Fig. 34

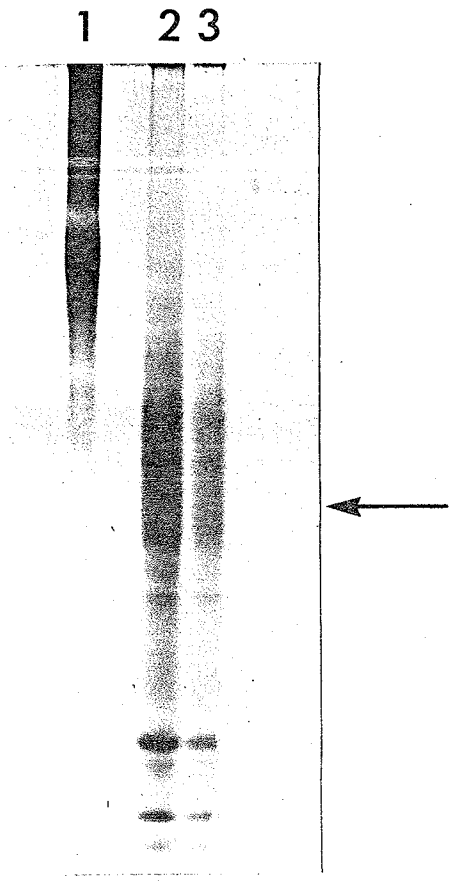
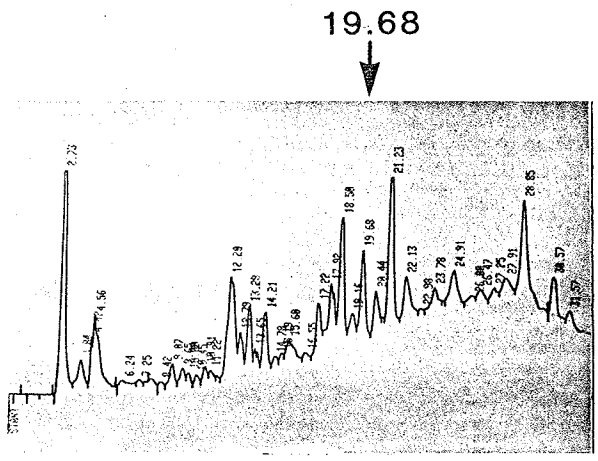


Fig. 35a,b,c:

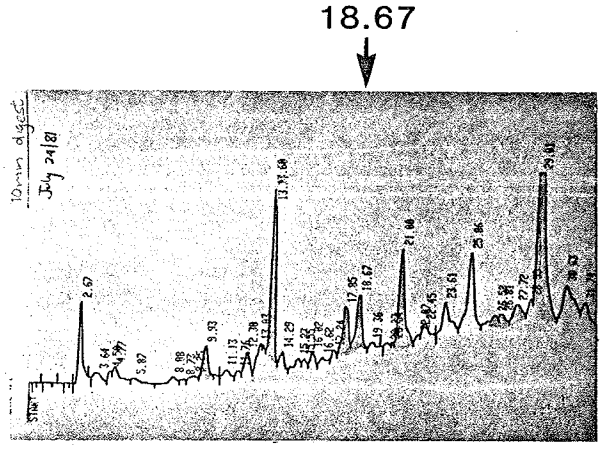
Tryptic cleavage of troponin: HPLC chromatography of protein samples digested at 37°C, with trypsin using a 1:20 enzyme/substrate ratio. The separation was performed as described in text. a - represents nitrated TN-I (40 μg); b - represents nitrated TN-C (40 μg); c - represents crosslinked TN-I:TN-C (40 μg). Those fractions indicated by arrows were pooled.

Fig. 35

a) TN-I



b) TN-C



c) TN-I:TN-C