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THE UNIVERSITY OF ALBERTA

SEQUENCE STUDIES ON RABBIT SKELETAL TROPOMYOSIN

by

© ROBERT STANLEY HODGES

A THESIS

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Sequence Studies on Rabbit Skeletal Tropomyosin" submitted by Robert Stanley Hodges in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Physical studies of rabbit skeletal tropomyosin have shown that it is a rod-like molecule of molecular weight about 70,000, consisting of two subunits of identical mass (35,000), and an α-helical content greater than 90 percent. X-ray diffraction studies provide evidence that the molecule consists of two α-helical chains arranged in an extended "coiled-coil" configuration. The possibility of non-identity of subunits has been suggested by N-terminal and C-terminal studies but these results were indecisive in proving the existence of two different polypeptide chains.

To shed further light on this question of subunit identity we have undertaken the elucidation of the number of unique sequences about the histidyl, methionyl, and cysteinyl residues. Amino acid analyses of tropomyosin have shown 4 histidines, 13-14 methionines and close to 3 cysteine residues per mole (70,000) of tropomyosin. The isolation of 2 unique histidyl peptides is consistent with two identical polypeptide chains of molecular weight 35,000. However, the presence of 3 cysteines and 3 unique cysteine sequences and at least 8 unique methionyl sequences per mole of 70,000 is indicative of either non-identical but similar chains or of more than one form of tropomyosin. This conclusion is further substantiated by the isolation and sequence analysis of two varieties of peptides arising from the C-terminus of the protein.

The previous report of an N-acetylated peptide has been confirmed and its sequence elucidated.

The limited amino acid sequences elucidated about the cysteine and histidine residues have indicated a possible periodicity of non-polar residues. To determine the sequence specificity required to maintain the "coiled-coil" structure an extension of these sequences was required. After CNBr treatment of the protein, a fragment was isolated whose molecular weight was approximately 17,000 by amino acid analysis and sedimentation equilibrium studies in 8 M urea. The fragment in benign media had a molecular weight of about 35,000 by sedimentation equilibrium and an α-helical content of approximately 60% by circular dichroism. The results of a limited chymotryptic digest of this fragment has given an extended sequence of 33 amino acids about the cysteine residues. Also, the sequence of 33 residues about the cysteine residues in the C-terminal cyanogen bromide fragment indicates a minimum of 4 different polypeptide chains. The sequence in this region of the tropomyosin molecule shows that the polypeptide chains are highly homologous and vary in ten positions with all the amino substitutions being highly conservative in nature. This sequence has not shown a simple regular periodicity of non-polar residues previously indicated. Elucidation of the complete amino acid sequence of the C-terminal cyanogen bromide fragment will hopefully yield sufficient information to provide an explanation of those features responsible for the unique structure of this protein.

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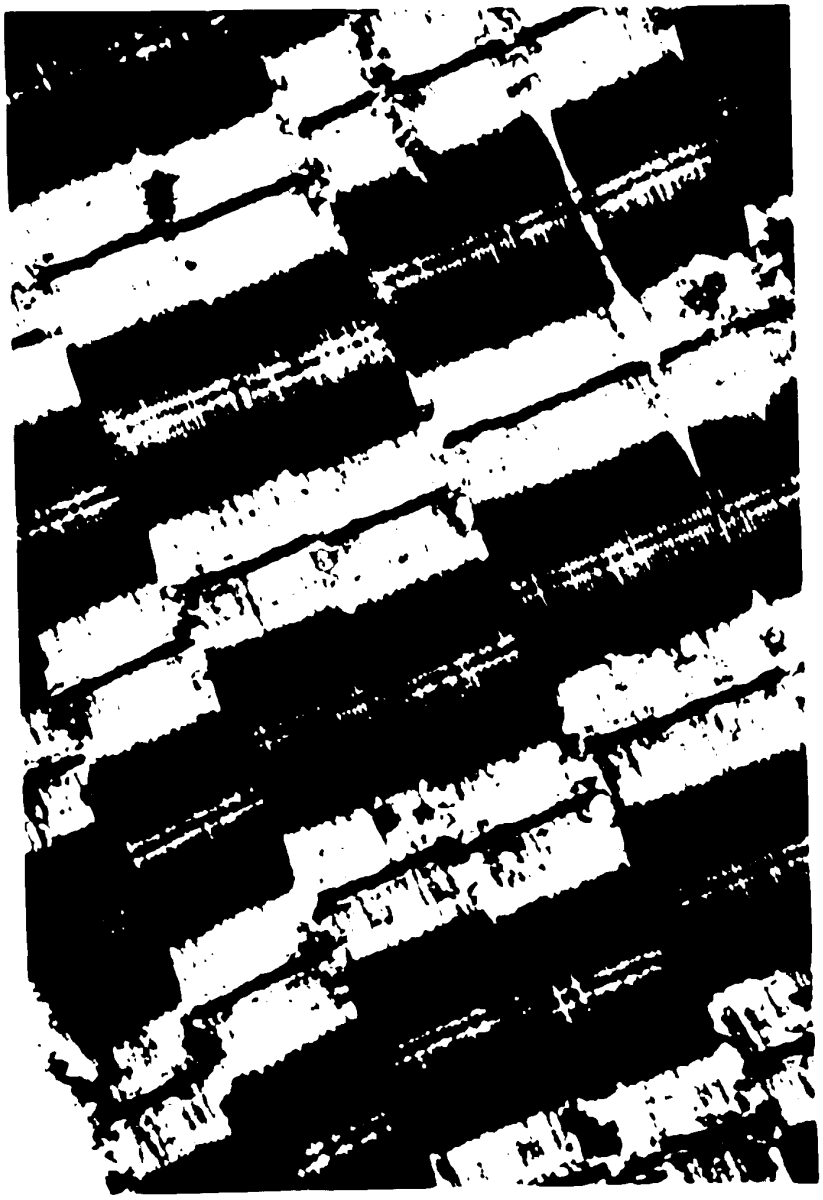
LIST OF ABBREVIATIONS

CM	Carboxymethyl-
CM-methionine	S-carbamoylmethylmethionine
CMC	S-carboxymethylcysteine
Cya	Cysteic acid
Dansyl chloride (DNS-Cl)	1-Dimethylaminonaphthalene-5-sulfonyl chloride
DNS-	1-Dimethylaminonaphthalene-5-sulfonyl
DNP-	2,4-Dinitrophenyl-
DEAE-	Diethylaminoethyl-
DTT	Dithiothreitol (Cleland's reagent)
$E^{1\%}_{1\ cm}$	Extinction coefficient of protein
EDTA	Ethylene diaminetetra-acetate
FDNB	Fluoro-2,4-dinitrobenzene
M_w	The weight-average molecular weight (g/mole)
NEM	N-ethylmaleimide
PITC	Phenylisothiocyanate
PTC-peptide	Phenylthiocarbamyl-peptide
QAE-Sephadex	Diethyl-(2-hydroxypropyl) aminoethyl-Sephadex
$s_{20,w}$	Sedimentation coefficient in water at 20° (in Svedberg units, S)
SDS	Sodium dodecyl sulphate
TFA	Trifluoroacetic acid
TPCK	L-1-tosylamido-2-phenylethylchloromethyl ketone

All temperatures are given in Centrigrade degrees.

Plate A

Electron micrograph of a longitudinal section of rabbit psoas muscle (by H. E. Huxley cited in Perry, 1960).



CHAPTER I

INTRODUCTION

Muscle contraction is one of the key processes of animal life. Research in this field will unfold answers to one of the most basic problems of biological science, the way in which chemical energy of ATP is transformed to mechanical energy through the co-ordinated movement of the structural elements of the contractile system.

Muscles are usually classified into two types: the "striated" muscles, which act quickly and are under voluntary control (Plate A), and the "smooth" muscles which act slowly and involuntarily and have no striations (Plate B). Of these two types of muscle, the structure and distribution of the contractile elements of the seemingly less complex system of smooth muscle still remains unclear (Shoenberg et al., 1966). The striated skeletal muscle has not only been the most extensively studied but has also yielded simpler answers to the fundamental problems of muscle contraction.

Striated muscles are made up of muscle fibres, which in turn consist of many small striated fibrils. These myofibrils are the functional units of muscle contraction. It is the collective and co-ordinated action of these fibrils that result in the shortening (contraction) followed by re-extension (relaxation) of the muscle. The myofibril is segmented into small longitudinal compartments, or sarcomeres at regular intervals by the Z-line. The myofibril is packed with microfilaments; the so-called thin filaments of actin and the thick filaments of myosin (Plate A). These filaments account for the characteristic striated pattern; the less dense filaments of actin

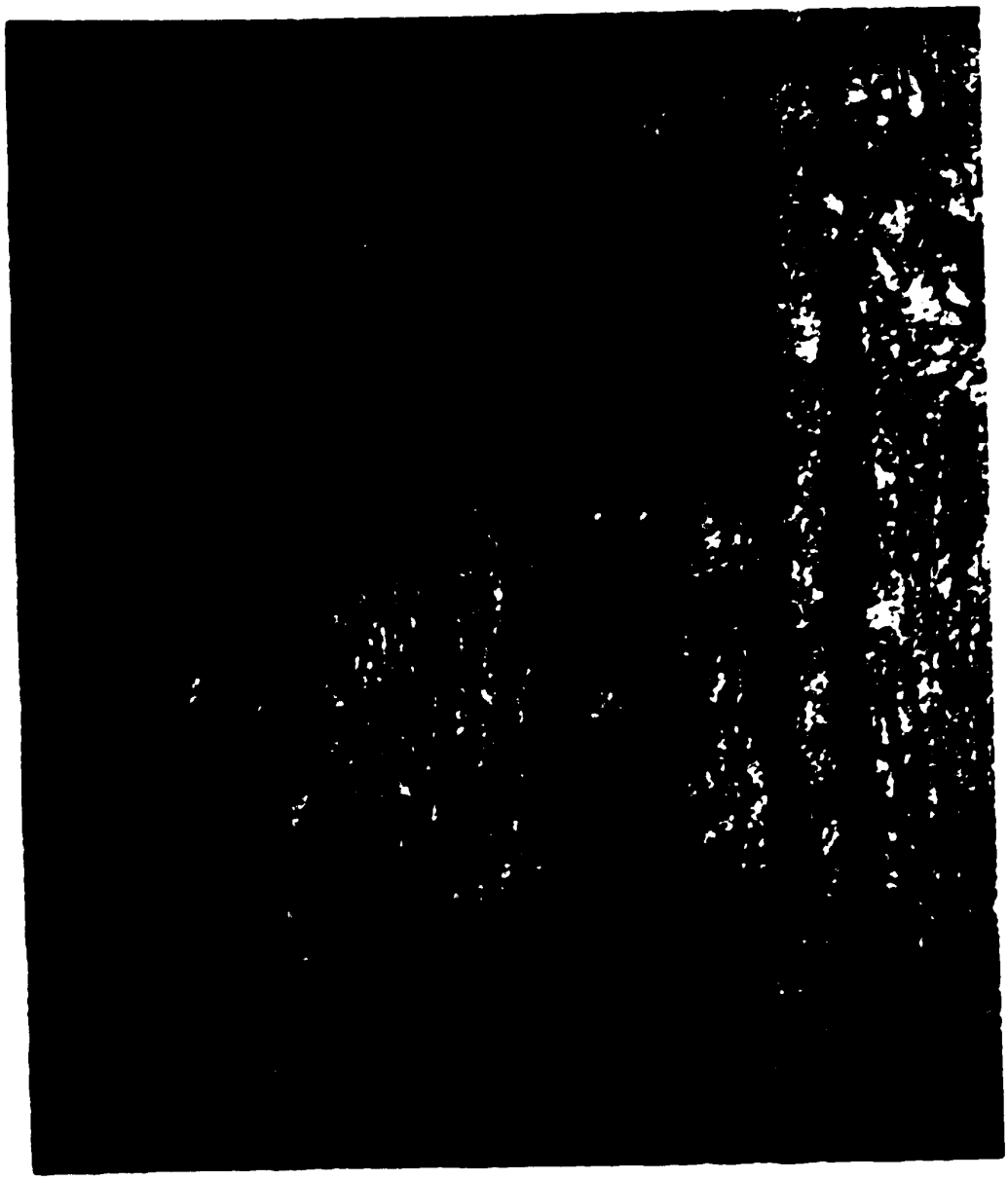


Plate B

Electron micrograph of a longitudinal section of smooth muscle (by Needham and Schoenberg, 1964).
←— thin filament ←←— thick filament

extending from either side of the Z-line to the border of the H-zone make up the I band and the dense region, the A band, consisting of the thick myosin filaments and the thin actin filaments extending from the I band (Figure 1).

During the various stages of muscle contraction it has been found that the A band remains at a constant length while the I band and the H zone in the middle of the A band vary. However, the myosin and actin filaments remain at a constant length during extension or contraction (Hanson and Huxley, 1955; Huxley and Niedergerke, 1954). These observations led to the sliding filament model of muscle contraction (Huxley and Hanson, 1954, 1960). A schematic representation of this model is shown in Figure 1. Huxley suggests that the process of muscle contraction occurs through the sliding movement of the actin filaments inward into the myosin filaments by the formation of cross-bridges between actin and myosin. Each bridge is part of a single myosin molecule. Chemical energy for this process comes from the splitting of ATP at the enzyme sites on the heads of the myosin molecules. Several reviews that cover all facets of the contraction process are given by Seifter and Gallop (1966); Bendall (1969); Huxley (1969); Young (1969); Davies (1965) and Gibbons (1968).

Location and Possible Function of Tropomyosin

There are two classes of muscle proteins known respectively as tropomyosin A and tropomyosin B. Tropomyosin A is generally referred to as paramyosin. It appears to be limited to a particular type of smooth muscle of invertebrates known as "catch" muscles. These muscles

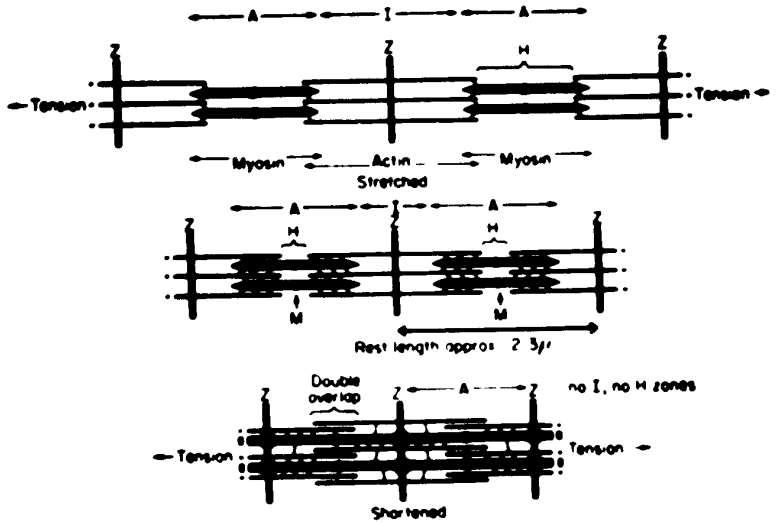


Fig. 1: Diagrammatic representation of the arrangement of filaments in striated muscle showing the behavior of the filaments in the I and A bands during changes in length of the myofibril according to the Huxley-Hanson sliding filament model (Davies, 1965).

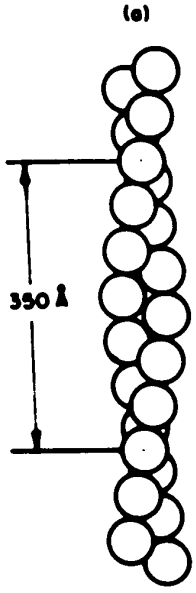


Fig. 2: A model of an actin filament. The model consists of spherical subunits (monomeric G-actin), 55 Å in diameter, arranged in two F-actin filaments twisted round each other forming an actin superhelix. The pitch of the helix lies between 350-400 Å (Hanson and Lowy, 1964).

differ greatly from the striated and smooth muscle of vertebrates and are characterized by their ability to resist stretch and to remain in a prolonged state of contraction with little expenditure of chemical energy. The content of paramyosin in these muscles is high and may constitute in certain muscles two-thirds of the fibrous proteins. An excellent discussion of all aspects of the paramyosin system is given by Gergely (1964).

Tropomyosin B, first discovered by Bailey (1948) is now generally referred to as tropomyosin and appears to be present in all vertebrate and invertebrate muscles. The two proteins paramyosin and tropomyosin are distinguishable by their solubility properties; paramyosin being insoluble in water and dilute salt solutions whereas tropomyosin is soluble. Tropomyosin is one of the three major structural proteins of the myofibril. Myosin, actin, and tropomyosin are found in the proportions of approximately 50-55% myosin, 20-25% actin, and 10-15% tropomyosin (Perry, 1965).

Tropomyosin crystals viewed in the electron microscope show a close resemblance to the Z-band of the myofibril and Huxley (1963) suggested that a part of tropomyosin in vertebrate striated muscle forms the filament lattice of the Z-line. Selective extraction techniques of the myofibril have also suggested that tropomyosin was located in the I-band (Perry and Corsi, 1958). The presence of tropomyosin in the I-substance has been further substantiated by Pepe (1966) who with the technique of antibody staining in fluorescent and electron microscopy has shown that antitropomyosin stains exclusively the I-band but not the Z-line.

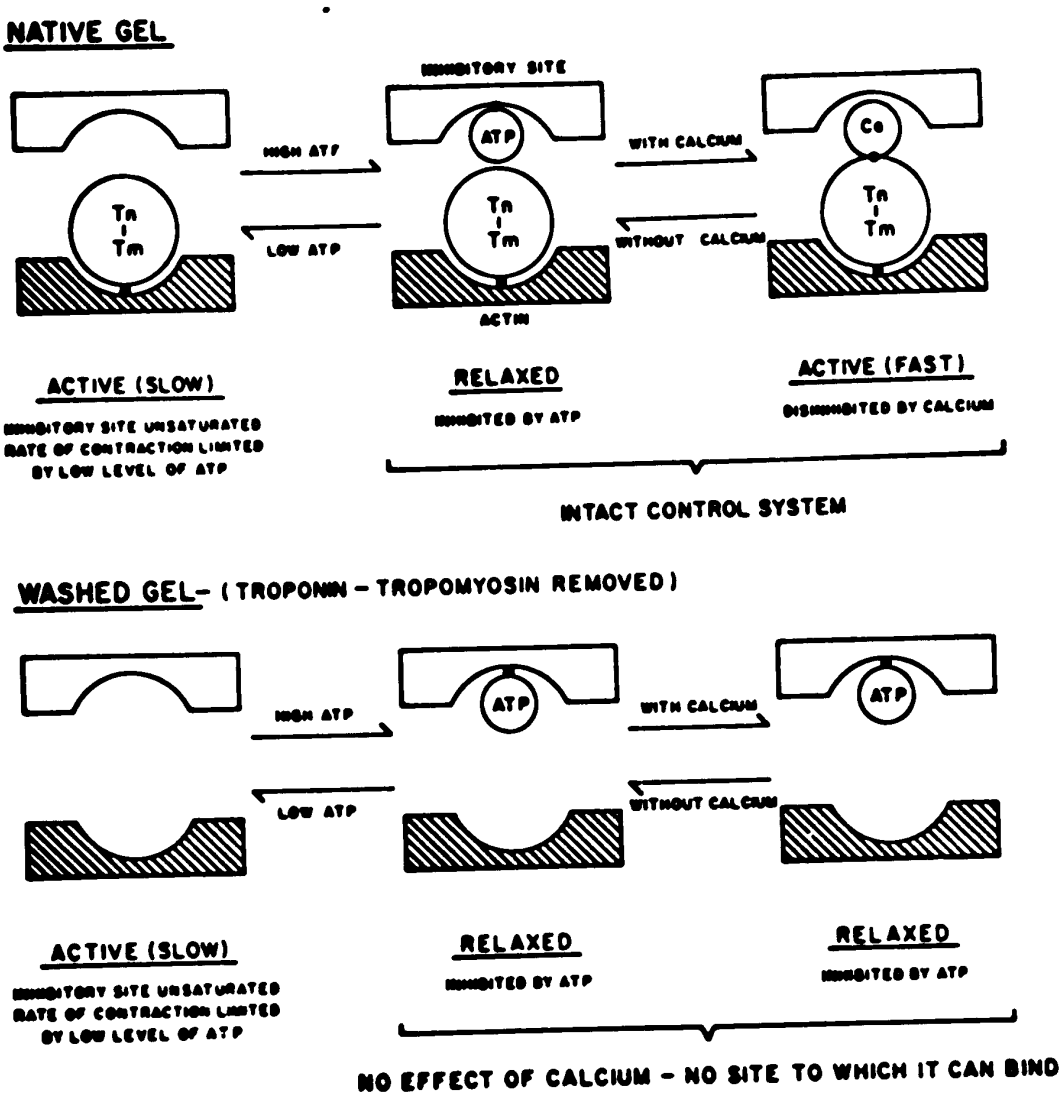


Fig. 3: A proposed mechanism for the control of muscular contraction by Stewart and Levy (1970). Tn-Tm, troponin-tropomyosin.

This protein has been shown to be a troponin-tropomyosin complex binding in a shoichiometric ratio of 1:1 by weight and often referred to as "native" tropomyosin or relaxing protein (Ebashi and Kodama, 1965 and 1966b; Hartshorne and Mueller, 1967; Ebashi et al., 1968; Yasui et al., 1968; Staprans and Watanabe, 1970). Neither component alone has any effect on actomyosin. This complex has been shown by several investigators to bind to F-actin (Ebashi and Kodama, 1965 and 1966a; Kominz and Maruyama, 1967).

Stewart and Levy (1970) have proposed a mechanism for the control of muscular contraction involving the tropomyosin-troponin complex (Figure 3).

The alignment of the components indicated in Figure 3 has considerable supporting evidence. For example, it is most probable that tropomyosin is bound to actin (Maruyama, 1964; Martonosi, 1962; Drabikowski and Gergeley, 1962; Laki et al., 1962; Drabikowski and Nowak, 1968 and Pragay and Gergely, 1968), that troponin binds to tropomyosin and tropomyosin-actin complex but not to F-actin (Ebashi and Kodama, 1966b and 1966a; Endo et al., 1966 Drabikowski and Nonomura, 1968), that neither troponin, tropomyosin nor the complex of the two proteins bind to myosin (Kominz and Maruyama, 1967; Ebashi and Kodama, 1966b), that calcium binds to troponin (Ebashi et al., 1967 and 1968) and that ATP inhibits contraction by binding to an inhibitory site that is distinct from the site of ATP hydrolysis (Levy and Ryan, 1965 and 1967). Figure 3 shows the existence of an inhibitory site, where the binding of ATP inhibits hydrolysis of ATP and contraction, and a control site comprised of the

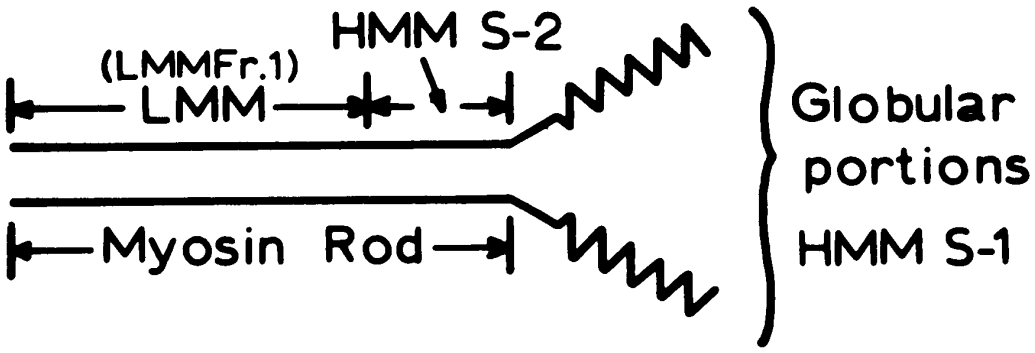


Fig. 4: Schematic representation of the myosin molecule and some of its proteolytic fragments.
LMM = light meromyosin
LMMFr.1 = alcohol fractionation of LMM (Szent-Györgyi et al.,1960).
HMM S-1 = heavy meromyosin sub-fragment 1
HMM S-2 = heavy meromyosin sub-fragment 2

troponin-tropomyosin complex. Whether this inhibitory site is on myosin, actin, or both remains unknown. Stewart and Levy (1970) suggest that in the presence of calcium, which binds to troponin, the inhibitory site is blocked to the access of MgATP, and the calcium-troponin-tropomyosin complex allows actin and myosin to come into proper alignment for ATP hydrolysis and the formation of new cross-bridges between actin and myosin. In the absence of calcium ions the inhibitory site is accessible to MgATP, which prevents contraction by moving the actin and myosin out of proper alignment so that new cross-bridges cannot be formed and relaxation occurs. If the troponin-tropomyosin complex is removed, the inhibitory site is still accessible to ATP even in the presence of calcium ions and relaxation occurs.

Since troponin has no direct effect on actin or myosin, it has been suggested by Ebashi et al. (1967) that calcium ions could possibly exert some conformation change in troponin which in turn may affect the actin-myosin interaction. Troponin has been shown to be distributed along the entire length of the thin filament at a periodicity of about 400 Å (Ohtsuki et al., 1967; Endo et al., 1966). Therefore an explanation of how troponin molecules exert their influence on those F-actin monomers which are located at some distance from them must await further investigation. Hanson and Lowy (1964a) suggested that the capacity of tropomyosin to bind to actin could be a means of control of the contractile function of actin. It seems probable that the effect of Ca ions is mediated via troponin through tropomyosin to actin. However the precise role of tropomyosin still remains to be elucidated.

TABLE I

OPTICAL PROPERTIES OF TROPOMYOSINS

Protein	b_o (deg.)	m'_{232} (deg.)
Vertebrate Tropomyosins[a]		
Sheep skeletal	-610	-14,930
OX skeletal	-650	-15,800
OX heart	-630	-16,000
Fowl gizzard	-650	-15,500
Rabbit skeletal	-615	-15,800
Beef heart[b]	-601	-14,870
Invertebrate Tropomyosins[c]		
Crayfish	-665	-16,100
Blowfly	-655	
Oyster	-640	
Abalone	-660	-16,400

[a] Woods (1969).

[b] McCubbin *et al.* (1967).

[c] Woods and Pont (1971).

Physicochemical Properties

Tropomyosin is a very asymmetric rodlike molecule in shape, with a length of 450 Å and a diameter of 20 Å (Holtzer et al., 1965; Woods, 1967; Caspar et al., 1969). A double-stranded coiled-coil has been proposed for tropomyosin, paramyosin and the rod-like portion of myosin. Tropomyosin shares with paramyosin and LMMFr.1 (Figure 4) the distinction of being more α-helical than any other known protein. All tropomyosins, vertebrate and invertebrate are almost completely α-helical as deduced from the constant b_o of the Moffit equation and the magnitude of the trough of the cotton effect at 232 nm. (Table 1). A value of near -600° for the constant b_o characterizes a fully-coiled, right-handed α-helix for synthetic polypeptides. A value of m'_{232} near -16,000° is suggestive of a 100% α-helix.

Amino acid compositions of various vertebrate and invertebrate tropomyosins are compared in Seifter and Gallop (1966); Woods (1969) and Woods and Pont (1971). Lowey (1965) has compared and contrasted certain features of the proteins paramyosin, tropomyosin and LMMFr.1 in relation to their compositions. These compositions show a very low proline content which is consistent with the extremely high content of α-helix in these proteins. The content of charged amino acid residues is remarkably high resulting in a high polyampholytic nature and charge density. Thus in tropomyosin, paramyosin and LMMFr.1, the total content of acidic and basic residues is 46%, 38% and 40% respectively.

Tropomyosin has the largest "zwitterion" density of any known protein. The residues of small size (glycine, alanine and serine) constitute only about 16% of the total residues in tropomyosin and 15% in LMMFr.1, in striking contrast to their high incidence in other structural proteins (eg. silk fibroin may have as much as 90% and collagen 50%). These three proteins are very similar with regard to hydrophobic residues (alanine, valine, proline, leucine, isoleucine, methionine, phenylalanine, tyrosine, and tryptophan) with contents of 35%, 35%, and 33% for paramyosin, tropomyosin, and LMMFr.1 respectively. Lowey (1965) found no significant differences among these proteins when comparing the reactivity of tyrosine residues and the stability of these proteins to conditions of extreme acidity and alkalinity. The tyrosine residues of all these proteins titrate in the normal pH range and none are "masked" or "hidden" which is consistent with the coiled-coil structure. These fibrous proteins were found to remain fully helical over a wide range of pH, (pH 2-9 for tropomyosin and 2-11 for LMMFr.1 and paramyosin) and were exceptionally stable in acid. Tropomyosin differed from the other α-helical proteins in the alkaline region in being less resistent to alkali. This instability may be related to tropomyosin's higher negative charge at any given pH. The unfolding of these proteins under basic conditions occurred as the negative charge increased and seemed to be an electrostatic phenomenon since the addition of 1 M KCl at pH 12.0 increased the helical content of tropomyosin by as much as 30%. The similarity of chemical properties of these proteins would suggest that they share a common structure.

Noelken and Holtzer (1964) studied the denaturation of tropomyosin in solutions of guanidine hydrochloride. At pH 7.4 the denaturation of tropomyosin (helix-coil transition) was complete at 3.5 M as determined by b_o values ($b_o \sim 0$), whereas at pH 2.0 appreciable helix remains until a guanidine hydrochloride concentration of 6.0 M is reached. This increased stability at pH 2.0 of the coiled-coil has been explained on the basis of carboxyl-carboxyl hydrogen bonds. This denaturation of tropomyosin in strong denaturing solvents was found to be 80% reversible.

Molecular weight determinations of protein and subunits

Tsao et al. (1951) and Kay and Bailey (1960) studying rabbit skeletal tropomyosin and McCubbin et al. (1967) and McCubbin and Kay (1969) studying cardiac tropomyosin have shown that tropomyosin is aggregated at neutral pH and low ionic strengths and concluded that dissociation to the monomer occurred on raising the ionic strength above 0.6. Woods (1967) has shown that tropomyosin does not dissociate completely to the monomer at high ionic strengths and concluded that tropomyosin consists of an associating-dissociating system in aqueous buffer at neutral pH and at all salt concentrations. Despite this disagreement similar molecular weight values have been obtained. The monomeric molecular weight obtained at low concentrations by low speed and high speed sedimentation equilibrium and osmotic pressure measurements was found to be 68,000 for rabbit skeletal tropomyosin (Woods, 1967). High speed sedimentation equilibrium experiments at high ionic strength have indicated that the minimum molecular weight for various vertebrate tropomyosins to be close to the value found for rabbit skeletal (Woods, 1969) and for invertebrate tropomyosins (Woods and Pont, 1971) to be in

TABLE II

MOLECULAR WEIGHTS OF RABBIT TROPOMYOSINS IN 8M UREA[a]

Preparation	Absence of -SH reagent	SH groups masked by reaction with NEM	0.1M mercaptoethanol added
1	84,700	37,900	34,800
2	67,200	51,800	33,500
3	82,000	70,400	34,100
4		35,500	
5		73,000	
6	80,000	35,000	
7		36,000	
8	84,000	33,300	
9[b]		34,000	

[a] Woods (1966).

[b] 0.01M mercaptoethanol added to extracting solution.

this region. McCubbin et al. (1967) found the molecular weight of monomeric beef cardiac tropomyosin to be about 70,000 by osmometry. Woods (1965) showed that tropomyosin could be dissociated into subunits of molecular weight one-half that of the monomeric value in the presence of 8M urea and 0.1 M β-mercaptoethanol. This result suggested the presence of a disulphide bond or bonds between the two chains of tropomyosin since urea alone does not decrease the molecular weight to this value (34,000). Woods (1966) found that tropomyosin in many preparations could be dissociated into subunits in the presence of 8M urea without the addition of reducing reagents. The molecular weights were determined after dissociation by 8M urea in the presence of N-ethyl-maleimide (NEM) to mask -SH groups and prevent formation of S-S bonds. The results for different preparations of skeletal tropomyosin from different rabbits are shown in Table II. When the -SH groups are masked most of the preparations gave molecular weights in the vicinity of 35,000 close to the value obtained in the presence of reducing agents. Preparations which gave higher molecular weights in 8M urea after reaction with NEM all gave values of 35,000 in the presence of 0.1 M mercaptoethanol and 8M urea. It was concluded from these results that disulfide bridges are present in some preparations and not in others, suggesting the possibility that the S-S bonds are formed during isolation and that the chains of tropomyosin in muscle are not covalently linked. This may explain many of the inconsistencies in earlier molecular weight determinations. A summary of subunit molecular weight determinations are shown in Table III. These results clearly indicate tropomyosins from various sources possess a common subunit molecular

TABLE III

SUBUNIT MOLECULAR WEIGHT OF TROPOMYOSIN

Source of Protein	Denaturing Media	Method	M.W.
Rabbit Skeletal			
S-sulphotropomyosin S-carboxymethyl tropomyosin	8M urea	Sed. Equil.	30,000-35,000[a]
-SH-tropomyosin	8M urea -0.1M β-mercaptoethanol	Sed. Equil.	34,000[b]
	8M urea	SDS-gel electrophoresis	36,000[c]
Sheep Skeletal Sheep Heart OX Skeletal OX Heart Fowl Gizzard	8M urea -0.1M β-mercaptoethanol and 8M urea 0.02% N-ethylmaleimide	Sed. Equil.	31,300-36,000[d]
Beef Heart	6.67M urea -0.1M β-mercaptoethanol	Osmometry	37,000[e]
Abalone	7M G·HCl -0.1M β-mercaptoethanol	Sed. Equil.	33,500[f]

[a] Woods (1966).

[b] Woods (1965).

[c] Weber and Osborn (1969).

[d] Woods (1969).

[e] McCubbin et al. (1967).

[f] Woods and Pont (1971).

weight of approximately 34,000. From this work it seems reasonable to conclude that the two polypeptide chains of all tropomyosins are identical in mass.

In 8M urea on polyacrylamide or starch gel electrophoresis the two subunits appear to migrate as a single band (Woods, 1967 and 1966 and Yasui et al., 1968). Mixtures of various tropomyosins also moved as a single band on gel electrophoresis (Woods, 1969). These results also provide a preliminary indication that the subunits are at least very similar if not identical. Carsten (1968) observed approximately 35 peptides from tryptic digests of skeletal tropomyosins against 110 basic residues per 70,000 g. This would be indicative of highly similar peptide chains or incomplete tryptic digestion.

Evidence for Non-identical Chains

Disc gel electrophoresis experiments of Bodwell (1967) on reduced and alkylated protein indicated two dissimilar subunits with distinct amino acid compositions. The protein had been exposed to dilute alkali for 12 hr and as pointed out by Bodwell in a footnote to the paper, the subunits could conceivably be two major fragments from alkali cleavage. Using very low concentrations of tropomyosin SDS-gel electrophoresis did not give a single band but indicated a doublet that was hardly separated (Weber and Osborn, 1969). The highly similar protein paramyosin yielded only a single band.

The N-terminal studies on tropomyosin are summarized in Table IV. Saad and Kominz (1961) suggested 1 mole of glutamic acid or glutamine per 60,000 g. However, Jen et al. (1965) were unable to confirm this result using similar conditions.

TABLE IV

N-TERMINAL STUDIES

Reference	Method	Result
Bailey (1951)	FDNB (Sanger's Method)	-
Chibnall and Spahr (1958)	Methoxycarbonylchloride	-
Jen and Tsao (1957)	FDNB	-
Saad and Kominz (1961)	FDNB (6M urea, 50°C 3 - 5 hrs)	1 mole of DNP - glutamic acid per 60,000 g.
Jen *et al*. (1965)	FDNB (6M urea, 50°C 4 hrs).	-
	Phenylisothiohydantoin	-

Alving *et al*. (1966) identified an N-acetylated peptide after pronase digestion of tropomyosin. This tripeptide had the sequence N-Ac-(Met,Asp)-Ala. This blocked N-terminal would explain the failure of previous workers to identify a N-terminal residue. The number of moles of N-acetylated N-terminal per monomer (70,000 g) has not been determined and the possibility remains that there is a glutamic or glutamine N-terminal which could be in a pyrrolidone carboxylic acid form through cyclization.

Kominz *et al*. (1957) and Alving *et al*. (1966) have reported that tropomyosin consists of two polypeptide chains terminating in the free carboxyl groups of serine and isoleucine. These results were obtained by carboxypeptidase digestion of tropomyosin. Kominz *et al*. (1957) reported one equivalent each of serine and isoleucine (60,000 g) were liberated within 5 min of enzymic hydrolysis. The amino acids liberated were quantitated by ion-exchange chromatography. Locker (1954) identified the C-terminal residue as isoleucine using two methods, carboxypeptidase digestion and hydrazinolysis. From the carboxypeptidase results Locker suggested the C-terminal sequence to be Ala-Ile-Met-Thr-Ser-Ile·COOH for the first six residues.

The results of N-terminal and C-terminal studies have been indecisive in proving the existence of two different polypeptide chains.

The Coiled-coil Structure

The X-ray diagrams of the keratin-myosin-epidermis-fibrinogen group of proteins exhibit an α-diffraction pattern (Astbury et al., 1948). Crick (1952) and Pauling and Corey (1953) suggested that the chain conformation of the k-m-e-f class of α-proteins could be based on a coiled-coil arrangement of α-helices; the α-helix being that proposed by Pauling et al. (1951). This coiled-coil proposal was advanced to account for the difference in meridonal spacings on the X-ray diffraction patterns of synthetic polypeptides in the α-form and the α-proteins. The polypeptides have a meridonal reflection at 5.4 Å, corresponding to the pitch of the α-helix; the α-proteins a meridonal 5.15 Å reflection. The two simplest models proposed were the two-stranded and three-stranded coiled-coils (Crick, 1953); however experimental evidence was not available to distinguish between these two cases. Basically Crick's model consists of inclining the axes of the α-helices to each other, deforming them slightly along their entire length to maintain contact between them. This results in the two (three) helices slowly winding around to form the coiled-coil. The proteins most likely to contain this coiled-coil structure are the three fibrous proteins tropomyosin, paramyosin, and the rod portion of the myosin molecule. Both physical studies and X-ray evidence (Fraser et al., 1965; Miller, 1965; Caspar et al., 1969; Cohen and Holmes, 1963; Szent-Gyorgyi et al., 1960 and Lowey and Cohen, 1962) strongly support the two-stranded coiled-coil for these three fibrous proteins (Woods, 1969a; McCubbin and Kay, 1968; Lowey et al., 1963; Cohen and Szent-Györgyi, 1957; Olander et al., 1967; Lowey et al, 1969;

TABLE V

PHYSICOCHEMICAL PARAMETERS OF HIGHLY α-HELICAL PROTEINS

Parameters	LMM[a]	HMMS-2[a]	Myosin Rod[a]	Paramyosin	Tropomyosin
Molecular Weight	140,000	62,000	220,000	220,000	70,000
Rotatory Dispersion Constant, b_o	-630[d]	-610	-660	-600	-620
% α-Helix[b]	95	92	100	91	94
Approximate Length (Å)	770	475	1,360	1,330	450
Number of Chains[c]	2.4	1.7	2.1	2.2	2.0

[a] See Figure 4.

[b] A value of -660 for constant b_o used to represent 100% α-helix.

[c] Determined on a basis of mass per unit length.

[d] LMMFr.1 has a value of -660 for b_o (Cohen and Szent-Györgyi, 1957).

Mueller, 1964; Mueller et al., 1964 and Lowey, 1965). It seems the only gross differences in molecular form between these molecules are their overall lengths (Table V). All three are rods of about 20 Å in diameter (Lowey et al., 1963; Rice, 1964; Zobel and Carlson, 1963; Huxley, 1963 and Lowey and Cohen, 1962).

X-ray diffraction studies on paramyosin by Elliott et al. (1968) have indicated that this simple coiled-coil concept might need modification. An alternative system known as the segmented rope, originally suggested by Fraser and MacRae (1961a,b) has recently been proposed by Parry (1970) as a conformation for α-fibrous proteins. This model consists of short lengths of straight α-helix inclined to one another, approximating the coiled-coil over a short range, and then an abrupt bend in the axes of the α-helices occurring every 20 - 30 Å. Caspar et al. (1969) have reported a further degree of coiling in crystals of tropomyosin so that they have described tropomyosin in muscle as a "coiled-coiled-coil". The term coiled-coil will be used loosely in what follows to denote the general structure of the α-fibrous proteins.

The biological significance of the coiled-coil is that this structure offers rigidity to highly charged polypeptides in an aqueous environment. It has been shown that highly charged synthetic polypeptides have little α-helix under conditions of neutral pH where the two-stranded coiled-coil is stable (Blout and Idelson, 1958). Probably the main force accounting for stability of the coiled-coil is hydrophobic bonding between the two chains rather than possible electrostatic interactions. This is evident from the unusual stability of

coiled-coils in acid and alkaline media despite the high percentage of charge residues present (Szent-Gyorgyi et al., 1960; Lowey, 1965 and Riddliford and Scheraga, 1962). This insensitivity to pH and ionic strength (Lowey, 1965) and the fact that the coiled-coils are more stable at pH 2.0 than neutral pH (Noelken and Holtzer, 1964) implies that electrostatic forces are not a major contributing factor to the stabilization. Thus the sequence specificity that might exist is one requiring non-polar residues between the two α-helices of the coiled-coil. The polar residues would be located at the "surface" of the coiled-coil to give the molecule its solubility properties.

The first objective in this study has been to provide more definitive chemical evidence on the question of identity or non-identity of the polypeptide chains of rabbit skeletal tropomyosin. To answer this question we have undertaken the elucidation of the number of unique sequences about the histidyl, methionyl, and cysteinyl residues. The second objective has been to determine the sequence specificity required to maintain the "coiled-coil" structure in this protein.

Since tropomyosin is the smallest and simplest of the "k-m-e-f coiled-coil" group of proteins, it was the logical choice for this study. Rabbit skeletal muscle was chosen as the tissue source since this tropomyosin has been the most fully characterized by physical and chemical methods.

It seems most probable that any information with regard to the coiled-coil conformation that applies to tropomyosin will also apply to the very similar proteins paramyosin and the rod-like portion of the myosin molecule. This concept of stabilization of the α-helix in

aqueous solution by side-chain interactions characterizes the globular proteins as well as the α-helical coiled-coils. Due to the uniformity of structure, the relationship between primary sequence and the stabilization of the coiled-coil may be more rewarding than it has been for the α-helices of globular proteins. Investigations on these fibrous proteins may well clarify the general problem of understanding the stability of secondary and tertiary structure of proteins in solution.

CHAPTER II

GENERAL MATERIALS AND METHODS

1. MATERIALS

A. General Chemicals and Solvents

Sephadex G-25 (20-80μ), G-50 (20-80μ) and QAE-Sephadex A-50 (40-120μ) and Blue Dextran 2000 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Cellex-D-(DEAE-Cellulose), AG1-X2 (Dowex 1-X2) (200-400 mesh, actual wet mesh size 80-200) were obtained from Bio-Rad Laboratories, Richmond, California, U.S.A. Technicon Chromo-Beads Type P (peptides) equivalent to Dowex 50-X4 was obtained from Technicon Chemical Company, Inc., Chauncey, New York, U.S.A. N-ethylmorpholine (practical) and α-picoline (practical) were purchased from Eastman Organic Chemicals, Rochester, New York, U.S.A. Both reagents were redistilled prior to use; N-ethylmorpholine (b.p. 134.0°C) and α-picoline (b.p. 125°C). Pyridine, spectrograde, and naphthalene were also products of Eastman Organic Chemicals. The scintillators, PPO (2,5-diphenyloxazole), POPOP (p-Bis[2-(5-phenyloxalzolyl)]-benzene), and the scintillation fluid, Aquasol were obtained from New England Nuclear, Boston, Massachusetts, U.S.A. Iodoacetic-1-^{14}C acid was obtained in 0.25 mc quantities from New England Nuclear. Iodoacetic acid was purchased from Mann Research Laboratories, Inc., New York, N. Y., U.S.A. and re-crystalized from petroleum ether. Ammonium sulphate (ultrapure), Tris (ultra-pure) and Urea (ultra-pure) were also products from Mann Research Laboratories. Dansyl amino acids were obtained from Calbiochem, Los Angeles, California, U.S.A. or were prepared by the

method described by Gray (1964). Dithiothreitol (Cleland's reagent) and DNS-Cl (5-dimethylamino-1-naphthalene sulfonyl chloride) B grade were also products of Calbiochem. All other chemicals in this study were reagent grade and were used without further purification.

B. Enzymes

α-Chymotrypsin (three times crystallized), pepsin and trypsin-TPCK were purchased from Worthington Biochemical Company, Freehold, New Jersey. Trypsin-TL (minimal chymotrypsin content) was obtained from Mann Research Laboratories, New York, N. Y. Thermolysin crystalline, B grade was purchased from Calbiochem, Los Angeles, California. An α-lytic protease preparation from Sorangium sp. was kindly donated by Dr. D. R. Whitaker.

2. METHODS

A. Preparation of Rabbit Skeletal Tropomyosin

SH-Tropomyosin was prepared from back and hind leg muscles of adult rabbits of the New Zealand White strain as described by Bailey (1948) in the presence of 0.5 mM dithiothreitol in all preparative solutions as recommended by Mueller (1966) and with an initial two precipitation steps to remove troponin as described by Yasui *et al.* (1968).

(1) Preparation of the Muscle Powder

Freshly excised rabbit muscle was minced, mixed with an equal volume of deionized water and homogenized for one minute in a Waring Blendor. One rabbit yields approximately 500 g of wet muscle. The juice was removed by centrifugation and discarded. After each of the following extractions (stirring the muscle fiber for 2-3 mins in each solvent), the liquor was expressed by being filtered through cheese cloth and squeezed out by hand:

- one extraction with an equal volume of 95% ethanol
- three extractions with four volumes of 1:1 ethanol (95%) water
- two extractions with four volumes of 95% ethanol
- two extractions with four volumes of ether.

These extractions remove all soluble proteins and lipid material. The fiber was then air dried at room temperature.

(2) Tropomyosin Extraction with 1M KCl

The muscle fiber was immersed in 1M KCl and 0.5 mM dithiothreitol (DTT) (700 ml per 100 g dry fiber), and the pH adjusted to pH 7.0 with 1N NaOH. The solution was incubated in an 18°C water bath for 16 hours. The supernatant was removed by filtering through cheese cloth and squeezing out the pulp by hand. The volume of supernatant was measured and the residue was re-extracted with this volume of 1M KCl and 0.5 mM DTT. The pH was adjusted to pH 7.0 with 1N NaOH. Re-extraction was allowed to proceed for 1-2 hours at 18°C. The supernatant was removed as above and combined with the first extraction.

(3) Precipitations at pH 4.6 for Troponin Removal

The combined extracts were acidified to exactly pH 4.6 with 1N HCl. The precipitate was collected by centrifugation and dissolved in a volume of 1M KCl - 0.5 mM DTT identical to the re-extraction volume. The supernatant containing troponin was discarded. As the precipitate dissolved the pH of the solution was maintained at pH 7.0 with 1N NaOH. Insoluble material was removed by centrifugation. The pH of the solution was again brought to pH 4.6, and the precipitate collected by centrifugation and redissolved as before in 1M KCl - 0.5 mM DTT. The dissolution of the tropomyosin precipitate was aided by breaking it up through gentle homogenization under hand pressure in a tissue grinder. The tropomyosin was then dissolved by gentle stirring of the precipitate for 3 hrs in the above solvent at 4°C.

(4) Alternating Isoelectric Point and
$(NH_4)_2SO_4$ Precipitations of Bailey (1948)

All isoelectric point precipitations were carried out corresponding to the point of maximal precipitation.

a. Isoelectric Point Precipitation

The supernatant was acidified with 1N HCl to approximately pH 4.3. The precipitate was collected by centrifugation and dissolved in 5 volumes of water containing 0.5 mM DTT (pH adjusted to pH 7.0).

b. Ammonium Sulfate Precipitation

The total volume of supernatant was determined and $(NH_4)_2SO_4$ added to bring the solution to 40% saturation at 0°C. The precipitate from the centrifugation was discarded. The supernatant volume was

measured and $(NH_4)_2SO_4$ added to give a 70% saturation at 0°C. The supernatant from centrifugation was discarded and the precipitate dissolved in water, 0.5 mM DTT. This solution was dialyzed overnight against water, 0.5 mM DTT with 3-4 changes of the dialysate.

c. Isoelectric Point Precipitation

The procedure is carried out as in 4(a) with the point of maximal precipitation near 4.8.

d. Ammonium Sulfate Precipitation

The procedure was repeated as in 4(b) except that the initial $(NH_4)_2SO_4$ concentration was at 45% saturation.

e. Isoelectric Point Precipitation

Repeat isoelectric point precipitation as outlined in 4(a) with the point of maximal precipitation near pH 5.3.

f. Ammonium Sulfate Precipitation

The final precipitate of tropomyosin was obtained by salting out to 70% saturation at 0°C. The precipitate was dissolved in 0.5 mM DTT solution and dialyzed against 0.5 mM DTT solution as before.

(5) Nucleic Acid Removal

The dialysate was made 0.2 M KCl and 0.05 M phosphate and adjusted to pH 7.0. This protein solution was passed through a DEAE-cellulose column equilibrated with 0.05 M phosphate - 0.5 mM DTT, pH 7.0. The protein was eluted with this buffer and dialyzed exhaustively against 0.01 M β-mercaptoethanol and freeze-dried. Absorbancy ratios $A_{280\ m\mu}:A_{260\ m\mu}$ were calculated to ensure that the preparation was free

of nucleic acid. The yield of tropomyosin was approximately 1.5 g per 500 g wet muscle. The protein was stored in the freeze-dried state under vacuo over a drying agent.

B. Preparation of ^{14}C-S-carboxymethylated Tropomyosin

In a typical experiment, 1 g of tropomyosin was reduced and carboxymethylated by modifications of the methods of Canfield and Anfinsen (1963) and Crestfield et al. (1963).

(1) Reduction

A 0.5 M Tris-HCl Buffer pH 8.6, 8 M urea and 0.2% EDTA was used for the reduction of tropomyosin. A slow stream of nitrogen was passed through the buffer in a 100 ml graduated cylinder for 20 mins. prior to the addition of the protein (10 mg/ml). The freeze-dried protein was dissolved in this solution (a few drops of octanoic acid added to prevent foaming) and the vessel flushed with nitrogen for a further 10 mins. A 150-fold excess of 2-mercaptoethanol to protein -SH groups was added. The calculation of protein -SH content was based on the assumption of 4 cysteine residues per 70,000 g of tropomyosin. The vessel was sealed and the reduction continued for 4 hrs at room temperature (22-25°C) under a nitrogen barrier.

(2) Isolation of the Reduced Protein

For precipitation of the reduced protein an organic solvent was used consisting of 95% ethanol, acetone and concentrated HCl (33:66:1 v/v). After incubation the solution was transferred under nitrogen to four centrifuge bottles containing a 30 fold excess of the organic solvent

HCl mixture (cooled in a dry ice-acetone bath) over protein solution (v/v). A fine precipitate of the reduced protein was produced and left to develop at -20°C overnight, then centrifuged. The supernatant was removed under nitrogen using a vacuum line. The precipitated protein was dissolved in 75 ml of an 8 M urea solution, pH 2.0 which had been flushed with nitrogen for 30 min.

(3) Carboxymethylation

In a typical experiment 25 ml of a 8 M urea solution of iodoacetic acid (specific activity 0.6 mc/mM) was added to the protein. The cold iodoacetic acid used for dilution was recrystallized from petroleum ether. A 20 fold excess of iodoacetic acid to protein -SH groups was used. The pH was then raised to 8.6 with 6N NH_4OH (constantly keeping the solution under nitrogen). After 30 min a 20 fold excess of 2-mercaptoethanol to iodoacetic acid was introduced and the pH maintained at 8.6 for a further 15 min under a nitrogen barrier. This assured the destruction of excess iodoacetic acid. The solution was dialyzed exhaustively against 10^{-3}N HCl and freeze-dried.

The incorporation of radioactivity into the protein (70,000 g) was determined in two ways. The protein was dissolved in 0.01 M KH_2PO_4 - KOH buffer, pH 7.0. The solution was allowed to stand overnight in the refrigerator and centrifuged to remove any undissolved material. The protein concentration was calculated by measuring the optical density at 277 mμ ($E^{1\%}_{1cm}$ of 3.3 Woods, 1967) and from the amino acid analysis using a leucine value of 95 residues per 100,000 g. The extent of alkylation was also determined by the quantity of S-carboxymethylcysteine on the amino acid analyzer relative to 95 residues of leucine per 100,000 g.

C. Digestion of Tropomyosin with Proteolytic Enzymes

Trypsin digests of SH-tropomyosin and ^{14}CM-tropomyosin were prepared by a five hour incubation at 37°C in 0.05 M N-Ethylmorpholine-acetate buffer, pH 8.0 (trypsin 0.2 mg/ml, protein 10 mg/ml). Digestion with pepsin of SH-tropomyosin and ^{14}CM-tropomyosin was in 5 per cent (v/v) formic acid for 24 hours at 37°C (pepsin 0.2 mg/ml, protein 8.0 mg/ml). Thermolysin digests of SH-tropomyosin were in 0.05 M N-Ethylmorpholine-acetate buffer, 5 mM $CaCl_2$, pH 8.0 (thermolysin 0.05 mg/ml, protein 5.0 mg/ml).

D. Peptide Purification Procedures

(1) High Voltage Paper Electrophoresis

High voltage electrophoretic separation of peptides at pH 3.5 and 1.8 was performed in a Gilson High Voltage Electrophorator Model D (Gilson Medical Electronics, Middleton, Wisconsin, U. S. A.) equipped with a large fiberglass tank, as described by Dreyer and Bynum (1967). The vertical strip high voltage electrophoresis apparatus similar to that described by Michl (1951) and Ryle *et al*. (1955) was used for the pH 6.5 system. The buffer systems and coolants at pH 6.5, 3.5, and pH 1.8 were similar to those described by Ambler (1963) except that the toluene was 8 per cent (v/v) with respect to pyridine for the pH 6.5 buffer system. The buffer systems were pyridine-acetic acid-water (100:3:900, by vol.), pH 6.5; pyridine-acetic acid-water (1:10:189, by vol.), pH 3.5; and formic acid-acetic acid-water (1:4:45, by vol.), pH 1.8. The peptides were purified by paper electrophoresis on Whatman No. 1 and 3 MM filter paper. Protein digests were applied as wide bands

TABLE VI

ELUTION SYSTEM FOR CHROMOBEAD TYPE P RESIN

Chamber	0.1M PyrAc* pH 2.75 (ml.)	0.2M PyrAc pH 3.1 (ml.)	2.0M PyrAc pH 5.1 (ml.)	2.0M PyrAc pH 6.5 (ml.)	Water (ml.)
1	90				
2	90				
3	90				
4		90			
5		90			
6		55	15		20
7		5	45		38
8			87		
9			65	22	

* The composition of all buffers are given in the Technicon Manual T-67-101, p. 25, except the pH 2.75 buffer, which has half the pyridine concentration of the pH 3.1 buffer but the same acetic acid concentration.

to the filter paper at a loading of about 1 mg per cm for Whatman 3MM paper and 0.3 mg per cm for Whatman No. 1 paper. For the separation of less complex peptide mixtures the sample loading was 0.02 μmoles of peptide per cm on Whatman No. 1 paper. Samples were dried in a stream of warm air during application. The papers were wet with the appropriate buffers, the bands sharpened and electrophoresis was carried out at 60 v per cm for the appropriate time as stated with the results. Peptides were located with the cadmium-ninhydrin reagent of Heilmann et al. (1957) and the Pauly reagent for histidine (Dent, 1947). When radioactive peptides were purified by paper electrophoresis, they were located by staining with cadmium-ninhydrin reagent and also for radioactivity by scanning electrophoresis side strips after staining in a Nuclear Chicago Actigraph III Strip scanner with 4π geometry.

(2) Ion-Exchange Chromatography

a. Separation of Peptides on Chromobead Type P

The cation exchange resin, Chromobead Type P (Dowex 50-X4), was used in the 0.6 cm x 110 cm column with volalite pyridine-acetate buffers as described in the Technicon Instruction Manual T-67-101 for peptide analysis. The resin equilibrated with starting buffer 0.1 M pyridine-acetate buffer, pH 2.75, was packed into the column and the column was equilibrated overnight. The appropriate peptide fractions or protein digests were dissolved in 1 ml of equilibration buffer and the solution was applied to the column. The column was connected to a 9-chambered autograd and the elution of the peptides at 38°C was performed using the buffer systems as indicated in Table VI at a flow rate of 25 ml

TABLE VII

VOLUME OF DEVELOPERS FOR DOWEX 1 CHROMATOGRAPHY

Developer*	Volume (ml.)
Buffer pH 9.4	40
Buffer pH 9.4	120
Buffer pH 6.5	160
Acetic acid 0.5N	240
Acetic acid 2N	400

* The composition of all developers are given in Schroeder (1967).

per hour. The flow rate was maintained by the use of a Beckman High Pressure constant volume pump. When the autograd was almost empty 2.0 M pyridine-acetate buffer, pH 6.5 was added to the last chamber of the autograd and the elution continued for 5 hours. Fractions of 3.0 ml were collected. After elution with the pH 6.5 buffer the column was re-equilibrated with starting buffer overnight before the next sample was added to the column. The 0.6 x 110 cm column gave good resolution of up to 7 μmole of protein digests.

b. Separation of Peptides on Dowex 1

The general procedure as described by Schroeder (1967) for the separation of peptides on Dowex 1 with volatile developers was used. The anion exchange resin Agl-X2 (200-400 mesh), was packed into a 0.9 cm x 169 cm column by the recommended procedure at 38°C. The sample was dissolved in 1-2 ml of pH 9.4 starting buffer and the pH raised to about 10.5 with sodium hydroxide. The gradient was produced with a 135 ml constant volume mixer and with a series of solvents successively introduced into the changing solution in the mixer. The volumes of each solvent used in the gradient are shown in Table VII. The changes of the developers were made manually at the appropriate time depending on the flow rate used for elution. Flow rates of 30 ml and 40 ml per hour were used and maintained by a Beckman constant volume pump. Fractions of 3.0 ml were collected. On completion of the chromatogram, the column was re-equilibrated with pH 9.4 starting buffer to be made ready for the next column separation.

c. Location of Peptides

The fractionations on the Dowex 50 and Dowex 1 ion-exchange columns were monitored using the Technicon autoanalyzer system of ninhydrin analyses before and after alkaline hydrolysis and where appropriate, by radioactivity counting.

i. Automated Ninhydrin Analysis

The general procedure was as outlined in the Technicon Instruction Manual T-67-101 for peptide analysis. The analytical system was modified as described by Welinder and Smillie (in press) to consume only 1.8 ml per hour of the column effluent for ninhydrin analyses before and after alkaline hydrolysis. This means that 7% of sample applied to Dowex 50 columns and 4.5-6% to Dowex 1 columns was consumed by the peptide analytical system.

ii. Radioactivity counting

For routine determinations of radioactive elution profiles, 20 µl aliquots of the effluent fractions were transferred to a scintillation vial, 10 ml of scintillation fluid added and the samples were counted in a Beckman LS-200B liquid-scintillation counter. The scintillation fluids used were either Bray's (1960) solution without ethylene glycol, or Aquasol from New England Nuclear.

iii. High Voltage Paper Electrophoresis

Aliquots (approximately 0.01 µmoles of peptide) from each effluent fraction shown to contain peptides from the radioactive and automated ninhydrin elution profiles were spotted on Whatman No. 1 filter paper for high voltage electrophoresis at pH 6.5. Electrophoresis at

pH 1.8 was carried out on the neutral peptides from the pH 6.5 electropherogram. The paper electrophoresis indicated the purity or complexity of the peaks shown on elution profiles of the column separations. It also enabled the selective pooling of peptide fractions and suggested possible methods of further purification. The peptides were located on the electropherograms as previously indicated.

(3) Paper Chromatography

Descending paper chromatography of peptides was performed on Whatman No. 4 paper at room temperature. The sample loads were about 0.02 μmoles of peptide per cm. After the sample was applied to the paper and dried with a stream of warm air, the chromatogram was equilibrated with the vapour of the developer that was to be used. Paper chromatography was used only as a final purification step after high voltage electrophoresis was unsuccessful. Conditions of chromatography varied according to the type of separation required and are therefore recorded with the results. After the peptides were located on side strips by the usual staining procedures the peptide band was cut out from the paper chromatogram and sewn on Whatman No. 1 paper for high voltage electrophoresis at pH 1.8. The pH 1.8 electrophoresis concentrated the peptides from paper chromatography and was necessary to prevent poor amino acid analyses which were obtained on elution of peptides from paper chromatograms (Ambler, 1963).

TABLE VIII

AMINO ACID ANALYSIS FOR HOMOSERINE

Analysis Conditions:	Acidic and Neutral Amino Acids	Basic Amino Acids
Resin nomenclature	Beckman UR30	Beckman PA35
Column dimensions	0.9 x 69 cm	0.9 x 23 cm
Resin height	0.9 x 56 cm	0.9 x 10 cm
Column temp.	52°C	52°C
Buffer (sodium citrate)	1st pH 3.00 (50 min.) 2nd pH 3.44 (40 min.) 3rd pH 4.35 (90 min.)	4th pH 5.25 (100 min.)
Flow rate		
Buffer	70 ml/hr	70 ml/hr
Ninhydrin	35 ml/hr	35 ml/hr
Sodium Citrate Buffer		
1st Buffer	0.2 N Na-citrate pH 3.00	
2nd Buffer	0.2 N Na-citrate pH 3.44	
3rd Buffer	0.2 N Na-citrate pH 4.35	
4th Buffer	0.35N Na-citrate pH 5.25	

E. Further Digestion of Peptides with Proteolytic Enzymes

Tryptic and α-lytic protease digests of peptides were carried out in 0.05 M N-Ethylmorpholine-acetic acid buffer, pH 8.0 at 37°C. The final concentration of peptide was 0.20 to 0.50 μmole per ml. The peptide-enzyme molar ratio and the digestion time are dependent on the extent of digestion desired and are reported with the results.

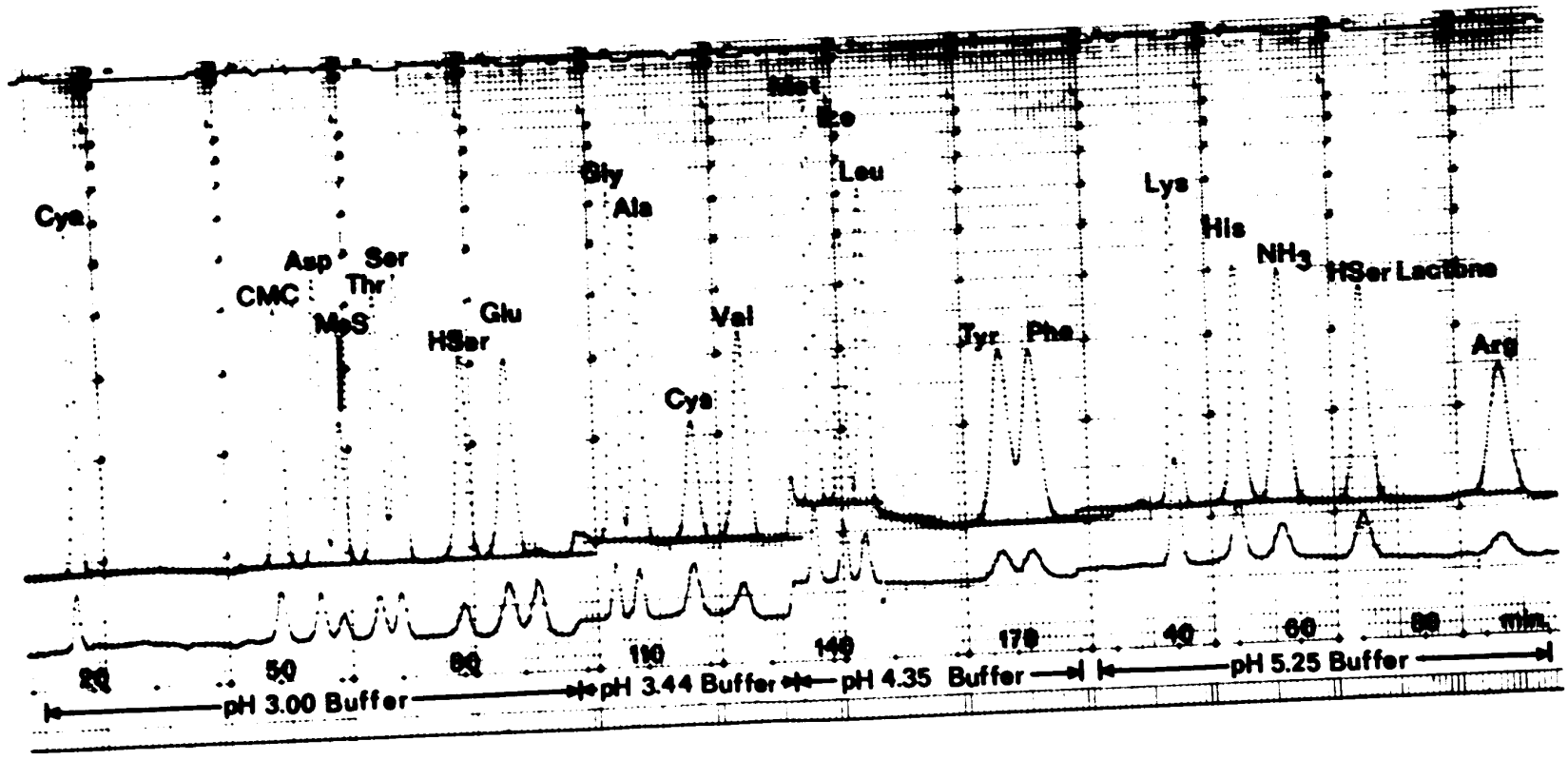
F. Amino Acid Analysis

(1) Peptides

Quantitative determination of the amino acid composition of peptides was made on the Beckman Spinco Model 120C automatic amino acid analyzer with expanded range card. The sample of peptide (about 0.03 to 0.05 μmole) was hydrolyzed in 0.25 ml constant boiling HCl (5.7N) for 16 to 24 hours at 110°C in evacuated sealed tubes (10 mm x 75 mm). The hydrochloric acid was then removed under vacuo in a vacuum dessicator at room temperature, and analyzed in the amino acid analyzer. Since recoveries of S-carboxymethylcysteine were often low in peptides eluted from paper electropherograms, the yield of this derivative was also calculated from measurement of the ^{14}C-content of the peptide. An aliquot of the radioactive peptides on dilution with pH 2.2 starting buffer for amino acid analysis was removed and transferred to a scintillation vial and the radioactivity was counted as previously described. Knowing the specific activity of iodoacetic-1-^{14}C acid (c.p.m. per μmole) and from the measurement of radioactivity the amount of S-carboxymethylcysteine on the analyzer column was

FIGURE 5

Resolution of Amino Acids on the Automatic Analyzer using the Homoserine System



calculated. The area constant used for S-carboxymethylcysteine was 0.905 times the value of the constant for aspartic acid. The area constant for aspartic acid was used for methionine sulphone and cysteic acid. Hydrolysates of homoserine peptides were evaporated to dryness, dissolved in 0.5 ml of electrophoresis buffer (pH 6.5 pyridine-acetate) and incubated for 1 hour at 110°C in sealed tubes. The samples are taken to dryness and not dissolved in the pH 2.2 starting buffer until immediately before application to the ion-exchange columns. This procedure converts homoserine lactone to homoserine (Ambler, 1965). The area constant used for homoserine was the average of that of serine and threonine. Amino acid analysis by the accelerated automatic system does not adequately resolve homoserine and glutamic acid. The procedure used is shown in Table VIII and the separation obtained in Figure 5. This buffer system not only resolves the homoserine and glutamic acid but maintains an excellent resolution between all other amino acids shown.

(2) Protein

The amino acid composition of proteins was determined on the Beckmann Spinco Model 120B amino acid analyzer with the normal range card. The analysis procedure is the same as described in Table VIII and shown in Figure 5. This system requires 0.05 to 0.10 μmoles of each amino acid for an adequate peak size. Protein samples containing about 6 mg were transferred into Pyrex glass tubes (15 mm x 150 mm) and hydrolyzed in vacuo at 110°C for 22 and 70 hours with 3 ml of constant boiling HCl. The cysteine content of the protein was determined as

cysteic acid (Moore, 1963) and S-carboxymethylcysteine by alkylation of cysteine with iodoacetic acid. It was a standard procedure to set up at least duplicate samples for each hydrolysis time. The different hydrolysis times allowed for correction of hydrolytic losses and incomplete hydrolysis at certain peptide bonds. The S-carboxymethylcysteine is sensitive to oxygen and the usual precautions during evacuation were observed as described by Moore and Stein (1963). The hydrolysates were taken to dryness in about 20 min on a rotary evaporator with the hydrolysate tube immersed in a water bath at 40°C. This rapid removal of the HCl by rotary evaporation prevents further decomposition of several amino acids (Moore and Stein, 1963).

To avoid the difficulty in estimating quantitatively the low levels of S-carboxymethylcysteine or cysteic acid, histidine, and phenylalanine in the presence of high levels of several of the other amino acids, dilute and concentrated aliquots of the hydrolysates were applied to the column. There was a 9 fold difference between dilute and concentrated aliquots. This method of analysis was also necessary for some of the large fragments obtained from cyanogen bromide cleavage and enzymatic digests of tropomyosin.

The recovery of all amino acids was calculated relative to 95 residues of leucine per 100,000 g. The yields of S-carboxymethyl-cysteine from measurements of ^{14}C-content were estimated in the same way.

G. N-terminal and Sequence Determination of Peptides

The general procedure of the 'Dansyl-Edman' technique (Gray, 1967) was used for the determination of N-terminal groups and sequence of peptides. Approximately 0.1 to 0.2 μmole of peptide in water or volatile buffer was evaporated to dryness in a glass stoppered 3 ml test tube. The peptide sample was dissolved in 150 μl water and the appropriate aliquot removed for dansylation.

(1) Dansylation

Approximately 0.005 to 0.01 μmoles of peptide was removed from the 150 μl of sample and placed in a small Durham tube (A. Gallenkamp and Company, Ltd., London, EC2). To this tube 20 μl of 0.2 M $NaHCO_3$ was added, the contents were thoroughly mixed, and the solution evaporated to dryness in a dessicator connected to a water aspirator. Deionized water (20 μl) and 20 μl of DNS chloride (2.5 mg per ml in acetone) were added, the solution mixed, and the tubes sealed with Parafilm and incubated in the 45° dessicator for 45 min. After drying under vacuum (water aspirator), 60 μl constant boiling HCl was added, and the tube was sealed under vacuum and incubated at 110°C for 6 to 16 hours. The tube was opened and the contents evaporated to dryness.

(2) Coupling with PITC

To the peptide sample 150 μl 5 per cent PITC in pyridine was added. The tubes were flushed with nitrogen and placed in a 45° dessicator (dessicator equipped with a heating mantle) for one hour. The tube was then transferred to a 60° dessicator with separate dishes

of NaOH and P_2O_5 in the bottom and evacuated for 30 min with oil pump equipped with lyophilizer trap.

(3) Cleavage of PTC-Peptide

The residue was dissolved in 200 µl anhydrous TFA, flushed with nitrogen, tubes stoppered and incubated for 30 min in the 45° dessicator. The TFA was removed in the 60° dessicator under vacuo over P_2O_5 and NaOH for 5 to 10 min.

(4) Extraction with n-butylacetate

To each tube 200 µl of water was added and extracted with three portions (1.5 ml) of n-butylacetate. The two phases were mixed thoroughly on a Vortex mixer and separated on a clinical centrifuge. The top layer was discarded. The remaining sample was taken to dryness over NaOH and P_2O_5 on vacuo for 30 min in the 60° dessicator and then dissolved in 150 µl of water for the next degradation step.

Some peptides were not soluble in water. For this reason the peptides were dissolved in 50 per cent pyridine (150 µl) instead of the 150 µl of water and after sampling for dansylation 50 µl of water and 100 µl of PITC (7.5% in pyridine) were added, otherwise the procedure was as described. This insolubility left the peptides precipitated at the interface during n-butylacetate extractions, however this resulted in no difficulties in sequencing these peptides.

(5) Identification of Dansyl Amino Acids

The dansyl amino acids were routinely identified by thin layer chromatography on polyamide sheets (Woods and Wang, 1967). The

solvent systems employed are described by Hartley (1970).

System 1: 1.5% (v/v) formic acid

System 2: Benzene-acetic acid (9:1, v/v)

System 3: Ethylacetate-methanol-acetic acid (20:1:1, by vol.)

The acid hydrolysate was dissolved in 50 per cent pyridine (10-20 µl) and half spotted on each side of the thin layer plate. Unequivocal identification of the N-terminal amino acid was obtained after as many as sixteen degradations. When the identification on polyamide sheets was ambiguous electrophoresis at pH 4.38 or pH 1.8 on the flat plate was performed using the methods described by Gray (1967), p. 139.

A 6 hr acid hydrolysis was routinely used for the dansylated peptides; however in many cases peptide bonds involving isoleucine and valine were incompletely hydrolyzed under these conditions. Both the dansyl amino acid and the dansyl dipeptide were usually present in the hydrolysate. However, little of the free dansyl amino acid was released for peptide bonds taking the form of DNS-Val-X and DNS-Ile-X when X was isoleucine or valine. Whenever the possibility of these dipeptides occurring in the peptide sequence was expected, the dansylated peptides were hydrolyzed for an extended period. The positions of dipeptides have been previously reported by Hartley (1970) and Johnson and Smillie (in press). The DNS-dipeptides were eluted from the thin layer plates after the initial separations. The spot was cut from the plate and placed in 0.5 ml of wash solution for the TLC plates (1:14:15, conc. NH_4OH:water:methanol) for 10 to 20 min. The solution was taken to

dryness on the water aspirator and constant boiling HCl added for an extended re-hydrolysis. The sample was taken to dryness and identified by TLC. This procedure along with the identification of the adjacent residue to the isoleucine or valine by further Edman degradations identified the DNS-dipeptides. When the assignment of amides could not be deduced from the electrophoretic mobilities of the peptides at pH 6.5 (Offord, 1966), the dansylated peptides were separated by pH 6.5 flat plate electrophoresis before hydrolysis in order that their mobilities could be determined (Gray, 1967, p. 469). In this way the assignment of amides was possible.

H. Disc-gel Electrophoresis

The general procedures and materials described in Canalco 1968 manual (Canal Industrial Corporation, 4935 Cordell Avenue, Bethesda, Maryland) and Davies (1964) were used for disc-gel electrophoresis at pH 9.5. The pH 9.5 system was in 7% polyacrylamide with the glycine-tris discontinuous buffer system, which stacks at pH 8.9 and runs at pH 9.5. For electrophoresis in 8 M urea, urea was added to the various solutions to give a final concentration of 8 M urea in the gels. Electrophoresis was carried out at a constant current of 3-5 m amp. per gel column until the bromophenol blue marker dye had almost reached the bottom of the column. After electrophoresis the gels were stained for 2 hr in a solution of 0.5% Analine Blue Black in 7% acetic acid and destained electrophoretically

and stored in 7% acetic acid. When the protein had to be run in the presence of reducing agent, DTT was added to the sample solution and in some cases to the gel solutions. The presence of reducing agent in the separating gel reacts with the ammonium persulfate which is a strong oxidant causing it to lose its effectiveness as a polymerization catalyst. However, at a concentration of 0.5 mM DTT polymerization occurred but was extended from the normal 30 min to 90 min. The full details of each disc-gel electrophoresis are presented with corresponding photographs in the result sections.

I. Isoelectric Focusing

The general procedures and materials as described in the LKB 8100 Ampholine Electrofocusing Equipment Instruction Manual from LKB-Produkter AB, Stockholm-Bromma 1, Sweden were used for isoelectric focusing. The electrofocusing column, Ampholine Column LKB 8101 (110 ml) and gradient mixer LKB 8121 (110 ml) for preparing the density gradient along with a peristaltic pump for filling and emptying the column were used. Full experimental details are given in Chapter III.

J. Ultracentrifuge Studies

Sedimentation velocity and sedimentation equilibrium studies were performed using a Spinco Model E analytical ultracentrifuge equipped with an electronic speed control. For sedimentation velocity experiments a rotor speed of 60,000 r.p.m., and a standard 12 mm single sector Kel-F centerpiece were used. The run temperature was

maintained at 20°C. Both the low speed sedimentation equilibrium technique and the high speed or meniscus depletion method of Yphantis (1964) were employed. Sedimentation equilibrium studies were performed with the Rayleigh interference optical system, according to the procedure of Richards and Schachman (1959). Here, the standard 12 mm double sector cells were used. Molecular weight measurements in 8 M urea were performed at 20°C, by the meniscus depletion method using a 7 mm column and layering the solvent onto the protein solution in a synthetic boundary interference cell. In this way the times to equilibrium were considerably reduced. Photographs were taken on Kodak spectroscopic plates, Type IIG, at frequent intervals to establish the conditions of sedimentation equilibrium. Interference and sedimentation velocity patterns were measured with a Nikon Model 6C microcomparator. Ultracentrifugation was carried out in the laboratory of Dr. C. M. Kay by Mr. V. Ledsham whose skilled assistance is gratefully acknowledged.

(1) Sedimentation Velocity

Distances from the maximum ordinate to the reference hole were measured in each photograph and converted to the true distance in the cell by dividing by the magnification factor of the schlieren optical system (2.204). The distance from the axis of rotation to the inner reference hole (5.72 cm) was added to the distance in the cell to give the total distance of the maximum ordinate from the axis of rotation, in centimeters. Sedimentation coefficients were calculated by the method described by Schachman (1957) according to the equation

$$S_{obs} = \frac{2.303}{\left(\frac{2\pi R}{60}\right)^2} \cdot \frac{d \log x}{dt}$$

where x = distance from the maximum ordinate in cm to the axis of rotation

t = time in seconds

R = operating speed in r.p.m.

$$S_{obs} = C \cdot \frac{d \log x}{dt}$$

where C = constant

The sedimentation coefficient for the experimental conditions used is obtained by plotting graphically the log x vs. t from which the slope of the line is calculated and multiplied by the Constant C. This sedimentation coefficient was converted to the standard reference of water at 20°C according to the equation:

$$S_{20,w} = S_{obs} \left(\frac{\eta_t}{\eta_{20}}\right)\left(\frac{\eta_{solv}}{\eta_w}\right)_t \left(\frac{1-\bar{v}\rho_{20,w}}{1-\bar{v}\rho_t}\right)$$

where $\frac{\eta_t}{\eta_{20}}$ is the ratio of the viscosity of water at t degrees relative to water at 20°C.

$\frac{\eta_{solv.}}{\eta_w}$ is the ratio of the viscosity of solvent to that of water at temperature t.

$\bar{v}$ is the partial specific volume

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

ρ_t is the density of the solvent at t°C.

The partial specific volume, $\bar{v}$, was assumed to remain unaltered by temperature and solvent composition and taken as 0.733 ml/g (Kay, 1960). Density and viscosity values were obtained by reference to a table of physical constants.

(2) Molecular Weight Determinations

Values of fringe displacement which are proportional to concentration were measured as a function of radial co-ordinates at regular intervals down the cell. The apparent weight average molecular weights were calculated from the expression:

$$\bar{M}_{app} = \frac{2RT}{(1-\bar{v}\rho)\,w^2} \cdot \frac{d\ln c}{d(r^2)}$$

where R = gas constant (8.314×10^7 ergs/mole/degree)

T = absolute temperature

ρ = density of the solvent

$\bar{v}$ = partial specific volume

w = angular velocity in radians per second

c = protein concentration measured in fringe displacement units at a distance r from the axis of rotation

r = distance from the axis of rotation.

In the case of low speed runs the initial concentration C_o was determined by an ultracentrifuge run using a synthetic boundary cell. In both low speed and high speed runs the apparent weight average molecular weight was calculated from the slope of ln y vs. r^2 plots (where y is the concentration in fringe displacement units).

The methods of calculation are described in "A Manual of Methods for the Analytical Ultracentrifuge", by C. H. Chervenka, published by Spinco Division of Beckman Instruments, Inc. These calculations were performed with the aid of an I.B.M. 360 computer with programs made available to us by Mr. W. T. Wolodko. The partial specific volume, $\bar{v}$, was assumed to remain unaltered by temperature and solvent composition and taken as 0.728 ml/g (Kay, 1960) for the calculation of molecular weights of the fragments of tropomyosin.

CHAPTER III

HOMOGENEITY OF RABBIT SKELETAL TROPOMYOSIN AND ITS SUBUNITS

1. INTRODUCTION

The preparation of rabbit skeletal tropomyosin was first described by Bailey (1948). The ammonium sulfate precipitations in the Bailey procedure have been shown to lead to a loss of -SH groups due to the presence of heavy metals in the usual commercially available ammonium sulfate (Drabikowski and Nowak, 1965 and Kominz et al., 1957). Woods (1966) has shown that tropomyosin could be dissociated into subunits by 8M urea alone without the addition of reducing agents. Drabikowski and Nowak (1965) have studied the number and reactivity of sulfhydryl groups in tropomyosin and concluded that this protein contains no disulfide bridges. Their studies have shown the -SH groups of tropomyosin to be easily auto-oxidizable and suggest that this protein exists in muscle with no disulfide bridges. The inconsistencies in the determination of molecular weights and other physical parameters as well as the variability in the sulfhydryl content in earlier studies on tropomyosin could be explained on the basis of the variation in disulfide content in the preparations examined. Mueller (1966) suggested the use of highly purified ammonium sulfate, free of trace contamination of heavy metals, as well as the incorporation of a sulfhydryl protecting agent, 0.5 mM DTT, in all preparative solutions. It was shown by Yasui et al. (1968) that if precautions were taken to

assure the -SH groups of tropomyosin were not permitted to oxidize during preparation (0.5 mM DTT in all preparative solutions) and pH 4.6 isoelectric point precipitations carried out to remove troponin (major contaminant of tropomyosin) tropomyosin appears to be nearly homogeneous. These precautions were followed in the preparation of rabbit skeletal tropomyosin used in this study.

The homogeneity of tropomyosin and its subunits have been examined by sedimentation velocity, column chromatography, disc-gel electrophoresis, isoelectric focusing, and amino acid analysis.

2. MATERIALS AND METHODS

A. Preparation of the Protein

The full experimental details of the materials and methods used for the preparation of rabbit skeletal tropomyosin and ^{14}C-CM-tropomyosin have been described in Chapter II, General Materials and Methods.

B. Methods for Determination of Homogeneity

The procedures for disc-gel electrophoresis, sedimentation velocity, isoelectric focusing and amino acid analysis have been described in Chapter II, General Materials and Methods.

(1) Column Chromatography

a. Gel-Filtration with G-150 and G-75 Sephadex

i. Preparation of the Column

The column (250 cm x 5 cm) was prepared in two sections, from two Sephadex glass columns 100 and 150 cm long, fitted with adaptors with nylon nets and bed support screens. The two columns were mounted at the same level and connected in series. The Sephadex G-150 (40-120 μ particle size) and G-75 (40-120 μ) were allowed to swell for three days in water with many decantations to remove the fines. The swollen gel was then added to the buffer and decanted repeatedly to equilibrate the gel. The gel slurry and solutions were de-aerated before packing the column according to the procedures outlined in the Sephadex Manual. The columns were operated by upward flow and the flow rate regulated by the use of a Beckman Accu-Flo constant volume pump. The samples were also applied by the use of the pump.

ii. G-150 Sephadex Chromatography

A 25 ml sample containing about 500 mg of tropomyosin dissolved in 0.6 M KCl - 0.5 mM DTT - 0.1 M KH_2PO_4-KOH buffer, pH 7.0 was applied to the column and then eluted with buffer at a flow rate of 58 ml per hour (approximately 3 ml per cm^2 per hour) by upward flow. The eluate was collected in 20.0 ml fractions. The fractionation was monitored by absorbancy measurements at 280 mμ.

iii. G-75 Sephadex Chromatography

A 5.0 ml sample containing about 98 mg of CM-tropomyosin dissolved in 0.2 M KCl - 8 M urea - 0.025 M KH_2PO_4-KOH, pH 6.0 buffer was applied to the column and then eluted with this buffer at a flow rate of about 40 ml per hour (approximately 2.0 ml per cm^2 per hour) by upward flow. The eluate was collected in 14.0 ml fractions and monitored by absorbancy measurements at 280 mμ.

b. QAE-Sephadex A-50

The strong anionic exchange resin, QAE-Sephadex A-50, capacity 3.0 ± 0.4 meq/g, particle size 40-120 μ, was allowed to swell in a large excess of the initial eluant buffer (0.15 M KCl - 8 M urea - 0.05 M Tris-HCl buffer, pH 7.5). Over a 24 hr period the supernatant was decanted two or three times. This ensured complete equilibration of the gel and the gel was used without further treatment.

The slurry of the gel was packed in a 1.2 cm x 50 cm (Chromaflex, Kontes Glass Company, Vineland, New Jersey, U.S.A.) column. The column was equilibrated further by pumping starting buffer through the column overnight at a flow rate of 3.0 ml/hr regulated by a Beckman constant volume pump. ^{14}C-CM-tropomyosin (10 mg) was dissolved in 250 μl of starting buffer and applied to the column. After application, a linear gradient elution was generated by using 200 ml volumes of equilibration buffer and 0.35 M KCl - 8 M urea - 0.05 M Tris-HCl buffer, pH 7.5, contained in a two-

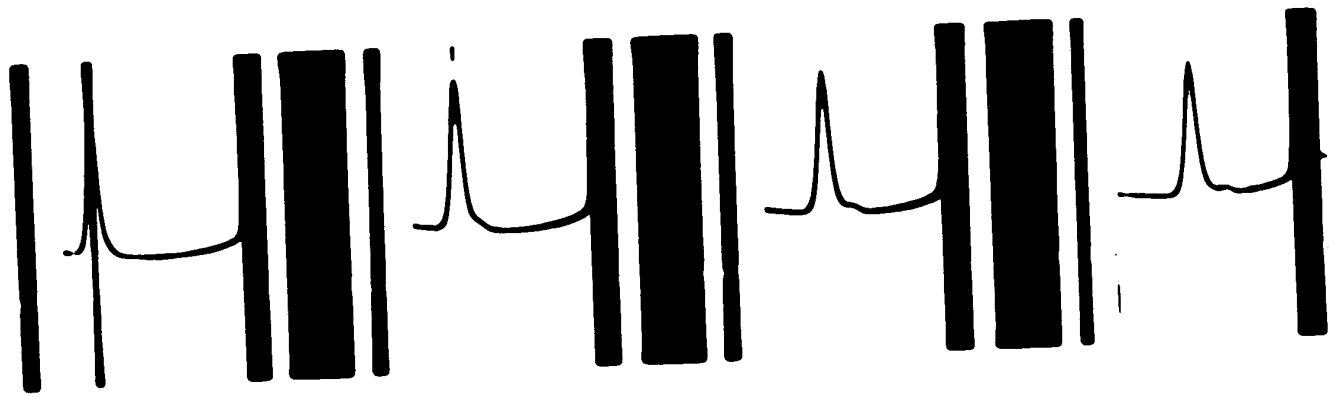


Fig. 6: Sedimentation velocity patterns of rabbit skeletal tropomyosin (c=14.2 mg/ml) in 0.5 mM DTT - 0.5 M KCl - 0.046 M phosphate buffer, pH 7.0, μ = 0.6 at 60,000 r.p.m. Photographs were taken at 16 min intervals after top speed was reached. The intervals shown are at 64, 96, 128, and 160 minutes. The direction of sedimentation is toward the right.

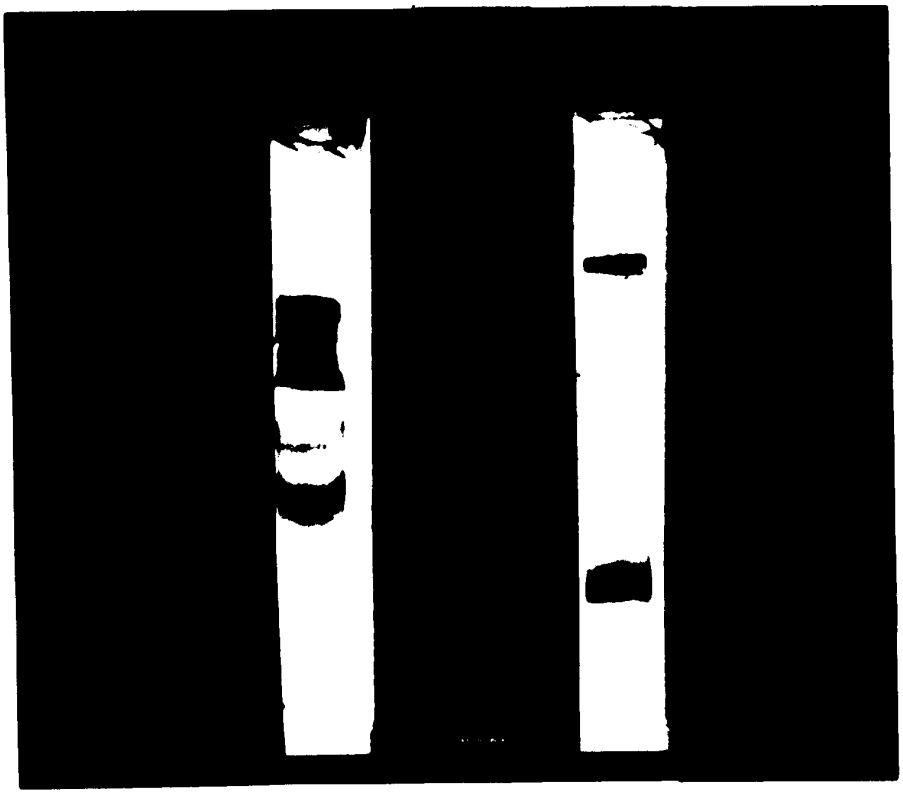
chambered apparatus of identical cross section. The column was eluted at a flow rate of 3 ml per hour, at room temperature and fractions of 1.0 ml were collected. The fractionation was monitored by radioactive counting on 250 µl aliquots of the effluent fractions and by absorbancy measurements at 230 mµ.

3. RESULTS

A. Sedimentation Velocity

In order to determine the extent of homogeneity of tropomyosin prepared by the method used in this study, tropomyosin was first examined for evidence of impurities by means of sedimentation velocity in the ultracentrifuge. Only a single symmetrical peak was detected at a protein concentration of 7.7 mg/ml. However, at a protein concentration of 14.2 mg/ml a very small amount of a faster moving component (Figure 6) was observed (approximately 5%). This component is most likely aggregated tropomyosin. Woods (1967) has shown similar results and obtained only 5 preparations of a total of 25 which showed a single boundary.

The sedimentation coefficients, $S_{20,w}$ values, were determined at two protein concentrations in the buffer system shown in Figure 6. At protein concentrations of 14.2 mg/ml and 7.7 mg/ml the $S_{20,w}$ was found to be 1.81S and 2.12S respectively. These values were comparable to those obtained for the rabbit skeletal system, denoted by the straight line relationship,



a b

Fig. 7: Disc-electrophoresis at pH 9.5 of SH-tropomyosin.

a. The protein was dissolved in pH 9.5 electrophoresis buffer and applied immediately to the column (urea and reducing agent were absent).

b. The protein was dissolved in pH 9.5 electrophoresis buffer, 8 M urea and 0.5 mM DTT. The gel also contained 8 M urea and 0.5 mM DTT.

$$\frac{1}{S_{20,w}} = 0.372 + 0.013\ c \qquad S^{\circ}_{20,w} = 2.69S$$

where c is concentration in milligrams per ml (Woods, 1967). From this equation $S_{20,w}$ values of 1.79S and 2.12S for the concentrations 14.2 mg/ml and 7.7 mg/ml are obtained.

B. Disc Electrophoresis

When tropomyosin was applied directly to the disc-gel without the protection of a reducing agent only one major band was observed (Figure 7,a). The incorporation of DTT and 8 M urea into the sample solution and gel results in tropomyosin being dissociated into its subunits. A homogeneous preparation is indicated by the appearance of a single band (Figure 7,b). Figure 8 shows the disc-gel patterns arising from two different tropomyosin preparations and clearly indicates that the slower moving band on pH 9.5 electrophoresis is an oxidation product of tropomyosin. Figure 8,b shows an increase in slower moving component indicating that the 1 mM DTT did not completely reduce the protein shown in Figure 8,a and that the amount of oxidized component increases on standing. Figure 8,c was obtained by dissolving the protein in 8 M urea without reducing agent and shows a complete reversal of the results in Figure 8,a where the oxidized component is now the major species present. Comparison of Figure 8,d and 8,e shows that on standing this particular sample contained less of the slower moving oxidized component, (Figure 8,e) suggesting a further reduction of the protein on standing. Figure 8,f

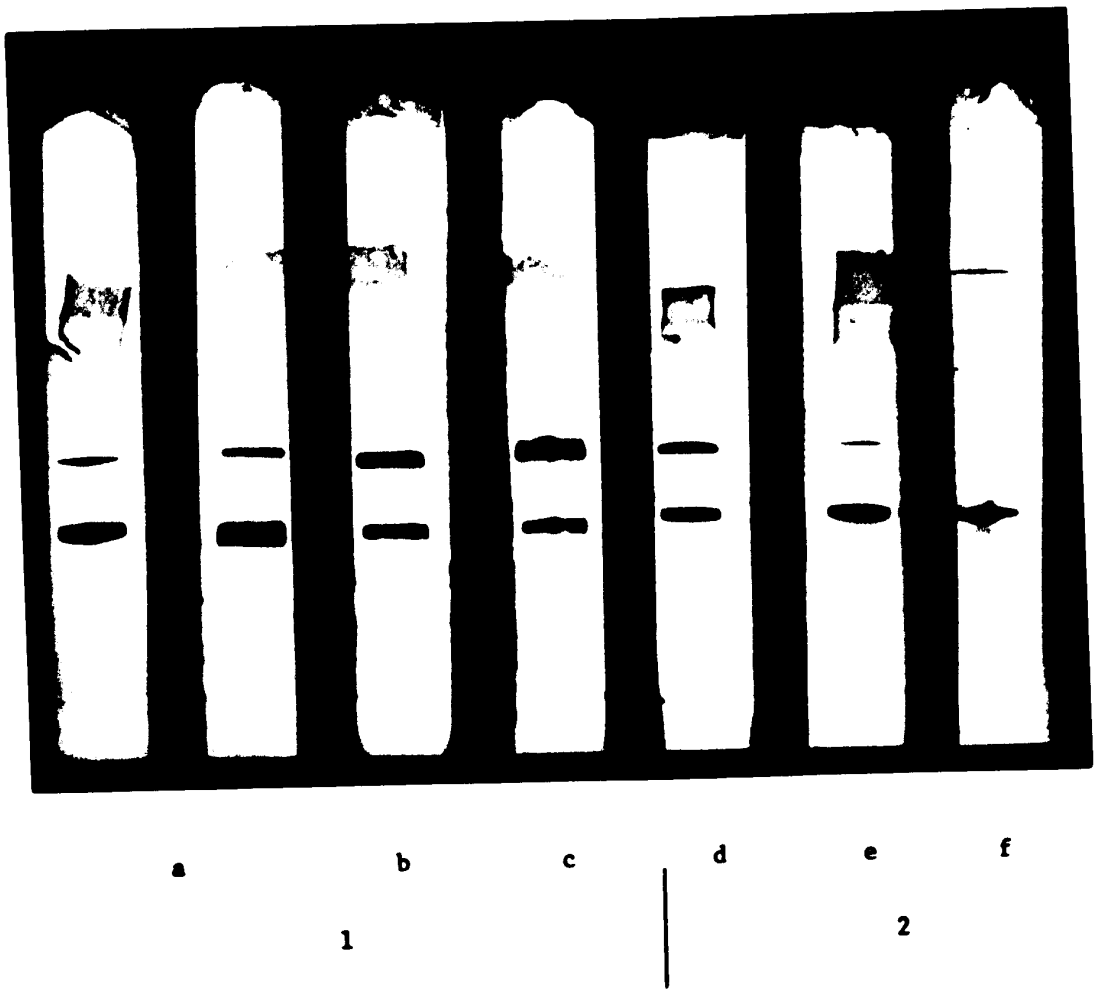


Fig. 8: Disc-gel electrophoresis at 9.5 in 8 M urea of SH-tropomyosin. Numbers 1 and 2 denote two different preparations.

a. 20 µg and 40 µg of protein dissolved in water, 8 M urea and 1 mM DTT and applied to the column.

b. 40 µg of sample (a) 24 hours later.

c. 40 µg protein in 8 M urea solution with the absence of DTT.

d. 20 µg protein dissolved in 8 M urea, 1 mM DTT and applied to the column.

e. 20 µg of sample (d) 24 hours later.

f. 20 µg of sample (d) with excess DTT added.

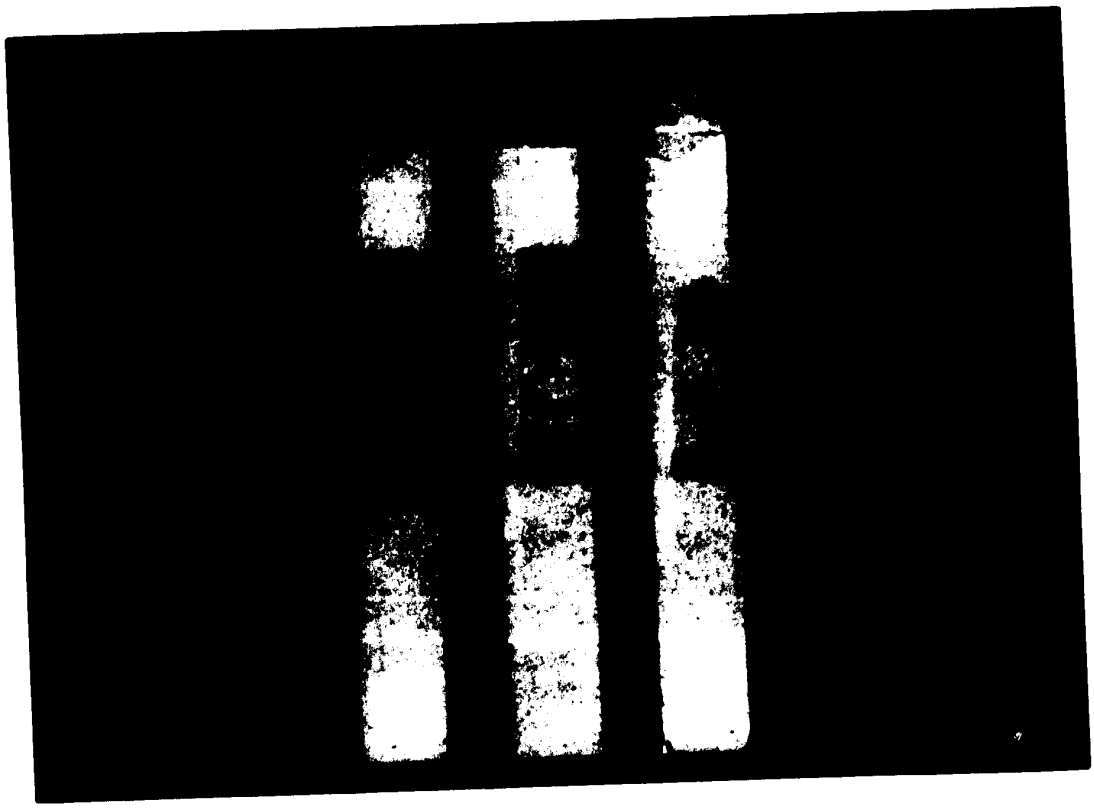


Fig. 9: Disc-gel electrophoresis of CM-tropomyosin at pH 9.5 - 7% gel in 8 M urea at three different protein concentrations. The concentrations from left to right were 200 µg, 100 µg, and 50 µg.

is obtained by adding excess reducing agent and contains only a single band. Since the catalyst used in disc-gel electrophoresis for polymerization is a strong oxidant it may be absolutely impossible to prevent the occurrence of trace oxidation products of a protein like tropomyosin.

The running of CM-tropomyosin at pH 9.5 shows only a single major band (Figure 9). The minor components probably arose from the aggregation of unreacted tropomyosin.

C. Isoelectric Focusing

Isoelectric focusing has proven itself capable of providing results not obtainable by other methods for characterizing and separating proteins. However, there are natural limitations to isoelectric focusing. Some proteins are only slightly soluble at their isoelectric point, especially in a salt-free milieu and precipitate in the electrofocused zones and cause disturbances by sedimenting through the column. For substances with low solubility, or substances which tend to precipitate at their pI's, a higher Ampholine concentration, the use of urea, and the use of a more shallow pH gradient may give better results.

Isoelectric focusing was chosen as a method to check the homogeneity of tropomyosin as well as to possibly provide an answer to the chemical identity or non-identity of its constituent polypeptide chains. For this reason the isoelectric focusing was carried out in 7 M urea. Isoelectric focusing in 6 M and 7 M urea had previously been

carried out successfully in the study of the subunit structure of alpha-crystallin (Bloemendal and Schoemaker, 1968 and Björk, 1968).

Preliminary results indicated that all precautions would have to be taken to prevent precipitation of tropomyosin during the isoelectric focusing run. The 7 M urea was already being used to dissociate tropomyosin into its subunits. The addition of neutral detergents Brij 58 (polyoxyethylene glycol) to the ampholine-sucrose gradients has been shown to help keep insoluble proteins in solution and have no effect on the migration rate (Godson, 1970). Jeppsson (1967) working with transferrins found that the use of a wattage above 1 W resulted in the isoelectric precipitation of the proteins. These precautions along with the use of a higher ampholyte concentration were used in the isoelectric focusing of tropomyosin. To obtain maximum resolving conditions a narrower pH range than the standard pH range (2 pH units) was used by a two-step isoelectric focusing run. The initial isoelectric focusing run was carried out with sample at a 4% ampholyte concentration of the standard 4-6 pH range in 7 M urea at a temperature of 30°C.

Solutions

Anode solution at the column bottom

Sulphuric acid	0.2 ml
Sucrose	12.0 g
Urea	9.2 g
Water to final volume	21.9 ml

Dilute sulphuric acid with water and add this solution last.

Cathode solution at column top

Ethylenediamine	0.2 ml
Water	10.0 ml

Light gradient solution

Urea	23.1 g
Ampholytes	2.75 ml of standard 40% solution
Water to final volume	55.0 ml

Dense gradient solution

Sucrose	24.0 g
Urea	21.0 g
Ampholytes	8.25 ml of standard 40% solution
Water to final volume	50.0 ml

Density Gradient

A linear gradient was prepared using the LKB gradient mixer (110 ml) as follows:

1) The light and dense gradient solutions were placed in the gradient mixer (50 ml of each) and the gradient pumped into the column at a flow rate of 2.0 ml per min carefully layering this solution on top of the anode solution.
2) After 30 ml of gradient was added to the column the pump was stopped, the line between the gradient vessels clamped off, 10 ml of the dense solution removed from the gradient vessel to dissolve the sample (8 mg ^{14}C-CM-Tropomyosin) and then replaced. The gradient was restarted.
3) At the end of the gradient 5 ml of the light solution was added to the top of the column before the cathode solution.

The above procedure keeps the protein from coming in contact with the electrode solutions and prevented the early precipitation observed when the standard procedure of dissolving the sample in the light gradient solution was used.

The column was run at a constant wattage of 0.75. This required increasing the voltage during the run. At 67 hours a very fine precipitated band was observed. The precipitate most probably represented only a small portion of the total protein. The column was stopped and elution was carried out after removing the upper electrode solution by means of pumping water into the top of the column at a flow rate of 0.5 ml per min. Fractions of 1.0 ml were collected, the

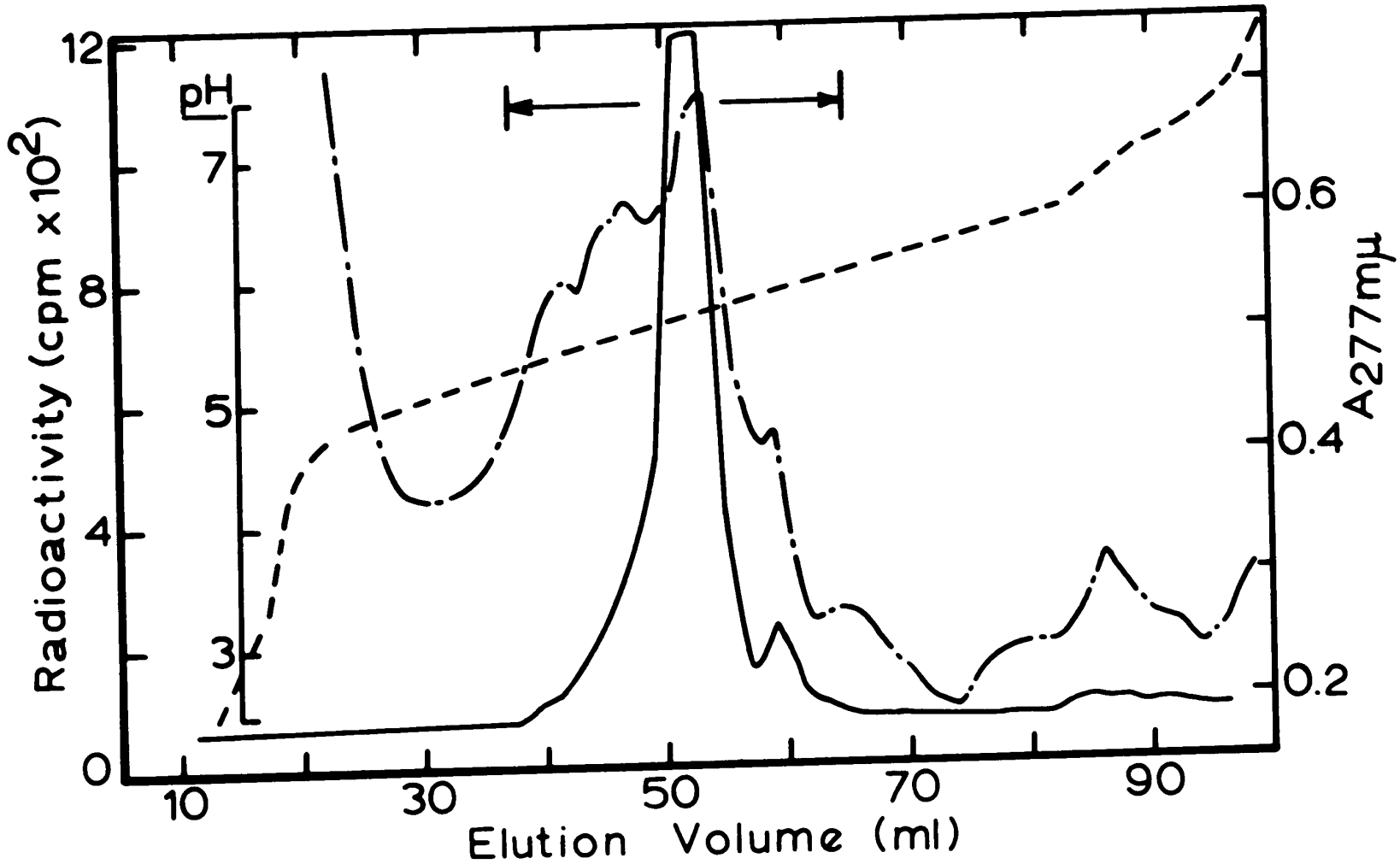


Fig. 10: The first run of a two-step isoelectric focusing run of ^{14}C-CM-tropomyosin in 7 M urea at a 4% ampholyte concentration of the standard 4-6 pH range and at a temperature of 30°C. The effluent was monitored for radioactivity (————) and absorbancy at 280 mμ (——·——). The pH gradient is indicated by the broken line.

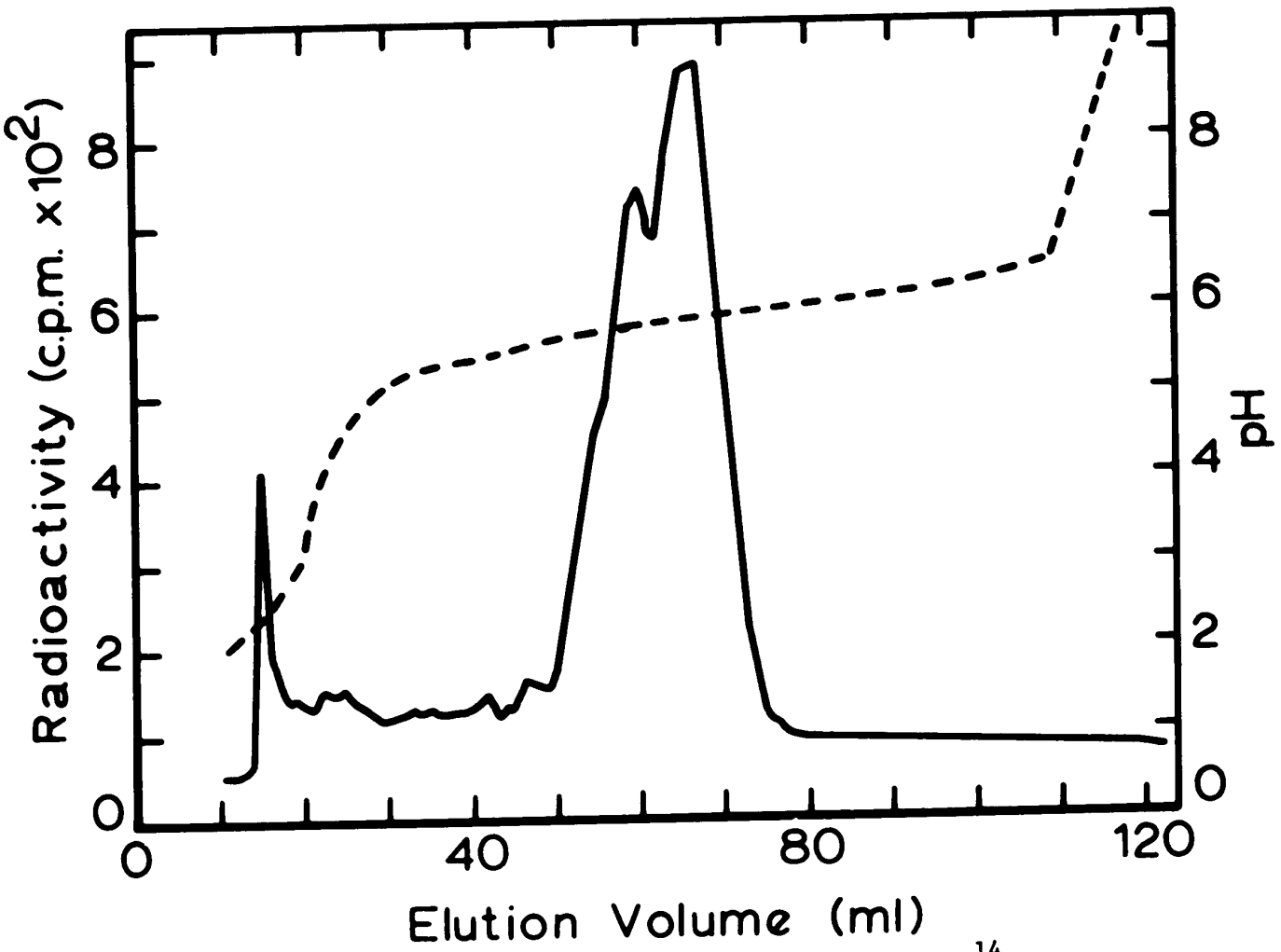


Fig. 11: The second run of a two-step isoelectric focusing run of ^{14}C-CM-tropomyosin in 7 M urea. The effluent was monitored for radioactivity (solid line) and the pH gradient is indicated by the broken line.

pH, A_{277} mμ and radioactivity of the fractions were determined. For radioactivity measurements 100 μℓ aliquots were taken from every second fraction. The results are shown in Figure 10.

Fractions 38-65 inclusive (24 ml) were pooled and re-run in a second isoelectrofocusing experiment. All solutions were identical except for the incorporation of 8% Brij 58 in the light gradient solution to help prevent precipitation.

Solutions

Light gradient solution

Urea	23.1 g
Brij 58	22.0 ml of 20% solution
Ampholyte fraction	6.0 ml
Water to final volume	55.0 ml

Dense gradient solution

Sucrose	27.5 g
Urea	21.0 g
Water to final volume	of 50 ml

From this solution 37 ml was taken and added to 18 ml of the ampholyte fraction of the previous run to give a final volume of 55 ml.

These solutions were pumped into the column from the gradient maker at a flow rate of 2 ml per min. Isoelectric focusing was carried out for 68 hr at a constant wattage of 0.75. No precipitation of the protein was observed during this run. The column was eluted as before and the pH and radioactivity (250 μℓ) of each

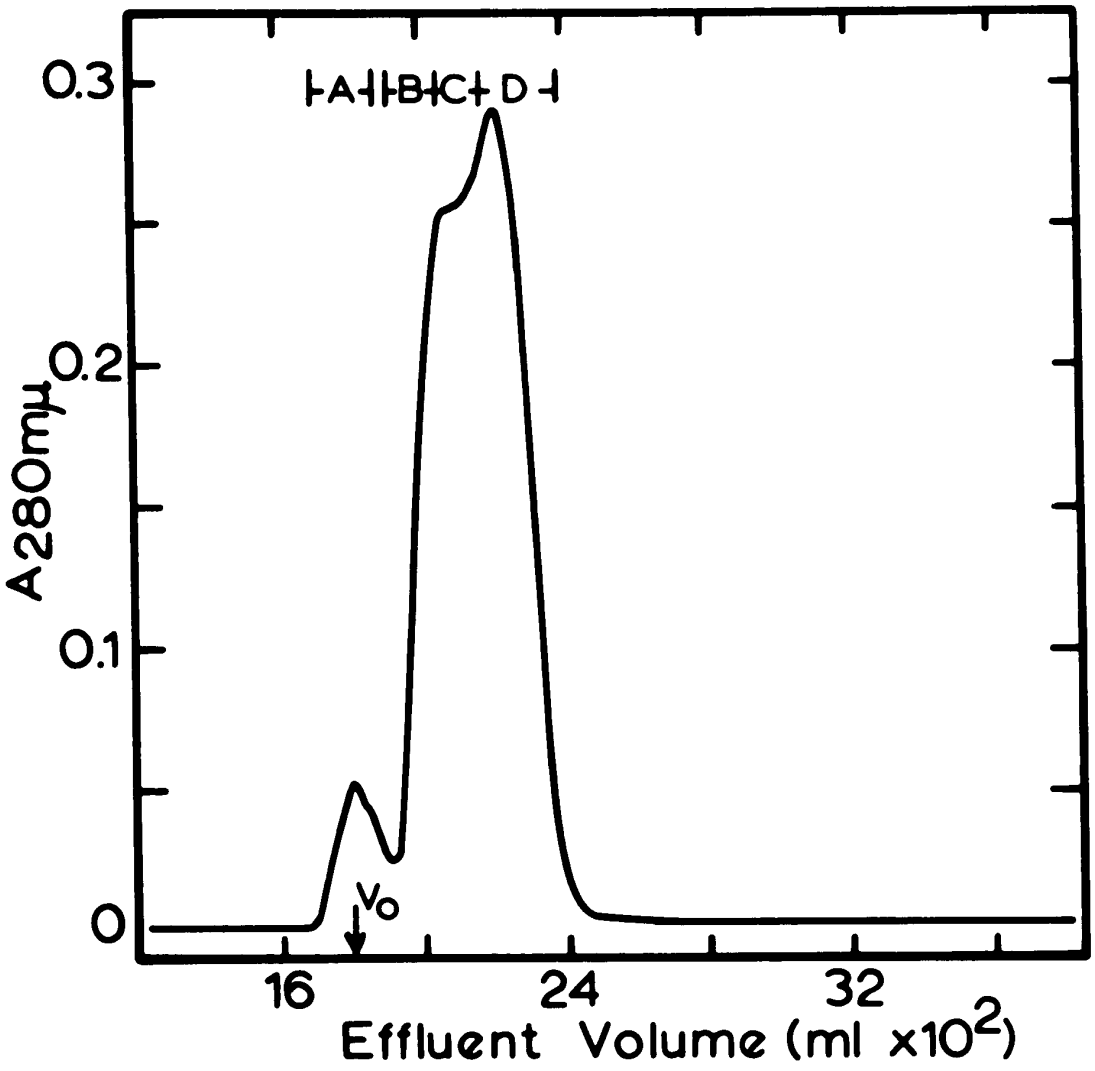


Fig. 12: Gel-filtration of SH-tropomyosin on a 250 cm x 5 cm Sephadex G-150 column in 0.6 M KCl - 0.5 mM DTT - 0.1 M phosphate buffer, pH 7.0. Fractions A to D were pooled as indicated.

A B C D

Fig. 13: Disc electrophoresis at pH 9.5 in 8 M urea of elution fractions A to D from G-150 Sephadex chromatography. Protein from each fraction was dissolved in 0.5 mM DTT - 8 M urea solution.

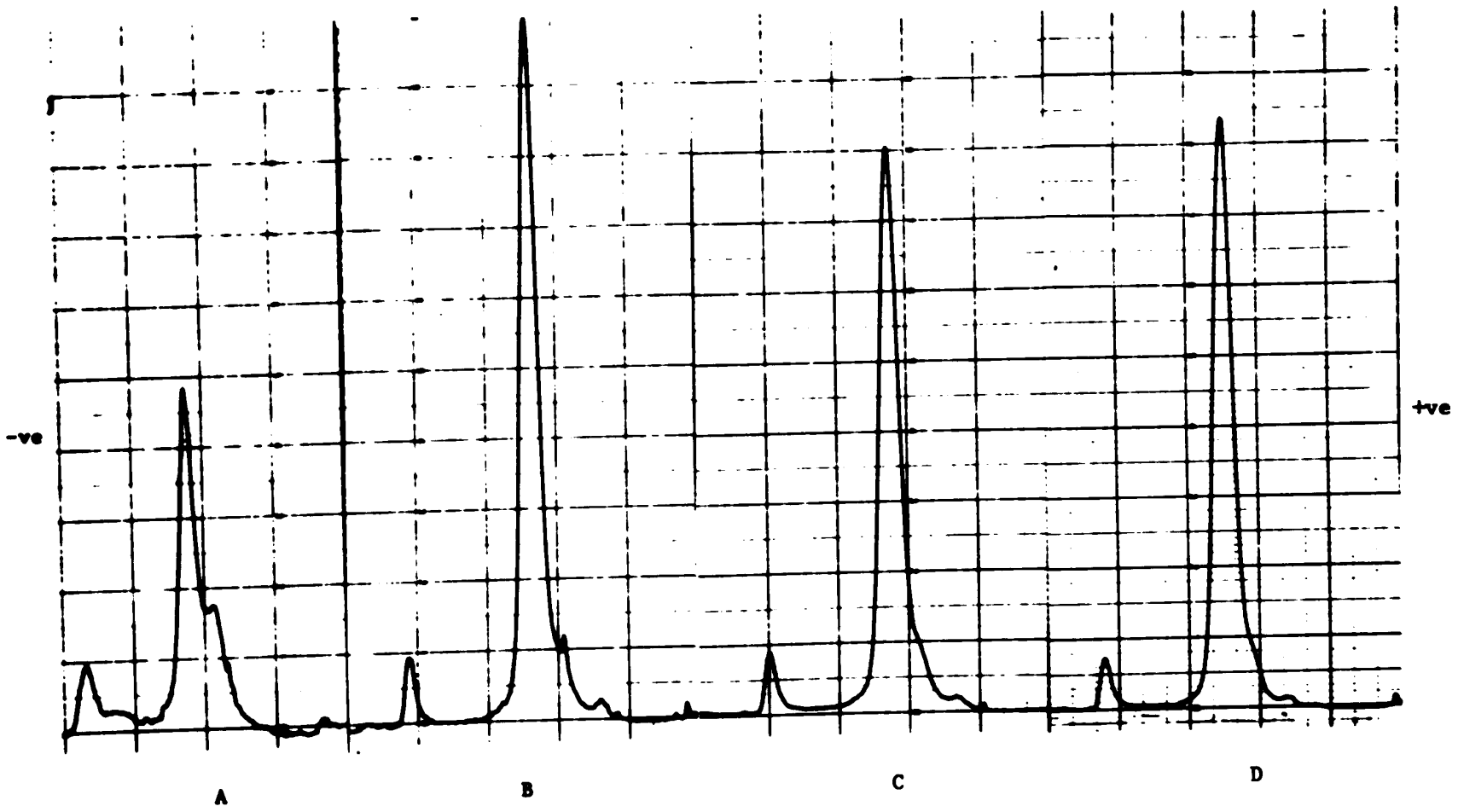


Fig. 14: Densitometer tracings of disc-gels of protein fractions A to D from G-150 Sephadex chromatography. Gels were scanned in a Gilford Spectrophotometer with disc-gel scanning attachment at 660 mμ with a recorder speed of 120 inches per hour and a scan rate of 1 cm per min.

fraction was determined. The results are shown in Figure 11. Brij 58 was found to precipitate out in the fractions at room temperature and for this reason the A_{277} mμ were not determined. These results gave an indication of two closely separated peaks and therefore the possibility that the subunits of tropomyosin may not be identical. However, the resolution with this technique has not been great enough to unequivocally answer the question of subunit identity.

D. Column Chromatography

When tropomyosin was examined by gel filtration through Sephadex G-150 at high ionic strength, part of the protein emerged with the void volume and heterogeneity was evident (Figure 12). The void volume peak consisted of about 5% of the ultraviolet-absorbing material. This heterogeneity shown in the elution profile was very probably the result of protein aggregation. These results are in direct agreement with those of Woods (1967) who examined NEM-tropomyosin on Sephadex G-200. Fractions A to D were examined by disc-gel electrophoresis in 8 M urea at pH 9.5 (Figure 13).

Observations of the disc-gels from each fraction A to D show one major peak and compare identically with those in Figure 8 of unfractionated tropomyosin. Densitometer tracings of these gels are shown in Figure 14. Fractions B, C, and D of the main component can be considered as identical in all respects. The void volume peak showed the same general pattern but indicated considerably more of the component on the trailing edge of the main peak in the densitometer tracings.

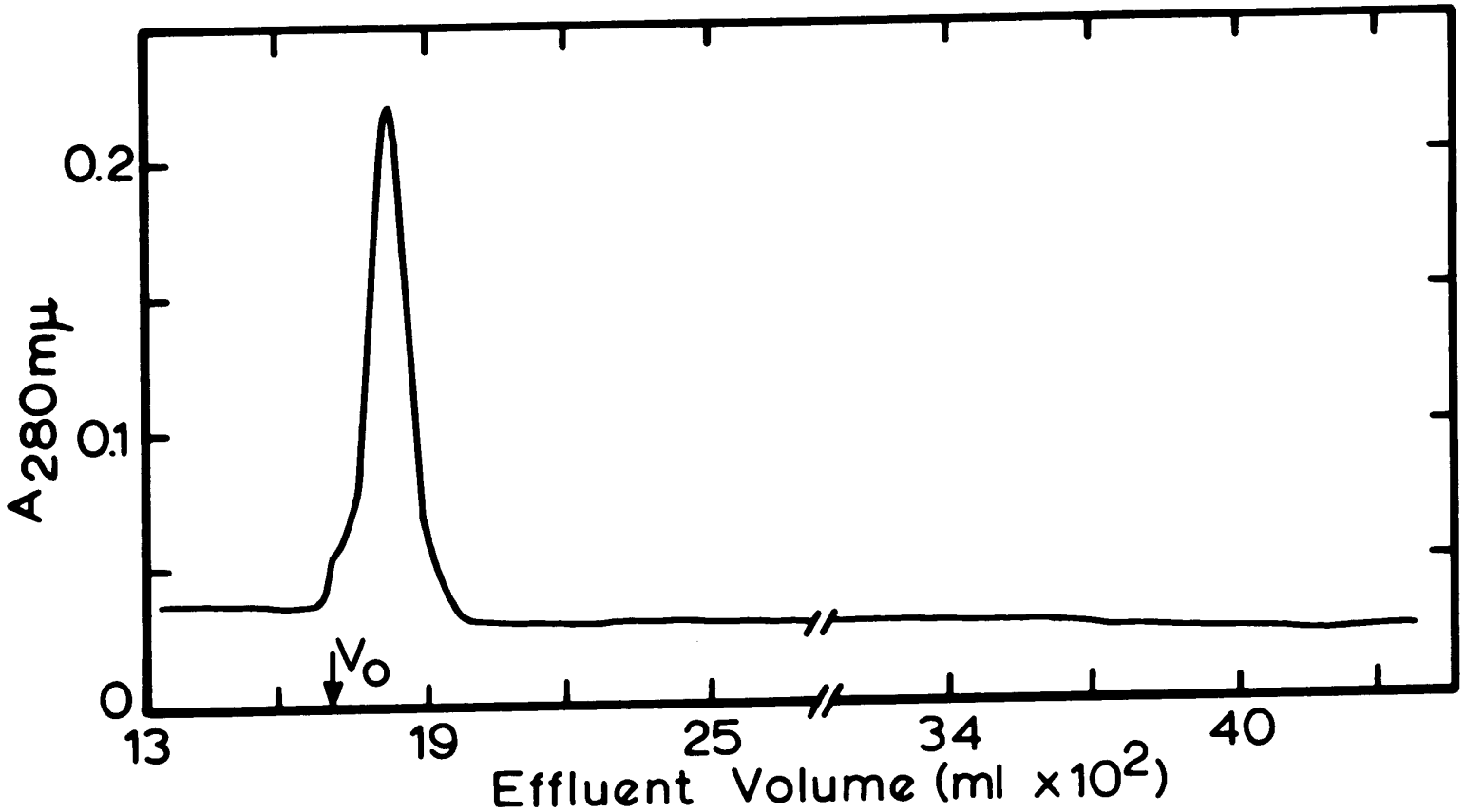


Fig. 15: Gel-filtration of CM-tropomyosin on a 250 cm x 5 cm Sephadex G-75 column in 0.2 M KCl - 8 M urea - 0.025 M phosphate buffer, pH 6.0.

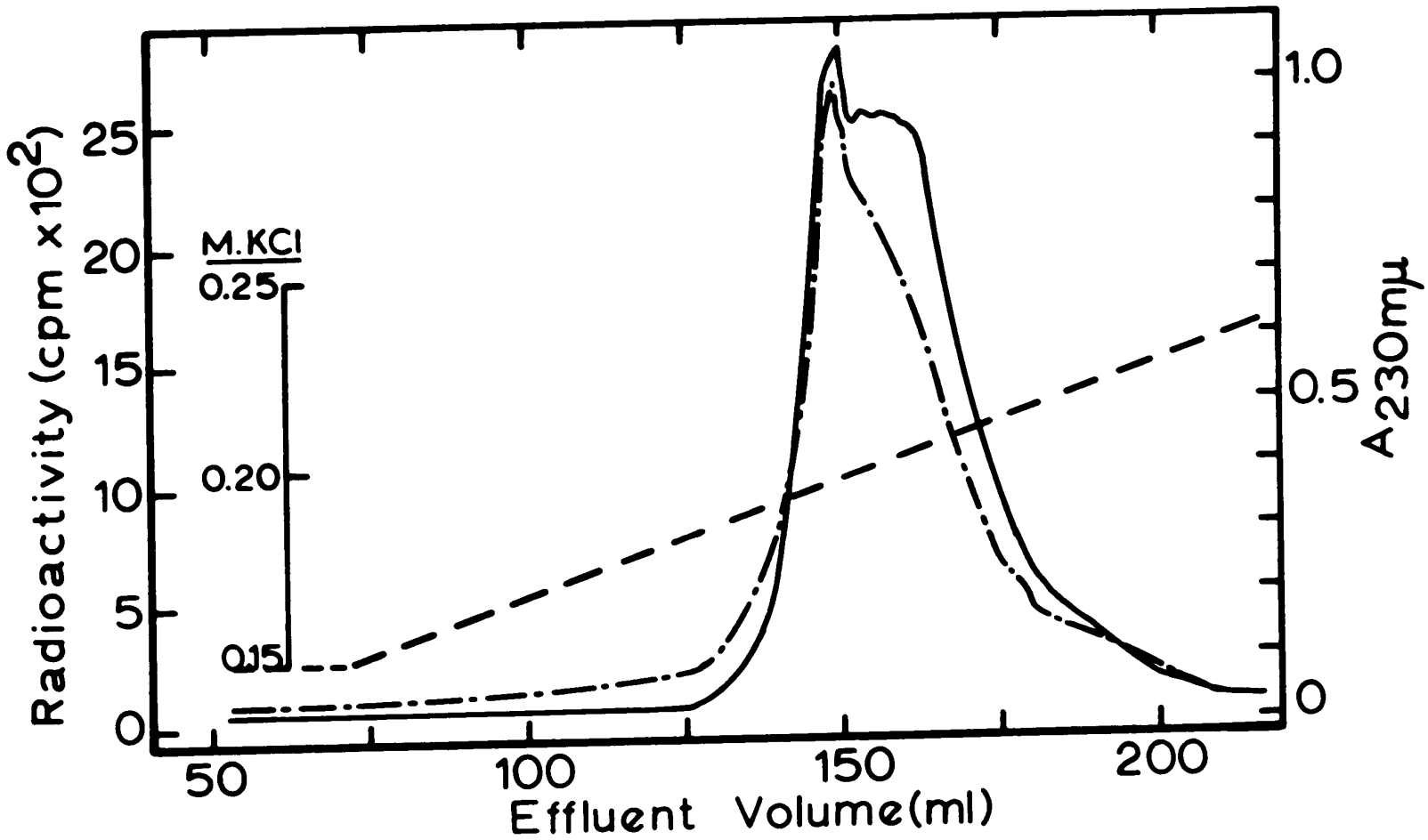


Fig. 16: Anion-exchange chromatography of ^{14}C-CM-tropomyosin on a QAE-Sephadex column (50 cm x 1.2 cm) in 8 M urea. The effluent was monitored for absorbancy at 230 mμ (——– – ——) and radioactivity (————). The salt gradient is indicated by the broken line.

Chromatography of CM-tropomyosin in 8 M urea on Sephadex G-75 (Figure 15) indicated a single component and did not show the heterogeneity of the major component seen on the G-150 Sephadex fractionation of tropomyosin. However, the symmetry of this peak cannot be taken as too meaningful due to its close proximity to the void volume.

The results of the anion exchange chromatography (Figure 16) indicated heterogeneity of the subunits of tropomyosin but an unequivocal answer as to the identity or non-identity of the subunits could not be made.

E. The Amino Acid Composition

The amino acid composition of the tropomyosin preparation used in this study is in close agreement with those obtained previously by others (Table IX). The proline content has been shown by Woods (1969) to be variable among different preparations of rabbit skeletal tropomyosin and could not be detected in one preparation purified by fractionation on DEAE-cellulose and Sephadex G-200. Fowl gizzard and crayfish tropomyosin have been shown to contain no proline (Woods, 1969 and Woods and Pont, 1971). It may be possible that this proline arises from a trace contamination of troponin which has been reported to contain 30 moles of proline per 100,000 g (Hartshorne and Mueller, 1968; Ebashi et al., 1968). The proline value obtained in this study of 1 mole per 70,000 does indicate that the troponin content is extremely low or negligible since the actual proline content of tropomyosins are uncertain. The pH 6.5 electropherograms of tryptic digests of CM-tropomyosin when stained with Ehrlich's reagent for

TABLE IX

AMINO ACID COMPOSITION OF TROPOMYOSIN

(MOLES/70,000 g)

Amino Acid	Kominz et al.* (1957)	Katz & Converse* (1964)	Carsten* (1968)	Woods* (1969)	This Study
Lys	75	80	74	81	81[a]
His	3.9	4.1	3.9	4.2	4.0[a]
Amide NH_3	(45)	-	-	-	-
Arg	29	30	29	30	29.5[a]
Asp	62	59	57	64	62[a]
Thr	18	16	15	16	16.6[b]
Ser	28	25	26	27	27[b]
Glu	148	150	135	154	151[a]
Pro	1.2	-	1.3	0-2.9	1.0[a]
Gly	9	8	10	9	8.0[a]
Ala	77	73	66	79	74[a]
Cys/2	4.6	-	-	-	-
$Cys(SO_3)$	-	-	-	-	2.9[a]
CMC	-	-	1.9	2.0-3.2	2.8[a,f]
Val	19	18	17	20	20.7[c]
Met	11	12	13	14	13.2[a]
$Met(O)_2$	-	-	-	-	13.7[a]
Ile	21	22	18	23	23.5[c]
Leu	67	66	57	69	67[a,d]
Tyr	11	12	9	11	11.5[a]
Phe	2.3	2.5	3.2	2.8	3.0[a]
Try[e]	0	-	-	-	-

* Analyses converted to residues per 70,000 g.

[a] Average of 22- and 70-hour hydrolysates.

[b] Extrapolated to zero time.

[c] Average of 70-hour hydrolysate.

[d] Leucine = 95.0 residues per 100,000 g.

[e] No tryptophan, determined by Bailey (1948), and Kominz et al. (1957).

[f] Value of 2.8 also obtained by radioactive incorporation.

tryptophan (Harley-Mason and Archer, 1958) were negative in agreement with Bailey (1948) and Kominz et al. (1957) who found no tryptophan in tropomyosin. Of particular interest for the purposes of this work were the histidine and methionine contents as well as the levels of cysteine as determined by cysteic acid determinations, incorporation of ^{14}C-labelled iodoacetic acid into the protein, and the analysis of S-carboxymethylcysteine after acid hydrolysis. These analyses are in good agreement with a value of 2.8-2.9 residues of cysteine per mole of 70,000 g. Previous estimates of cysteine content have been variable among different laboratories, possibly arising from troponin contamination in the earliest work and from the difficulty of estimating quantitatively the low level of S-carboxymethylcysteine or cysteic acid in the presence of high levels of several of the other amino acids. To avoid the latter problem, dilute and concentrated aliquots of the hydrolysates were applied to the analyzer columns in the present work. The good agreement obtained among the three methods of estimation lends some confidence to the reliability of these analyses.

4. DISCUSSION

The inclusion of this chapter in this thesis has been twofold. First, to indicate the homogeneity of the tropomyosin preparations used in this study and secondly, to indicate the homogeneity of the subunits themselves. Sedimentation velocity at high protein concentrations showed the presence of a slower moving component representing about 5% of the total material. Similarly, the separation of tropomyosin on G-150 Sephadex revealed a peak in the void volume of about 5%. This peak on

disc-gel electrophoresis showed a pattern similar to that of the main component. From these results it is most likely that this material represents aggregated tropomyosin. The heterogeneity of the main component on Sephadex G-150 can best be explained as arising from protein-protein interactions. The disc-gel patterns from three different portions of this peak were identical displaying only one major band. Disc-gel electrophoresis on two different preparations of tropomyosin under varying states of oxidation clearly showed an interrelationship of the various bands and that a single band can be obtained if the protein is completely reduced. Protection of the -SH groups from oxidation by carboxymethylation of the protein gave only a single band on disc-gel electrophoresis. The aggregation of tropomyosin may also be affected by the possible presence of trace amounts of troponin which has been shown to promote the aggregation of tropomyosin (Ebashi and Kodama, 1965). The fractionation of CM-tropomyosin on QAE-Sephadex in 8 M urea gave a heterogeneous peak in agreement with the results of isoelectric focusing where two unresolved peaks were observed. However, on Sephadex G-75,CM-tropomyosin showed no asymmetry in the main component. These results are in agreement with previous workers, that the subunits of tropomyosin are highly similar in mass and charge. They also show that if the chains are dissimilar their separation will be extremely difficult.

CHAPTER IV

THE NUMBER AND AMINO ACID SEQUENCES OF HISTIDINE AND METHIONINE PEPTIDES FROM RABBIT SKELETAL TROPOMYOSIN

1. INTRODUCTION

These studies were carried out to determine the number of unique histidine and methionine sequences in tropomyosin and to relate this information to the subunit structure of the molecule. The amino acid composition of tropomyosin (Table IX, page 53) shows 4 histidine and 14 methionine residues per monomer of tropomyosin (70,000 g). If the two subunits of tropomyosin are identical one would expect to find not more than 2 unique and 7 unique histidyl and methionyl sequences respectively. On the other hand, if they are non-identical this should be reflected in the number of unique sequences about these residues, obtaining possibly 3 or 4 unique histidine and 8 to 14 unique methionine containing sequences.

The amino acids chosen for a study of this kind should fulfill two criteria. Firstly, the numbers of these residues per monomer should be small. Secondly, a method of selective detection from all other peptides should be available. Both histidine and methionine meet these requirements. The histidine containing peptides can be selectively identified by the use of a stain known as Pauly reagent (Dent, 1947). Also, the unique properties of the histidine moiety in having a pK near pH 6.5 for the imidazole usually allow easy purification from other peptides by pH 6.5 and pH 1.8 high voltage electrophoresis. The methionine containing peptides can be

selectively located and purified using the diagonal electrophoretic peptide mapping procedure of Tang and Hartley (1967). Obtaining the sequences about the methionine residues has the added feature of enabling the 'overlap' of cyanogen bromide fragments and thus arranging these larger pieces in the correct sequence.

The methionine diagonal procedure relies on the change in electrophoretic mobility when methionine peptides are alkylated with iodoacetamide to give the S-carbamoylmethylmethionine peptides. The pH 6.5/pH 6.5 'diagonal technique' was used in our first attempts to isolate the methionine peptides. This method has been shown to work successfully with other proteins in our laboratory, as for example, α-lytic protease, horse-radish peroxidase, and Streptomyces griseus Protease A. With these proteins only methionine containing peptides came off the diagonal. However, with tropomyosin a large number of misleading spots were obtained off the pH 6.5/pH 6.5 diagonal peptide "map". This is probably the result of alkylation of a small portion of the carboxyl groups of the protein. This conclusion is further substantiated by the results of the pH 1.8/pH 1.8 diagonal, which show considerably fewer peptides off the diagonal. At pH 1.8 the carboxymethylation of carboxyl groups would not result in any change in their electrophoretic mobility. Carboxymethylation at carboxyl groups is not a normal reaction, but has been shown to occur slowly at one glutamic acid residue in ribonuclease T_1 (Takahaski *et al.*, 1967). This reaction was very specific occurring with the γ-carboxyl group of glutamic acid in the active site and only with iodoacetic acid

and not iodoacetamide. To our knowledge this reaction has been the only one ever reported. The reaction of the carboxyl groups with iodoacetamide suggested in this study would seem to be more non-specific because of its high frequency of occurrence. Tropomyosin contains approximately 25% of its amino acid residues as free acidic groups and has the largest "zwitterion" charge density of any known protein, which may well explain the occurrence of this rare event. The haloacetate (or amide) is one of the most important alkylating agents used for the modifications of proteins for biochemical study. Thus this type of reaction definitely warrants further investigation but is beyond the scope of this thesis. The final procedure utilized for the isolation of the methionine containing peptides is as outlined in the Method section of this chapter.

Trypsin,because of its specificity, was used for the digestion of CM-tropomyosin in this study. The digest was applied to Whatman 3MM paper and high voltage electrophoresis used as the main purification method of methionine and histidine peptides. Once isolated,these peptides were sequenced by the Dansyl-Edman procedure as outlined in the General Methods, Chapter II.

The results of this study have been reported briefly elsewhere (Hodges and Smillie, 1970) and the experimental evidence for these findings is presented in this chapter.

2. MATERIALS AND METHODS

A. Materials

Iodoacetamide was purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A. and used without further purification. The N-acetyl amino acids, N-acetyl-dℓ-methionine, N-acetyl-ℓ-alanine, and N-acetyl-ℓ-aspartic acid were also products of Sigma Chemical Company. N-acetyl-dℓ-methionine sulfone was prepared by performic acid oxidation of N-acetyl-dℓ-methionine by the method of Moore (1963). Bromocresol Green (pH 4.0 yellow to pH 5.4 blue) was obtained from Fisher Scientific Company. All other chemicals and enzymes have been described in this thesis (Chapter II) or were reagent grade and were used without further purification.

B. Methods

(1) Isolation of Histidine Peptides

The histidine peptides were located on paper electropherograms by staining 1 cm guide strips with Pauly reagent (Dent, 1947). The histidine peptides were purified from the tryptic digest of CM-tropomyosin by high voltage electrophoresis at pH 6.5 followed by electrophoresis at pH 1.8, elution and purification on Dowex 1 ion-exchange chromatography.

(2) The Diagonal Purification Procedure

The major experimental procedure employed in this study for the purification of methionine containing peptides from tryptic digests of CM-tropomyosin was the diagonal electrophoretic procedure described by Tang and Hartley (1967).

a. Preparative Separation of the Peptide Mixture by Electrophoresis

A small portion of the preparative tryptic digest of CM-tropomyosin was applied as a band over 11 cm to Whatman No. 1 filter paper at a loading of approximatley 0.3 mg per cm and subjected to electrophoresis at pH 1.8 (60 v/cm;40 min). The remainder of the digest was applied as a band to the middle of Whatman No 3MM filter paper at a loading of approximately 1 mg per cm and submitted to electrophoresis at pH 6.5 (60 v/cm: 40 min) for the preparative isolation of the methionine containing peptides. Guide strips (1 cm) were stained with cadmium-ninhydrin reagent (Heilmann et al., 1957) to reveal the position of the peptides.

b. Alkylation of the Methionine Peptides on the Paper Strips

A 3 cm band from an electropherogram was cut parallel to the direction of electrophoresis at pH 1.8 or pH 6.5. The dry strip was sprayed evenly in a fume hood with 0.1 M iodoacetamide in pyridine-acetate electrophoresis buffer, pH 3.5, until thoroughly wet but not running. It was then arranged on a glass rack, care being taken to avoid contact between different parts on the paper. The rack was placed in a dessicator containing a tray of pH 3.5 buffer and the strip incubated at room temperature for 14-16 hours. The strip was

dried in the fume hood and then dipped in acetone several times to remove excess iodoacetamide.

c. Diagonal Electrophoretic Peptide "Maps"

Diagonal electrophoretic peptide "maps" of CM-methionine peptides were prepared as follows: The iodoacetamide treated strips were stitched across a full sheet of Whatman No. 3MM or Whatman No. 1 paper and subjected to electrophoresis at pH 6.5 (60 v/cm; 40 min) or at pH 1.8 (60 v/cm; 35 min) at right angles to the original direction. The sheets were stained with cadmium-ninhydrin reagent to reveal the positions of CM-methionine peptides which migrate off the diagonal towards the negative pole (cathode).

d. Isolation of CM-Methionine Peptides

The CM-methionine peptides were located on the pH 1.8/pH 1.8 diagonal peptide "map" and a comparison was made to the pH 6.5/pH 6.5 diagonal peptide "map". There were many deceptive spots off the diagonal on the pH 6.5/pH 6.5 diagonal "map". The minimum number of spots off the diagonal were observed with the pH 1.8/pH 1.8 diagonal. The following procedure enabled one to interrelate the spots off each diagonal, thereby ruling out all the deceptive spots off the pH 6.5/pH 6.5 diagonal. One of the original pH 6.5 electropherograms was arbitrarily divided into 7 horizontal bands, each between 5-8 cm high and 5 cm wide. Each band was subjected to pH 1.8 electrophoresis. Guide strips of 1 cm were stained with cadmium-ninhydrin. The remaining 3 cm strip was reacted with iodoacetamide and stitched on a full sheet and exposed to electrophoresis at pH 1.8 at right angles to the original direction. This will give 7, pH 6.5, pH 1.8/pH 1.8

diagonals. Comparison of these with the pH 1.8/pH 1.8 diagonal of the total digest revealed the location of each methionine peptide off the 1.8/1.8 diagonal at pH 6.5 on the preparative electropherograms. After location of the CM-methionine peptides, the corresponding bands from the original pH 6.5 preparative electropherograms were cut out, stitched to a new sheet and electrophoresis at pH 1.8 performed or reacted with iodoacetamide and stitched to a new sheet of paper for electrophoresis at pH 6.5. The corresponding bands at pH 1.8 were cut out and reacted with iodoacetamide, and then stitiched to a new sheet of paper for electrophoresis at pH 1.8. These two methods are summarized below.

Further purification of the CM-methionine peptides was frequently necessary, because of diagonal contaminants or because of contaminating peptides of similar mobilities. The purification was achieved by electrophoresis at pH 1.8, 3.5, and 6.5. The pure peptides were then eluted from the paper with water or volatile buffer.

e. Acid Hydrolysis and Sequence of CM-methionine Peptides

The general methods of Tang and Hartley (1967) were used.

i. Conversion of CM-methionine into Homoserine

The CM-methionine is not stable to acid hydrolysis and peptides containing these residues were heated in water for 2 hr at 100°C in sealed tubes to convert CM-methionine into the more stable homoserine lactone. Acid hydrolysis was then carried out.

ii. Conversion of Homoserine Lactone to Homoserine

The acid hydrolysates for amino acid analysis or of DNS-peptides for sequence were evaporated to dryness, dissolved in 50 µl of aqueous 2N-NH_3, and incubated for 1 hour at 37°C to convert the homoserine lactone to homoserine. Amino acid analysis and sequence could now be performed. The molar yields of homoserine by amino acid analysis were low (0.5-0.8) using this procedure. Later studies in this thesis used the method of Ambler (1965) and considerably higher yields of homoserine were obtained (0.9-1.0). This procedure is described in Chapter II, page 30.

f. Isolation of Methionine Sulfone Peptides

Because of the poor yields of some methionine sulfonium salt peptides, the methionine peptides were also isolated as their sulfones after their location by the diagonal "mapping" procedure. The methionine containing bands from the preparative pH 6.5 electrophoresis were cut out and subjected to electrophoresis at pH 1.8. The methionine peptides were oxidized with performic acid to convert methionine to methionine sulfone. The procedure for performic acid

oxidation of paper strips is given in Chapter V of this thesis. The methionine sulfone peptides were further purified by 1,8, 3.5, or pH 6.5 high voltage electrophoresis and in some instances by column chromatography. The methionine sulfone peptides were identified during purification procedures by amino acid analysis.

(3) Detection of N-Acetyl Amino Acids with Bromocresol Green

This reagent was prepared as described by Kirchner et al. (1951). Bromocresol green was 0.3% in 80% by volume methanol to which had been added 8 drops of 30% NaOH per 100 ml. Acidic components appear as yellow spots on a blue-green background. The method is insensitive after paper electrophoresis unless electropherograms are dried extremely well to remove all volatile acidic components from the paper. N-acetyl amino acids were separated on paper chromatography using 1-butanol:formic acid:ethyl butyrate:water (400:60:50:100 v/v). Removal of the last traces of formic acid was facilitated by the inclusion of ethyl butyrate in the solvent (Cheftel et al., 1951). Concentrations of 0.1-0.2 μmole per spot could be easily detected. The removal of volatile acidic components from paper chromatograms and electropherograms was accomplished by air drying in a fume hood for several days.

3. RESULTS

A. Peptide Nomenclature

The sequence of purification steps for the isolation and characterization of the unique methionine and histidine peptides was the following:

1) The CM-tropomyosin was digested with trypsin and all peptide designations were begun with the letter T.
2) The digest was separated at pH 6.5 electrophoresis. Four bands were designated T-1 to T-4 in numerical order to indicate their relative mobility on electrophoresis at pH 6.5 in the first dimension. Peptides from bands T-1 and T-2 are basic and those from band T-3 and T-4 are acidic.
3) Diagonal purifications for unique methionine peptides:

a. pH 6.5, pH 1.8/pH 1.8 diagonal "mapping" procedure

Bands T-1 to T-4 were subjected to the diagonal "mapping" procedure at pH 1.8/pH 1.8. The CM-methionine peptides from bands T-1 to T-4 are denoted T-CM1 to T-CM4. When more than one CM-methionine peptide arose from each band T-1 to T-4 they were assigned a capital letter in consecutive order, according to their relative mobility at pH 1.8 after alkylation, the most basic peptide having the letter A.

b. The CM-methionine peptides purified by the pH 6.5/pH 6.5 diagonal "mapping" technique through the alkylation of bands T-1 to T-4 were denoted T-CM1Y to T-CM4Y where Y designates the pH 6.5/pH 6.5 diagonal

purification. When more than one CM-methionine peptide arose from each band T-1 to T-4 they were assigned a capital letter in consecutive order, according to their relative mobility at pH 1.8, the most basic peptide having the letter A. This inter-relates the peptides from the pH 6.5/pH 6.5 and the pH 6.5, pH 1.8/pH 1.8 diagonal "mapping" procedures. For example, peptide T-CM3C and peptide T-CM3YC are identical differing only in the isolation procedure.

4) The methionine sulfone peptides

Bands T-1 to T-4 from preparative pH 6.5 electrophoresis were separated at pH 1.8. When more than one methionine peptide was separated at pH 1.8 they were assigned a capital letter in consecutive order, according to their relative mobility at pH 1.8, the most basic peptide having the letter A. Oxidation of the methionine peptides from the pH 1.8 electrophoresis was denoted by the capital letter M. Therefore, the methionine sulfone peptides arising from each band T-1 to T-4 were designated T-M1 to T-M4.

5) The histidine peptides arising from band T-4 were denoted T-4A and T-4B after pH 1.8 electrophoresis where A is the most basic peptide. Further purification of band T-4B gave two histidine peptides T-4B1 and T-4B2 where the lowest number is assigned to the most basic peptide.

6) Peptides subsequently digested with α-lytic protease have been designated by the symbol α. Peptides cleaved in water at 100°C have been assigned the letter w. Partial acid hydrolysis of peptides was denoted by the letter h. The products of the above cleavages are assigned numbers in numerical order to indicate their relative mobility at pH 6.5, the most basic peptide of the cleavage having the number 1. In cases where a further separation of a peptide band from pH 6.5 at pH 1.8 or pH 3.5 resulted in more than one peptide, the small letters in alphabetical order were assigned with the most basic peptide being given the letter a.

The mobilities at pH 6.5 were measured relative to that of lysine for basic peptides, indicated by a positive sign (m = +1.0) and relative to aspartic acid for acidic peptides, indicated by a negative sign (m = -1.0). The mobilities of peptides at pH 1.8 were measured relative to lysine (m' = 1.0). For pH 6.5 mobilities distances were measured from the center of the neutral band to the front of the spots and at pH 1.8 from the origin to the front of the spots.

An example illustrates the system: the peptide T-CM3YCw3 was obtained from band T-3 of the preparative pH 6.5 electrophoresis, CM denotes the peptide was isolated as the S-carbamoylmethyl methionine peptide, Y indicates purification by the pH 6.5/pH 6.5 diagonal method, and C designates the third most basic peptide off the pH 1.8/pH 1.8 diagonal of fraction T-3. After water cleavage at 100°C(w) of the pure peptide T-CM3YC, it was recovered as the third most basic peptide upon electrophoresis at pH 6.5.

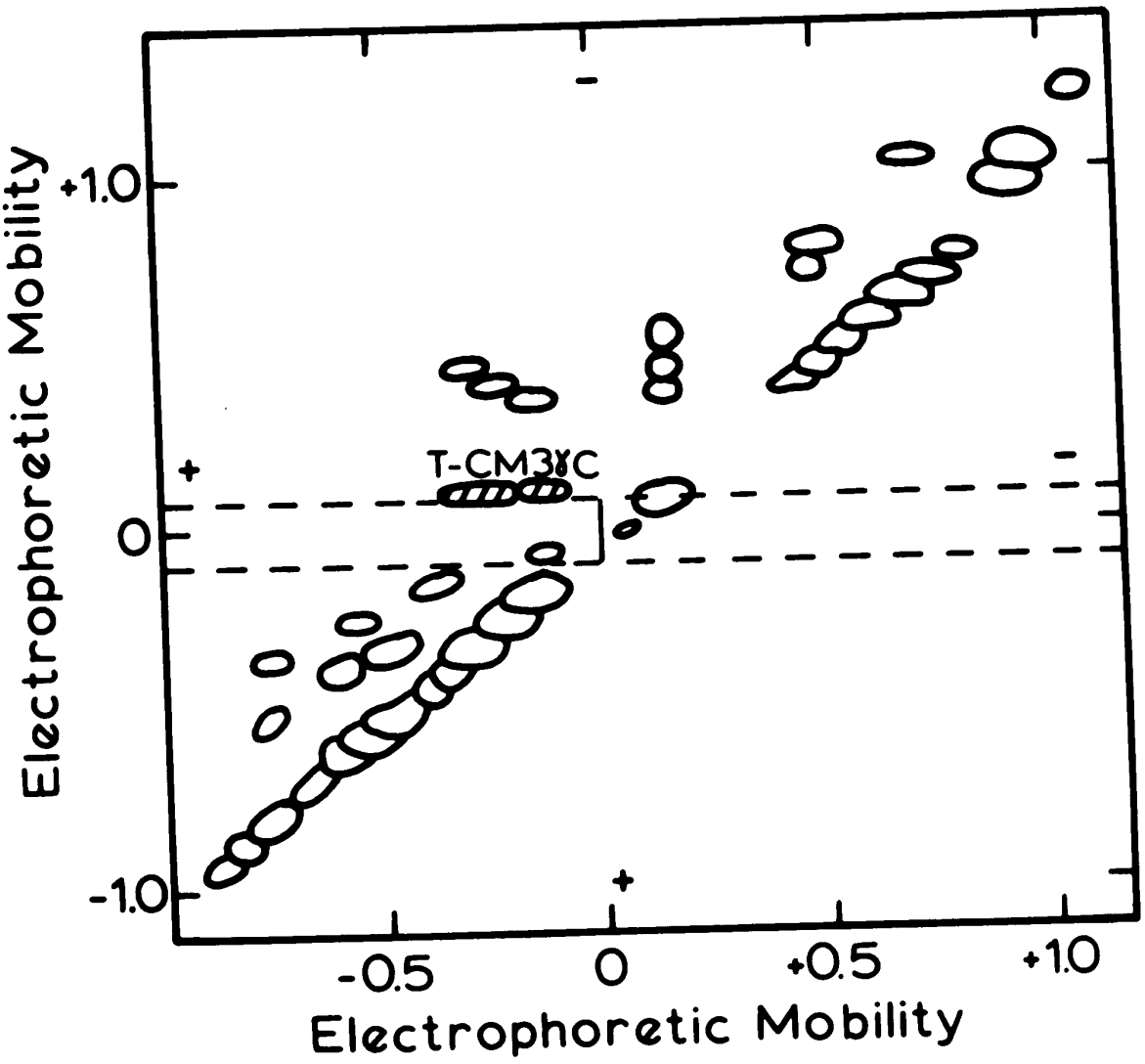


Fig. 17: A pH 6.5/pH 6.5 diagonal peptide "map" of a tryptic digest of CM-tropomyosin. The hatched spot denotes the CM-methionine peptide isolated from this diagonal procedure. The conditions and nomenclature are as described in the text.

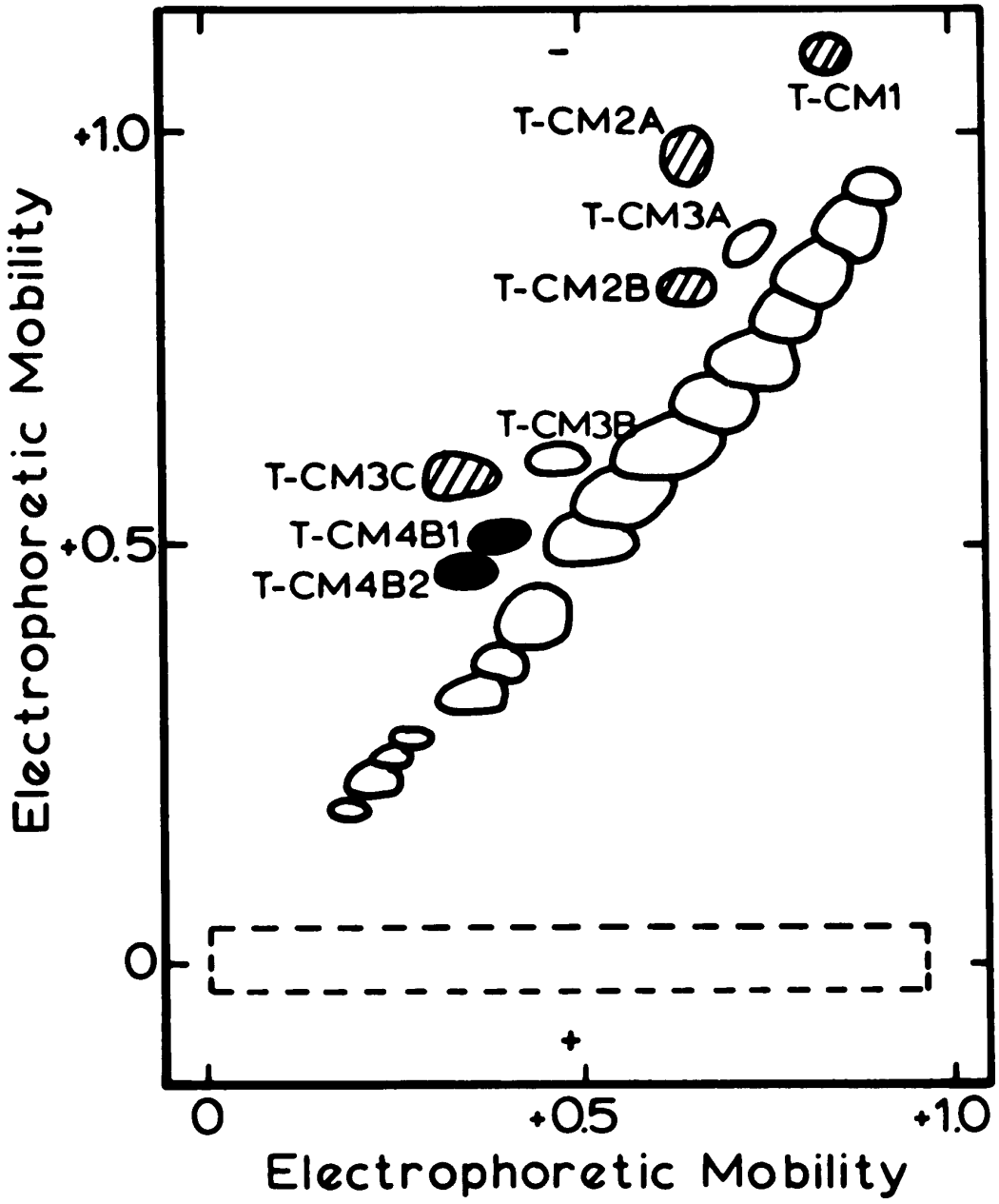


Fig. 18: A pH 1.8/pH 1.8 diagonal peptide "map" of a tryptic digest of CM-tropomyosin. The hatched spots denote the major CM-methionine peptides and the solid spots denote both CM-methionine and histidine containing peptides.

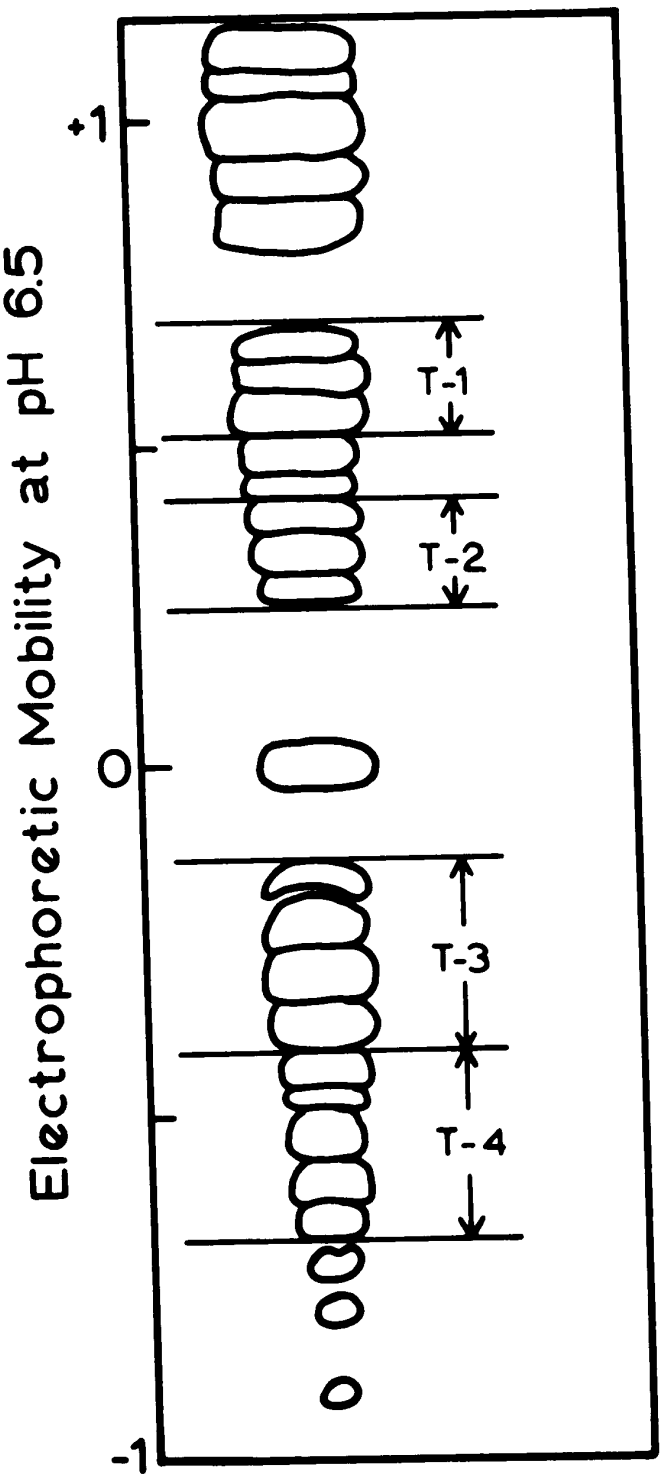


Fig. 19: Peptide bands obtained from a preparative pH 6.5 electrophoresis of a tryptic digest of CM-tropomyosin used for location and purification of histidine and methionine peptides.

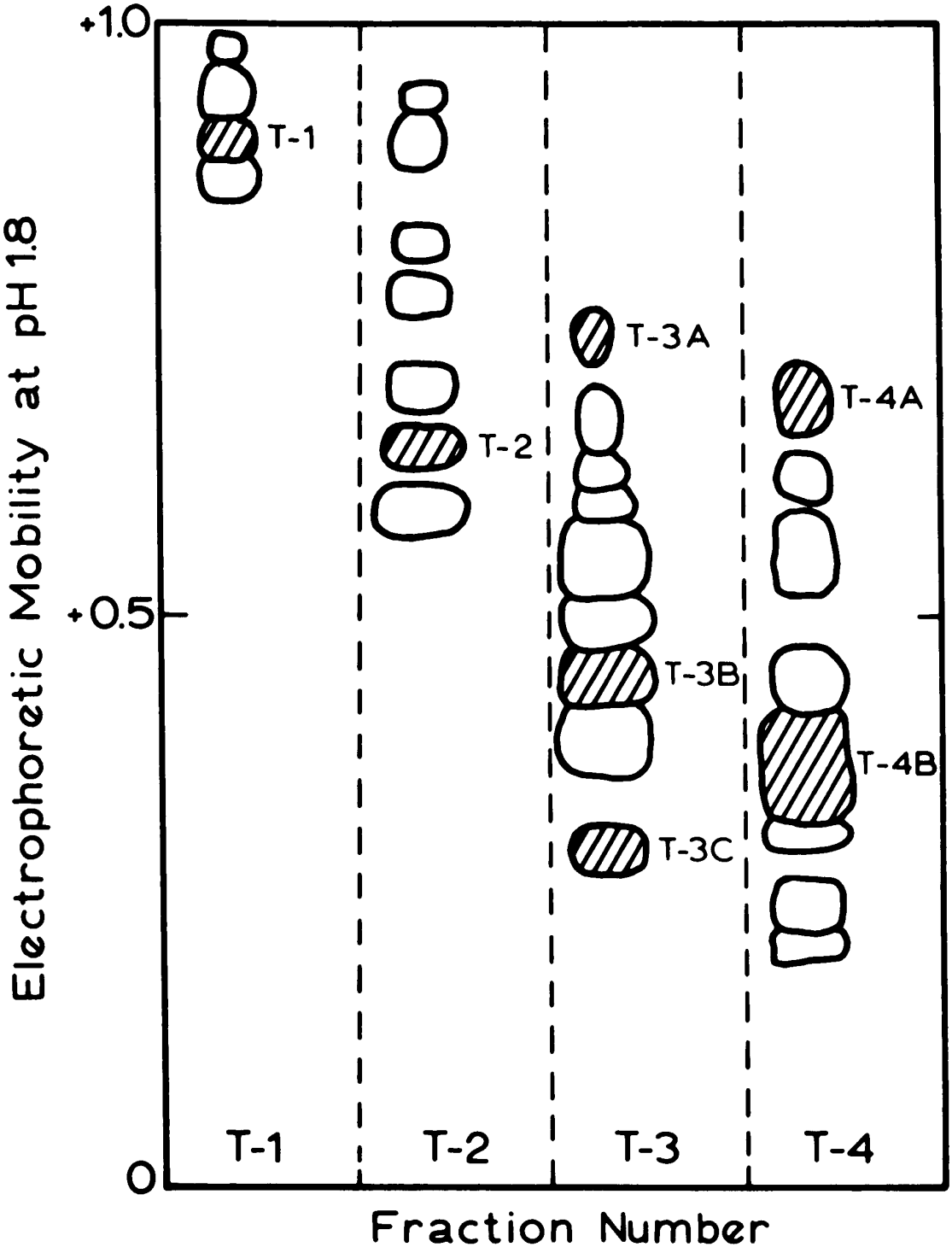


Fig. 20: Peptide bands arising from fractions T-1, T-2, T-3, and T-4 of preparative pH 6.5 electrophoresis obtained by electrophoresis at pH 1.8. Separations at pH 1.8 of bands T-1, T-2, T-3 and T-4 are first dimension pH 1.8 electrophoresis of pH 1.8/pH 1.8 diagonals shown in figures 21A, 21B, 21C, and 21D respectively. The hatched spots represent peptide bands taken for purification of histidine and methionine containing peptides.

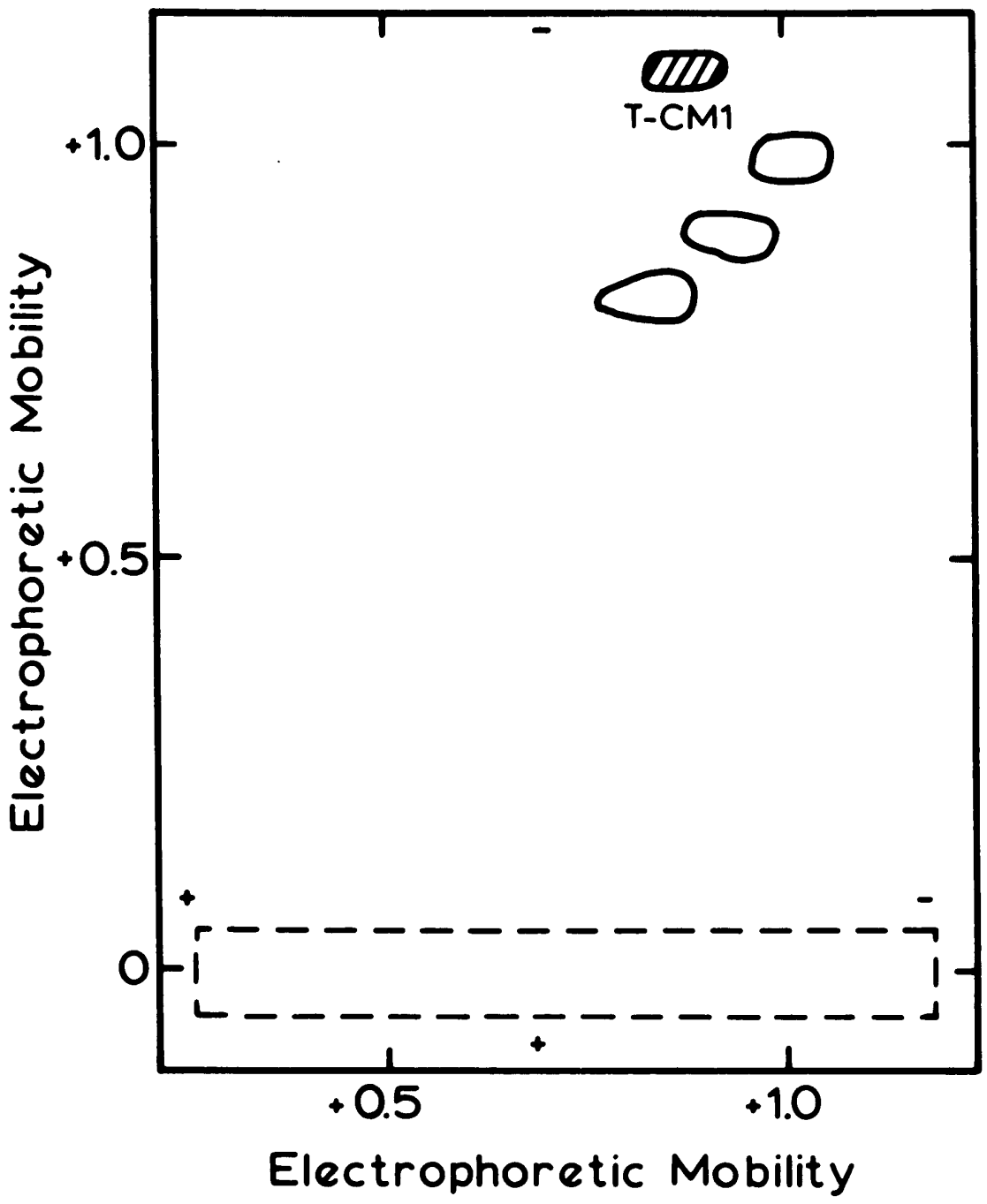


Fig. 21A: A pH 1.8/pH 1.8 diagonal peptide "map" of peptide band T-1 from preparative pH 6.5 electrophoresis. The hatched spot denotes the CM-methionine peptide. The conditions and nomenclature are as described in the text.

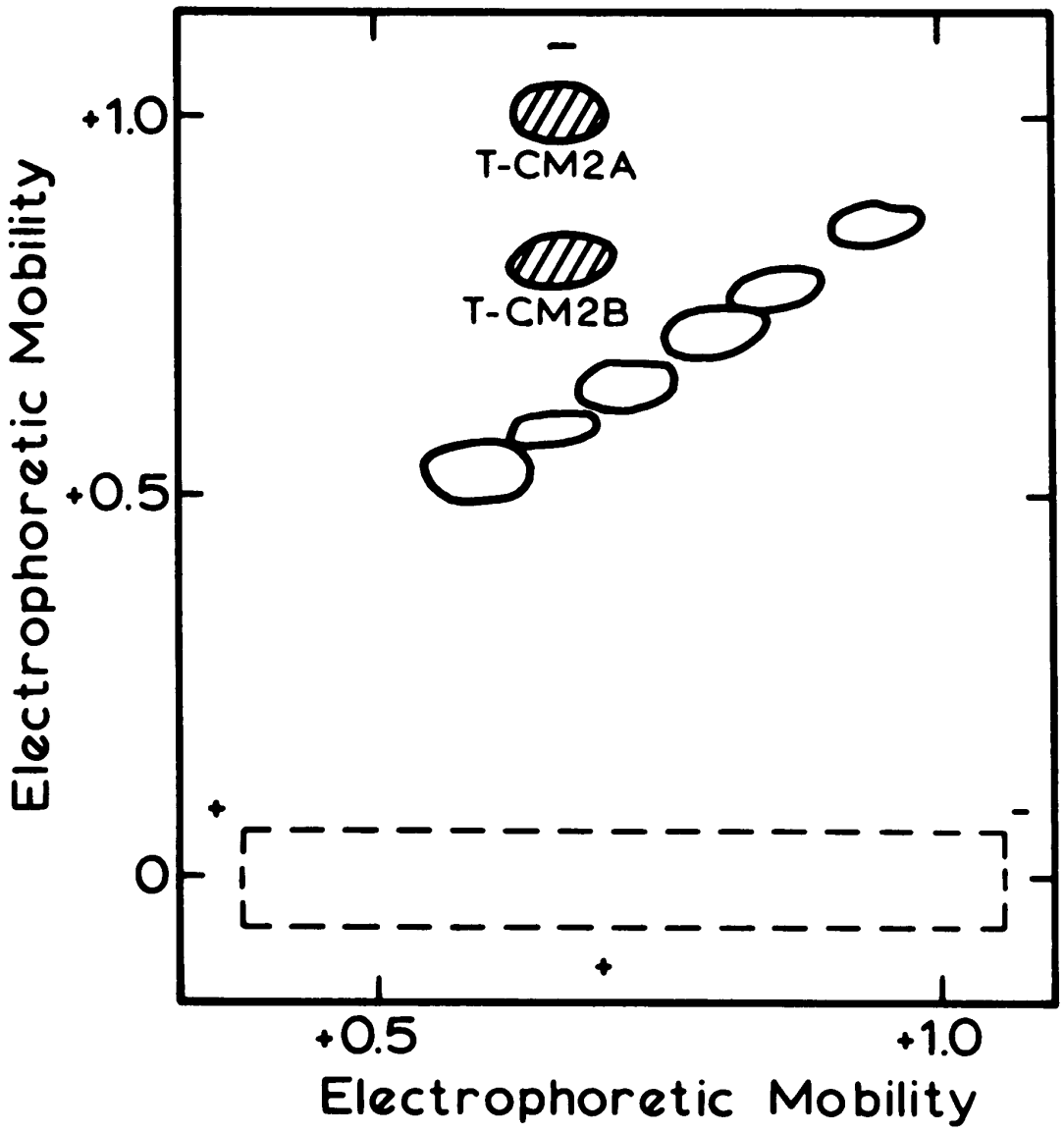


Fig. 21B: A pH 1.8/pH 1.8 diagonal peptide "map" of peptide band T-2 from preparative pH 6.5 electrophoresis. The hatched spots denote the CM-methionine peptides and the nomenclature corresponds to the designations used in text.

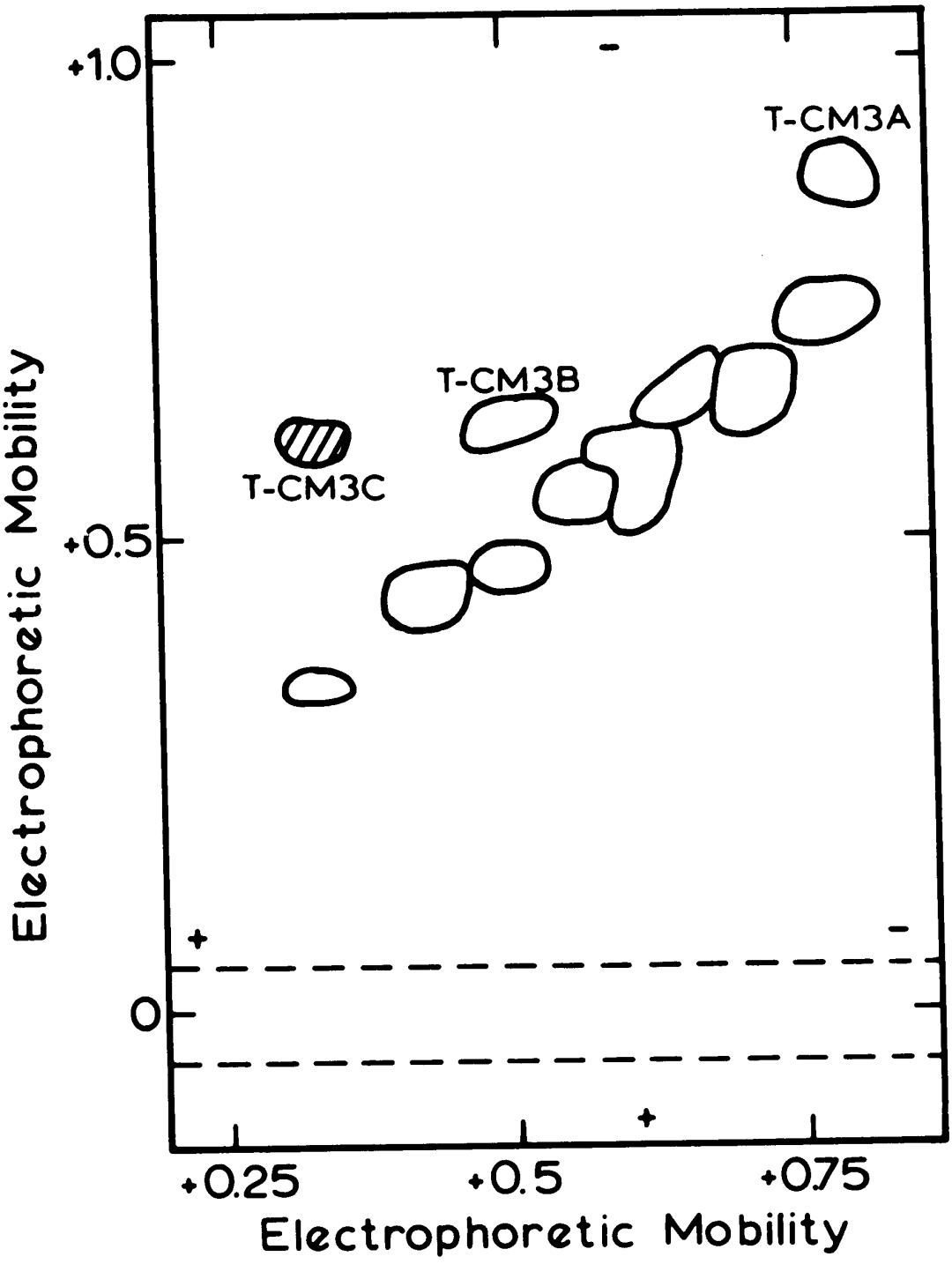


Fig. 21C: A pH 1.8/pH 1.8 diagonal peptide "map" of peptide band T-3 from preparative pH 6.5 electrophoresis. The major CM-methionine peptide is indicated by the hatched spot and symbols correspond to the designations used in the text.

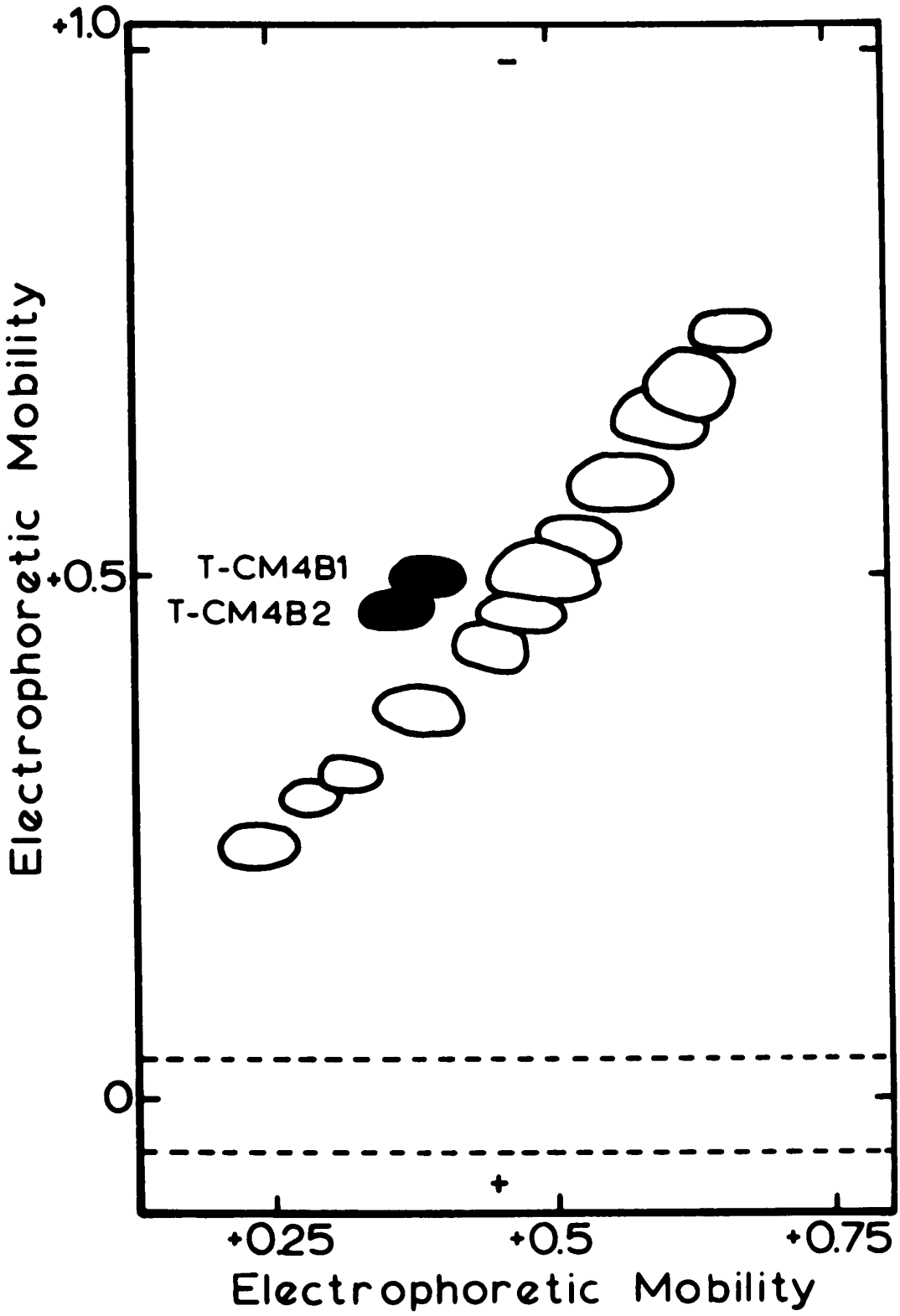


Fig. 21D: A pH 1.8/pH 1.8 diagonal peptide "map" of band T-4 from preparative pH 6.5 electrophoresis. The solid spots denote both CM-methionine and histidine containing peptides.

B. Purification, Isolation, and Characterization of the Unique Methionine and Histidine Peptides

The initial studies of this work used the pH 6.5/pH 6.5 diagonal "mapping" technique (Figure 17) for the isolation of the methionine containing peptides. This method was found unsatisfactory because of the large number of deceptive spots off the diagonal which necessitated their analysis for methionine derivatives and contributed to the contamination of methionine containing peptides making their isolation difficult. Only one CM-methionine peptide was isolated by this method.

The pH 1.8/pH 1.8 diagonal procedure of the preparative digest showed only 8 spots off the diagonal (Figure 18). These same 8 spots were located on the pH 6.5 preparative electropherograms by running pH 1.8/pH 1.8 diagonals of the fractions T-1, T-2, T-3, and T4 (Figure 19). The first dimension pH 1.8 separations are shown in Figure 20. The diagonals of each of these fractions are shown in Figures 21A, 21B, 21C, and 21D respectively.

(1) Histidine Peptides

Spraying the pH 1.8/pH 1.8 diagonals with Pauly reagent indicated that two of the CM-methionine peptides T-CM4A and T-CM4B also contained histidine (Figures 18 and 21D). For this reason these methionine containing peptides were not isolated as CM-methionine peptides but as histidine peptides with the use of Pauly reagent. To prevent low recoveries of these peptides from partial oxidation of methionine during isolation, the methionine was oxidized to methionine sulfone.

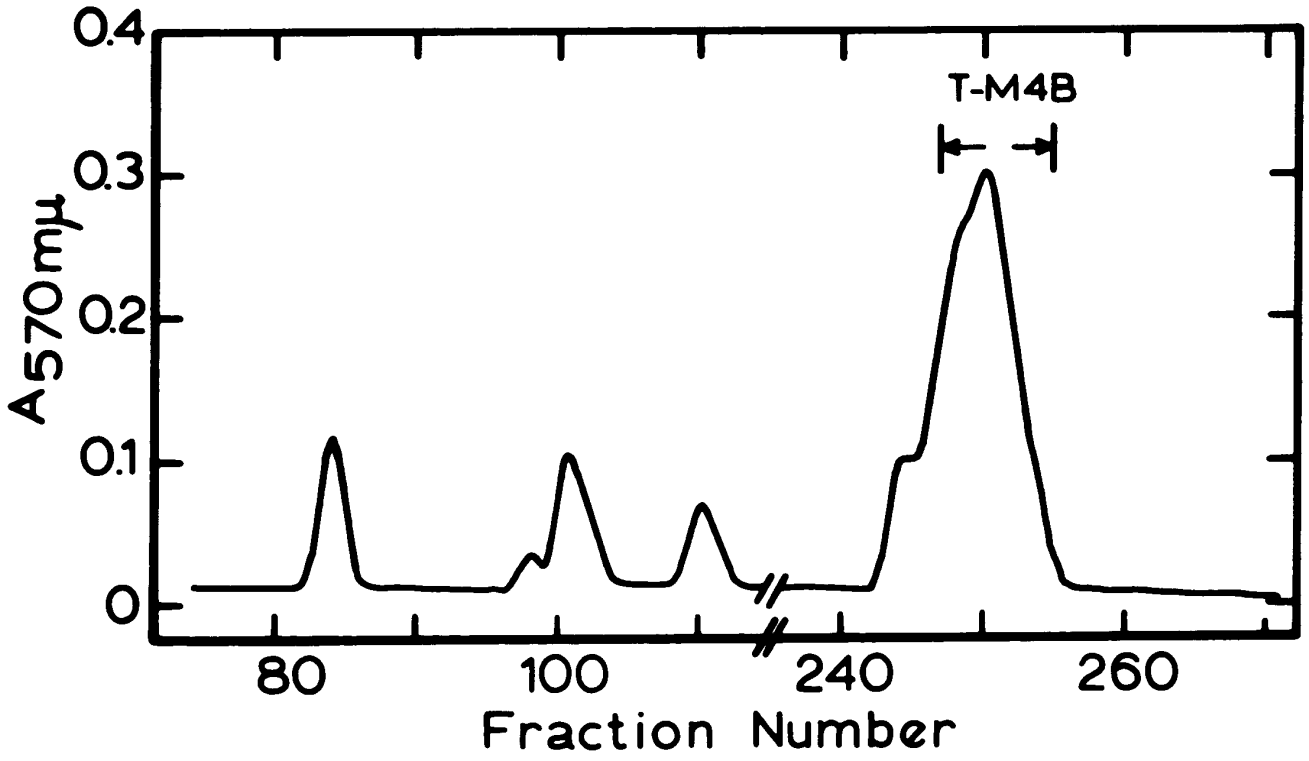


Fig. 22: Dowex 1 chromatography of fraction T-M4B from pH 1.8 electrophoresis. The flow rate was 1.0 ml per min and 3.0 ml fractions were collected. The effluent was monitored by automatic ninhydrin analysis (without alkaline hydrolysis) using 10% of the elution volume.

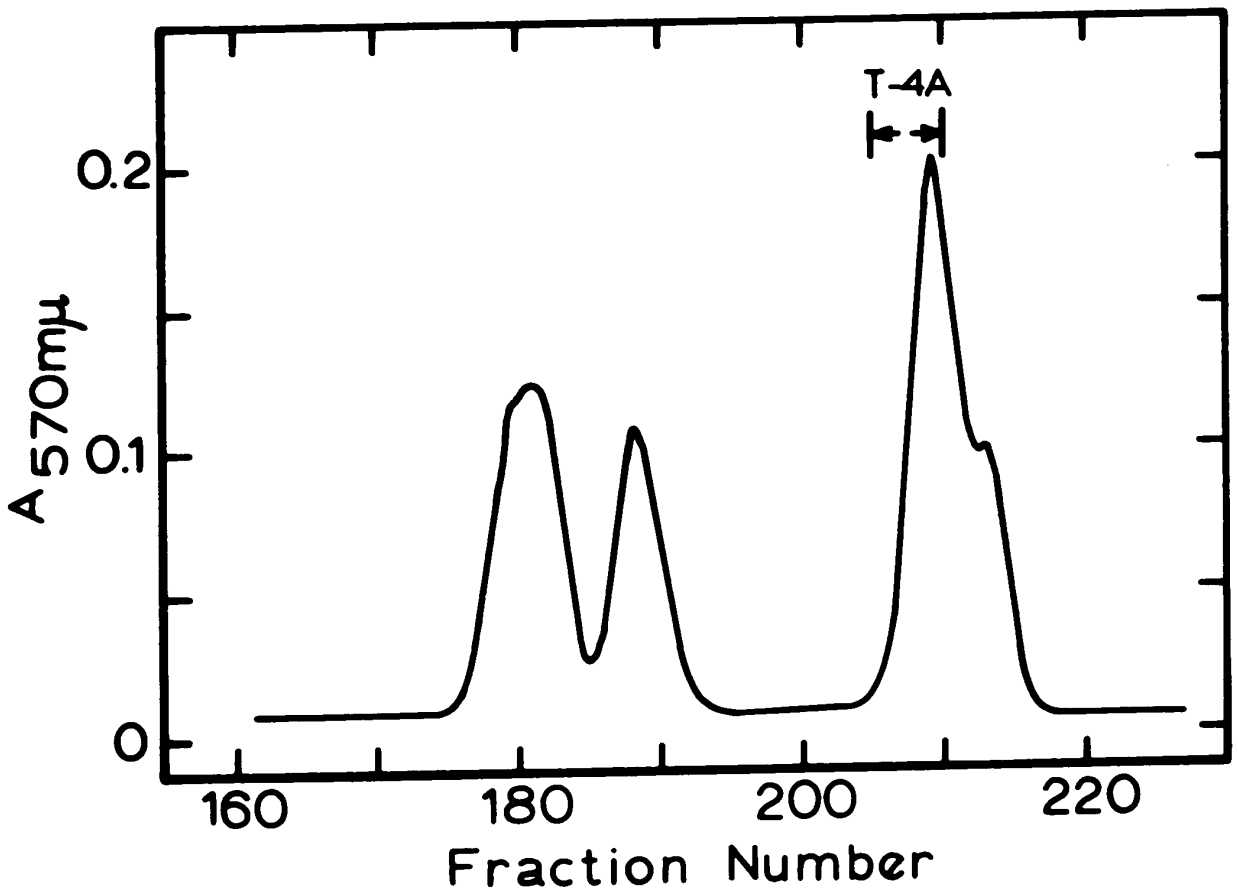


Fig. 23: Dowex 1 chromatography of fraction T-4A from pH 1.8 electrophoresis. The flow rate was 0.5 ml per min and 3.0 ml fractions were collected. Peaks were identified by automatic ninhydrin analysis (without alkaline hydrolysis) using 10% of the elution volume.

TABLE X

AMINO ACID COMPOSITION OF HISTIDINE PEPTIDES

ISOLATED FROM FRACTION T-4

(The Values are Expressed as Mole Ratios)

Amino Acid	T-M4B	T-M4B1	T-M4B2	T-4A
Histidine	0.86	0.86	0.92	1.00
Arginine				1.03
Asparatic Acid	2.90	3.14	3.06	1.98
Glutamic Acid	2.37	2.20	2.28	1.13
Threonine	0.94	1.00	0.89	
Serine	1.37	1.97	0.85	
Alanine	2.04	2.12	2.10	1.90
Leucine	2.02	1.97	2.01	
Isoleucine	1.37	1.84	0.96	0.98
Methionine Sulfone	0.91	0.94	0.94	
Number of residues	-	16	14	8
Mobility at pH 6.5 (m)	-	-0.53	-0.57	-0.49
N-terminal	Ala	Ala	Ala	His
Percentage Yield	17%	4.4%	3.9%	12.6%

All histidine containing peptides were located in band T-4 of the preparative pH 6.5 electropherograms (Figure 19).

a. Peptides from Band T-4

i. Purification of Peptides

The histidine peptides were further purified by cutting out band T-4 from the original preparative pH 6.5 electrophoresis and re-run on Whatman 3MM paper (60 v/cm; 50 min) at pH 1.8 (Figure 20). The histidine peptides T-4A and T-4B were still impure at this stage of purification. The band T-4B was oxidized with performic acid to convert methionine to methionine sulfone and this fraction was denoted T-M4B. These bands were eluted with pH 3.5 electrophoresis buffer and the samples freeze-dried. Each band was then applied to a Dowex 1 column (0.9 cm x 169 cm). The general operation procedures for Dowex 1 chromatography are described in Chapter II, page 26. The technicon automated analytical system was set up to sample 0.1 ml per min or 10% of the column effluent for ninhydrin analysis without alkaline hydrolysis. Sample T-M4B was eluted from the column at a flow rate of 60 ml per hour and sample T-4A at a flow rate of 30 ml per hour. Fractions of 3.0 ml were collected on each column run. Pure T-M4B was obtained by pooling fractions 248 to 256 inclusive (Figure 22) and pure T-4A by pooling fractions 205 to 210 inclusive (Figure 23). The early portions of the chromatograms are not shown since they contain only an ammonia peak.

TABLE XI

AMINO ACID COMPOSITION AND SEQUENCE OF α-LYTIC PROTEASE

PEPTIDES FORMED BY DIGESTION OF PEPTIDE FRACTION T-M4B

BY α-LYTIC PROTEASE

(Values are Expressed as Mole Ratios)

Peptide	Electrophoretic Mobility at pH 6.5 (m)	Amino Acid Composition and Sequence	% Yield	Ninhydrin Colour	Pauly Reagent	M.W.	Calculated Net Charge *
T-M4B 1	-0.53	Ala-Ile-Ser-Glu-Glu-Leu-Asp-His-Ala-Leu-Asn-Asp-Met(O)$_2$(Thr,Ser,Ile)	26	red	+	1791	-3.5
T-M4B 2	-0.57	Ala-Ile-Ser-Glu-Glu-Leu-Asp-His-Ala(Leu,Asn,Asp,Met(O)$_2$ Thr)	23	red	+	1603	-3.5
T-M4Ba1	-0.06	Ser-Ile (0.85 1.05)	19	yellow	-		
T-M4Ba2	-0.35	Leu-Asn-Asp-Met(O)$_2$-Thr-Ser-Ile (1.02 1.03 1.03 1.06 — 1.02 1.02 0.79)	8	red	-	814	-1.2
T-M4Ba3	-0.42	Leu-Asn-Asp-Met(O)$_2$-Thr (0.91 1.05 1.05 0.98 — 1.00)	22	red	-	614	-1.2
T-M4Ba4	-0.54	Ala-Ile-Ser-Glu-Glu-Leu-Asp-His-Ala-Leu (0.85 0.89 0.88 1.08 1.06 0.87 1.06 1.05 0.85 0.87)	14	red	+	1097	-2.5
T-M4Ba5a	-0.56	Ala-Ile-Ser-Glu-Glu-Leu-Asp-His-Ala (0.87 0.87 0.93 1.01 1.01 1.04 1.02 1.10 0.87)	19	red	+	984	-2.4
T-M4Ba5b	-0.56	Asn-Asp-Met(O)$_2$-Thr (1.02 1.02 1.01 — 0.96)	18	yellow	-	501	-1.4

*Offord (1966).

ii. Sequence Determinations

Peptide T-M4B

The yield of this peptide fraction was 1.7 μmole (17%) after purification by Dowex 1 chromatography. Approximately 0.5 μmoles of this fraction was spotted on Whatman No. 1 paper and electrophoresis at pH 6.5 (60 v/cm) was carried out for 80 min. Two cadmium-ninhydrin positive peptides were separated. These peptides had identical amino acid compositions except for peptide T-M4B1 which had two more residues (Ser and Ile). The results of amino acid composition, N-terminal analysis by the "dansyl" method, and electrophoretic mobilities of these two peptides and the mixture are summarized in Table X. These two peptides were submitted to the 'Dansyl-Edman' degradation and the following partial sequences may be written for:

T-M4B1 Ala-Ile-Ser-Glx-Glx-Leu-Asx-His-Ala-Leu-Asx-Asx-Met(O)$_2$(Thr, Ser, Ile)

T-M4B2 Ala-Ile-Ser-Glx-Glx-Leu-Asx-His-Ala(Leu,Asx,Asx,Met(O)$_2$,Thr).

Peptide T-M4B2 is clearly a smaller variety of the peptide T-M4B1. The final purification by paper electrophoresis for these peptides resulted in more than 50% losses, probably mainly during elution of the peptides from the filter paper. For these reasons, the peptide mixture T-M4B was subjected to further enzymatic cleavage to obtain the complete sequence. Fraction T-M4B from Dowex 1 chromatography (0.50μmoles) was digested with α-lytic protease (.005 μmole) in 0.05 M N-ethylmorpholine acetate buffer pH 8.0 at 37°C for 5 hr. The digest (1.85 ml) was applied

TABLE XII

pH 6.5 MOBILITY AND CALCULATED NET CHARGE[1] OF PEPTIDES

T-M4B1 AND T-M4B2 DURING EDMAN DEGRADATIONS

No. of Degradation	N-terminal Residue	Pauly Reagent	M.W.	T-M4B1 Mobility (-m)	Calc. Net Charge	M.W.	T-M4B2 Mobility (-m)	Calc. Net Charge
0	Ala	+	1791	0.53	-3.5	1603	0.57	-3.5
1*	Ile							
2*	Ser							
3	Glx	+	1391	0.65	-3.6	1202	0.71	-3.5
4	Glx	+	1261	0.50	-2.5	1073	0.54	-2.5
5	Leu	+	1148	0.32	-1.5	960	0.35	-1.5
6	Asx	+	1033	0.34	-1.5	845	0.37	-1.4
7	His	+	896	0.23	0 to -1.0	708	0.26	0 to -1.0
8	Ala	-	759	0.31	-1.0	571	0.36	-1.0

* Mobilities not determined.

[1] Offord (1966).

as a 20 cm band (approximately 0.025 μmole per cm) at a distance of 20 cm from the anode on Whatman No. 1 paper. After electrophoresis at pH 6.5 for 90 min, the digestion products were separated into five major cadmium-ninhydrin positive bands. Bands T-M4Bα4 and T-M4Bα5 were cut out together and separated further by electrophoresis at pH 1.8 (60 v/cm; 100 min). Pure peptides T-M4Bα4, T-M4Bα5a and T-M4Bα5b were obtained. The results of amino acid composition, amino acid sequence by the 'Dansyl-Edman' procedure, and electrophoretic mobilities of the α-lytic protease peptides formed by α-lytic protease digestion of peptide fraction T-M4B are summarized in Table XI.

The net negative charges calculated from Offord (1966) presented in Table XI for each peptide are consistent with peptides T-M4B1 and T-M4B2 containing all acidic residues except one amidated residue in the C-terminal half of the peptides. The partial negative charges of -3.5, -3.5, -2.5, -2.4 for peptides T-M4B1, T-M4B2, T-M4Bα4, and T-M4Bα5a respectively are attributable to the presence of histidine in these peptides which contains approximately one-half a positive charge on the imidazole group at pH 6.5. The negative mobility of peptide T-M4Bα5b is inconsistent with the presence of two aspartic acid residues and can be accounted for by an abnormally low pK for the α-amino group of this peptide. This is a common observation with peptides containing N-terminal asparagine. To obtain an unequivocal result the net charge was determined on peptides T-M4B1 and T-M4B2 during Edman degradations by their mobility at pH 6.5 (Table XII). The data shows the presence of one amide in the C-terminal half of the peptides. To determine the exact position of the amidated residue the

TABLE XIII

pH 6.5 MOBILITY AND CALCULATED NET CHARGE[1] OF DANSYLATED PEPTIDES T-M4Bα5b, T-M4Bα2 AND T-4Aα2 DURING EDMAN DEGRADATIONS

Peptide	No. of Degradation	N-terminal Residue	M.W.	Mobility	Calculated Net Charge
T-M4Bα5b	0	DNS-Asx	734	1.00	-2.0
	1	DNS-Asx	620	1.13	-2.0
T-4Aα2	0	DNS-Glx	838	1.21	-2.6
	1	DNS-Asx	709	0.96	-1.9
	2	DNS-Ala	594	0.61	-1.0
	3	DNS-Asx	523	0.62	-1.0
T-M4Bα2	0	DNS-Leu	-	-	-
	1	DNS-Asx	934	0.83	-2.0

[1] Gray (1967).

net charge of the dansylated peptides T-M4Bα5b and T-M4Bα2 during Edman degradations was calculated (Table XIII). The sequence -Asn-Asp- is indicated. From the data presented, the following sequence may be written for peptides T-M4B1 and T-M4B2:

T-4B1 Ala-Ile-Ser-Glu-Glu-Leu-Asp-His-Ala-Leu-Asn-Asp-Met$(O)_2$-Thr-Ser-Ile.

T-4B2 Ala-Ile-Ser-Glu-Glu-Leu-Asp-His-Ala-Leu-Asn-Asp-Met$(O)_2$-Thr.

Peptide T-4A

This peptide was pure as judged by its amino acid composition shown in Table X. The total recovery of this peptide was 1.26 μmole (12.6 per cent). The major loss occurred from the Dowex 1 fractionation where just over 50% of T-4A was recovered without contamination.

The 'dansyl' method yielded N-terminal histidine. The complete sequence of this peptide was determined from the sequence of the α-lytic protease fragments obtained by digestion of T-4A with α-lytic protease as follows: Peptide T-4A (0.40 μmole) was digested with α-lytic protease (0.008 μmole) in 1.4 ml 0.05 M N-ethylmorpholine acetate buffer, pH 8.0 (ratio of α-lytic protease to peptide, 1:50) at 37°C for 16 hours. The digest separated by pH 6.5 electrophoresis (60 v/cm; 45 min) gave two major cadmium-ninhydrin positive bands. Peptide T-4AαLP1 was further purified by electrophoresis at pH 3.5 (60 v/cm; 50 min). The amino acid composition, electrophoretic mobilities and amino acid sequence of the α-lytic protease peptides obtained are given in Table XIV. The relatively low positive mobility of

TABLE XIV

AMINO ACID COMPOSITION AND SEQUENCE OF α-LYTIC PROTEASE

PEPTIDES FORMED BY α-LYTIC PROTEASE DIGESTION OF PEPTIDE T4A

(Values are Expressed as Mole Ratios)

Peptide	Electrophoretic mobility at pH 6.5 (m)	Amino Acid Composition and Sequence	Cadmium Ninhydrin Color	Pauly Reagent	M.W.	Net Charge
T-4A	-0.49	His-(Ile,Ala,Glu,Asp,Ala,Asp,Arg) ⟶	orange	+	926	-2
T-4Aα1	+0.07	His-Ile-Ala 0.95 1.03 1.03 ⟶ ⟶ ⟶	orange	+	339	0
T-4Aα2	-0.76	Glu-Asp-Ala-Asp-Arg 1.02 1.00 0.94 1.00 1.03 ⟶ ⟶ ⟶ ⟶ ⟶	orange	-	605	-2

peptide T-4Aα1 compared to the histidine containing peptides of T-M4B is attributable to a lower pK for imidazole when histidine is in the N-terminal position of a peptide. Thus at pH 6.5, peptides with N-terminal histidine will bear a lesser positive net charge than peptides with an internal histidine. Peptide T-4Aα2 has a mobility with respect to aspartic acid of m = -0.76, suggesting that there was a net negative charge of 2 (Offord, 1966) and that no amidated residues were present. This result substantiates the conclusion of little positive charge on the imidazole group of the N-terminal histidine of T-4A where the calculated net charge was -2. The conclusion of no amide groups in peptide T-4A was further verified by the calculation of the net charge of the dansylated T-4Aα2 during Edman degradations (Table XIII).

(3) Methionine Peptides

a. Peptides from Band T-1

1. Purification of Peptides

Band T-1 from the original pH 6.5 electrophoresis was shown to contain one methionine peptide by the pH 1.8/pH 1.8 diagonal fingerprint technique (Figure 21A) and is denoted T-CM1 on the "map". This CM-methionine peptide is shown on the pH 1.8/pH 1.8 diagonal "map" in Figure 18 of the total digest. The methionine peptide of band T-1 was subjected to electrophoresis at pH 1.8 (60 v/cm; 45 min). A 3 cm wide strip cut parallel to direction of pH 1.8 electrophoresis was alkylated with iodoacetamide and run at pH 1.8 at right angles to

TABLE XV

AMINO ACID COMPOSITION OF METHIONINE PEPTIDES ISOLATED FROM FRACTIONS T-1, T-2 AND T-3 OF PREPARATIVE pH 6.5 ELECTROPHORESIS

(Values are Expressed as Mole Ratios)

Amino Acid	Amino Acid Composition				
	T-CM1	T-M1	T-M2	T-CM3YC	T-M3C
Lysine	1.03	0.96	1.03	0.99	0.99
Histidine					
Arginine					
Aspartic Acid				1.12	1.04
Threonine					
Serine					
Glutamic Acid			1.06		
Glycine	0.97	1.00			
Alanine				0.98	1.04
Isoleucine				0.92	0.97
Leucine			0.94		
Methionine Sulfone		1.04	1.96		0.97
Homoserine	0.80			0.67	
Number of Residues	3	3	5	5	5
Mobility at pH 6.5 (m)	+1.05	+0.60	+0.34	0	-0.40
Mobility at pH 1.8 (m')	1.15	0.90	0.67	0.63	0.34
N-terminal	Gly	Gly	$Met(O)_2$	Negative	Negative
Percentage Yield	22%	10%	55%	20%	54%

the original direction of 1.8 electrophoresis. The result of such a pH 1.8/pH 1.8 diagonal "map" is shown in Figure 21. This diagonal fingerprint allowed the location of the methionine containing peptide on the original pH 1.8 electropherogram (Figure 20). This band was either alkylated to isolate the CM-methionine peptide, T-CM1 or oxidized with performic acid to give the methionine sulfone peptide, T-M1.

Peptide T-CM1

The CM-methionine peptide, T-CM1, obtained after pH 1.8 electrophoresis (60 v/cm; 35 min) was further purified at pH 3.5 (60 v/cm; 35 min). This peptide was pure as ascertained by its amino acid composition shown in Table XV. This peptide gave an orange colour with cadmium-ninhydrin stain, which is consistent with glycine as the N-terminal residue of a lysine containing peptide as determined by the "dansyl" method. The following structure is obtained:

Gly-(CM-Met,Lys).

This peptide arose from a tryptic digest and therefore should contain the basic residue at the C-terminal position. Further evidence for the sequence of T-CM1 was obtained by heating the peptide in water at 100°C for 2 hours in a sealed tube. This procedure converts CM-methionine to homoserine lactone and cleaves the peptide on the C-terminal side of CM-methionine. N-terminal analysis of T-CM1w showed both glycine and lysine as N-terminal residues. From the data presented, the following sequence may be written for T-CM1:

CM-
Gly-Met-Lys.

Peptide T-M1

This peptide was further purified at pH 3.5 (60 v/cm; 50 min; origin at 11.5 cm from the anode) and pH 6.5 (60 v/cm; 100 min; origin 11.5 cm from anode). The amino acid composition and N-terminal of T-M1 are shown in Table XV. The amino acid sequence of this peptide by the 'Dansyl-Edman' procedure was found to be:

$$\underrightarrow{\text{Gly}}\text{-}\underrightarrow{\text{Met}}(\text{O})_2\text{-}\underrightarrow{\text{Lys}}.$$

b. Peptides from Band T-2

1. Purification of Peptides

Band T-2 from the original pH 6.5 electropherogram was shown to contain two methionine peptides by the pH 1.8/pH 1.8 diagonal 'mapping' procedure (Figure 21B). These peptides are denoted T-CM2A and T-CM2B. Attempts to isolate these peptides from the pH 6.5/pH 6.5 diagonal method resulted in impure peptides in poor yields. For this reason, these peptides were isolated as methionine sulfone containing peptides.

Band T-2 was subjected to electrophoresis at pH 1.8 (60 v/cm; 45 min). The diagonal fingerprint gave the position of the methionine containing peptides on the pH 1.8 electrophoresis (Figure 20). This band T-M2 was oxidized with performic acid and purified further by pH 3.5 electrophoresis (60 v/cm; 50 min). The amino acid composition of T-M2 is shown in Table XV.

ii. Sequence determinations

Peptide T-M2

The sequence as determined by the Dansyl-Edman method of this peptide is shown below:

$$\underrightarrow{\text{Met(O)}_2\text{-Gln-Met(O)}_2\text{-Leu-Lys.}}$$

The basic mobility of this peptide at pH 6.5 is consistent with the amidated residue. The finding of 2 methionine residues in peptide T-M2 explains the presence of the two CM-methionine spots T-CM2A and T-CM2B off the pH 1.8/pH 1.8 diagonal. T-CM2A has two methionine residues alkylated while T-CM2B has only one.

c. Peptides from Band T-3

i. Purification of peptides

The pH 1.8/pH 1.8 diagonal fingerprint of band T-3 from the original pH 6.5 electrophoresis (Figure 19) indicated three spots off the diagonal denoted T-CM3A, T-CM3B, and T-CM3C (Figure 21C). After location of these three bands on the preparative pH 1.8 electrophoresis of T-3 (60 v/cm; 45 min) bands T-3A, T-3B, and T-3C were oxidized with performic acid (Figure 20) and peptides T-M3A, T-M3B, and T-M3C were purified by pH 3.5 electrophoresis (60 v/cm; 50 min). Peptide T-M3C was still impure and was eluted and rerun on Whatman No. 1 paper at pH 6.5 for 2.5 hours at 60 v/cm (origin at 20 cm from cathode).

Peptides T-CM3A and T-CM3B were isolated by alkylation of bands T-3A and T-3B with iodoacetamide and pH 1.8 electrophoresis in the second

dimension (60 v/cm; 45 min), followed by pH 3.5 electrophoresis (60 v/cm; 50 min) after elution and re-applying to Whatman No. 1 paper.

The CM-methionine peptide, T-CM3γC was isolated by the pH 6.5/pH 6.5 diagonal electrophoretic technique (Figure 17) from band T-3 on the original pH 6.5 electrophoresis. The second dimension pH 6.5 electrophoresis was carried out at 60 v/cm for 1 hour. This peptide was neutral after pH 6.5/pH 6.5 electrophoresis and was further purified at pH 3.5 (60 v/cm; 50 min) and pH 1.8 (60 v/cm; 45 min).

ii. Sequence determinations

Peptides from bands T-3A and T-3B

The amino acid compositions of peptides T-CM3B, T-M3B, T-M3A, and T-CM3A after the final purification step indicated that they were still impure. The mole ratios of homoserine or methionine sulfone were low and variable, 0.22, 0.64, 0.25 and 0.36 respectively. The peptides seemed to be identical whether isolated as methionine sulfone or CM-methionine peptides, that is T-CM3A is equivalent to T-M3A and T-CM3B is equivalent to T-M3B. The yields of these peptides were between 1 to 1.7 per cent and it seems most likely that they are either impurities or minor varieties of the major methionine sequences already obtained. No further attempts were made to isolate these peptides.

TABLE XVI

AMINO ACID COMPOSITION AND SEQUENCE OF FRAGMENTS

ARISING FROM CLEAVAGE OF T-CM3VC IN WATER AT 100°C

(Values are Expressed as Mole Ratios)

Peptide	Electrophoretic Mobility at pH 6.5 (m)	N-Terminal	Amino Acid Composition and Sequence	Incubation Time (hours)	Percentage Yield	Cadmium-Ninhydrin Color	M.W.	Calculated Net Charge
T-CM3VC	0	-	N-acyl-(HSer,Asp,Ala,Ile,Lys) 067 1.12 0.98 0.92 0.99			red	688	0
T-CM3VCw1	+0.62	+	Ala Ile Lys 1.00 1.00 1.00 ⟶ ⟶ ⟶	2 5	5 21	red		
T-CM3VCw2	0	+	Asp Ala Ile Lys 1.02 1.00 0.98 1.01 ⟶ ⟶ ⟶ ⟶	2 5	58 50	red	445	0
T-CM3VCw3	-0.41	-	N-acyl-(HSer,Asp,Ala,Ile,Lys) 0.55 1.09 1.02 0.84 1.05	2 5	37 29	red	594	-1

Peptide T-CM3YC

The amino acid composition of this peptide is shown in Table XV. The 'dansyl' method for determination of the N-terminal was negative suggesting a blocked N-terminal residue. The reaction with cadmium-ninhydrin stain is not inconsistent with a blocked N-terminal, the red colour being produced from the ε-lysine group. The neutral mobility of T-CM3YC at pH 6.5 verifies the presence of a blocked N-terminal and that the acidic residue is not amidated.

The peptide was heated for 2 hours at 100°C in water in a sealed tube. The solution was cooled, a drop of 2N NH_3 added, and incubated at room temperature for 15 min to convert homoserine lactone to homoserine. The sample was applied to Whatman No. 1 paper and electrophoresis at pH 6.5 (60 v/cm; 40 min) was carried out. This specific cleavage resulted in three major cadmium-ninhydrin positive bands. Band T-CM3YCw2 was neutral at pH 6.5 and purified at pH 1.8 (60 v/cm; 45 min). The peptides were eluted with 1% NH_4OH. The results of the amino acid composition, N-terminal analysis and sequence by the Dansyl-Edman method, and electrophoretic mobilities are shown in Table XVI.

The mobility of T-CM3YCw3 with respect to aspartic acid was $m = -0.41$, suggesting that there was a net negative charge of 1 (Offord, 1966) and that aspartic acid rather than asparagine was present along with the blocked N-terminal. The 'dansyl' method for

TABLE XVII

AMINO ACID COMPOSITION OF FRAGMENTS ARISING

FROM PARITAL ACID HYDROLYSIS OF PEPTIDE T-M3C

(Values are Expressed as Mole Ratios)

Peptide	Electrophoretic mobility pH 6.5 (m)	pH 1.8	Amino Acid Composition	Percentage Yield Hydrolysis Time 12 Hr.	1 Hr.
T-M3C	-0.40	+0.33	N-acetyl(Met(O)$_2$,Asp,Ala,Ile,Lys) $_{Q99}$ $_{1.04}$ $_{1.04}$ $_{Q97}$ $_{Q97}$	0	15
T-M3Ch1	+0.62	+0.86	(Ala,Ile,Lys) $_{.097}$ $_{1.02}$ $_{1.01}$	67	36
T-M3Ch2	0	+0.38	Met(O)$_2$	65	1.6
T-M3Ch3	-0.88	-	N-acetyl-Met(O)$_2$	3[a]	21[a] 15[b]
T-M3Ch4	-1.00	+0.41	Asp	89	20

Elution of spot corresponding to position of N-acetyl methionine sulfone standard on:
[a]pH 6.5 electrophoresis, [b]paper chromatography

N-terminal determination was negative indicating the amino group was blocked. Peptide T-CM3YCw2 being neutral at pH 6.5 also showed the presence of aspartic acid rather than asparagine. The presence of peptide T-CM3YCw3 indicates that conversion of CM-methionine to homoserine had taken place without complete peptide bond cleavage. The appearance of peptide T-CM3YCw1 in low yields (5 per cent) suggests that some partial acid hydrolysis had occurred around the aspartic acid residue. This conclusion was further supported by a 5 hour incubation of peptide T-CM3YC where T-CM3YCw1 increased to 21 per cent with an equal decrease in the amounts of T-CM3YCw2 and T-CM3YCw3.

The data presented is consistent with the following sequence:

N-acyl-Met-Asp-Ala-Ile-Lys

Peptide T-M3C

The N-terminal methionine containing peptide was isolated as the methionine sulfone peptide T-M3C to obtain an unequivocal identification of N-acetyl methionine sulfone as the N-terminal residue. The acetyl group was previously identified by Alving *et al.* (1966). The amino acid composition of this peptide is shown in Table XV.

The sequence obtained by cleavage of T-CM3YC in water at 100°C was verified by a partial acid hydrolysis of peptide T-M3C in 0.03 N HCl for 12 hours at 110°C in an evacuated sealed tube and separation at pH 6.5 (60 v/cm; 30 min) of the digestion products. The neutral band at pH 6.5 was purified by pH 1.8 electrophoresis. The results are shown in Table XVII. The sequence may be written as follows:

N-acetyl-Met(O)$_2$-Asp-Ala-Ile-Lys

1 2 3

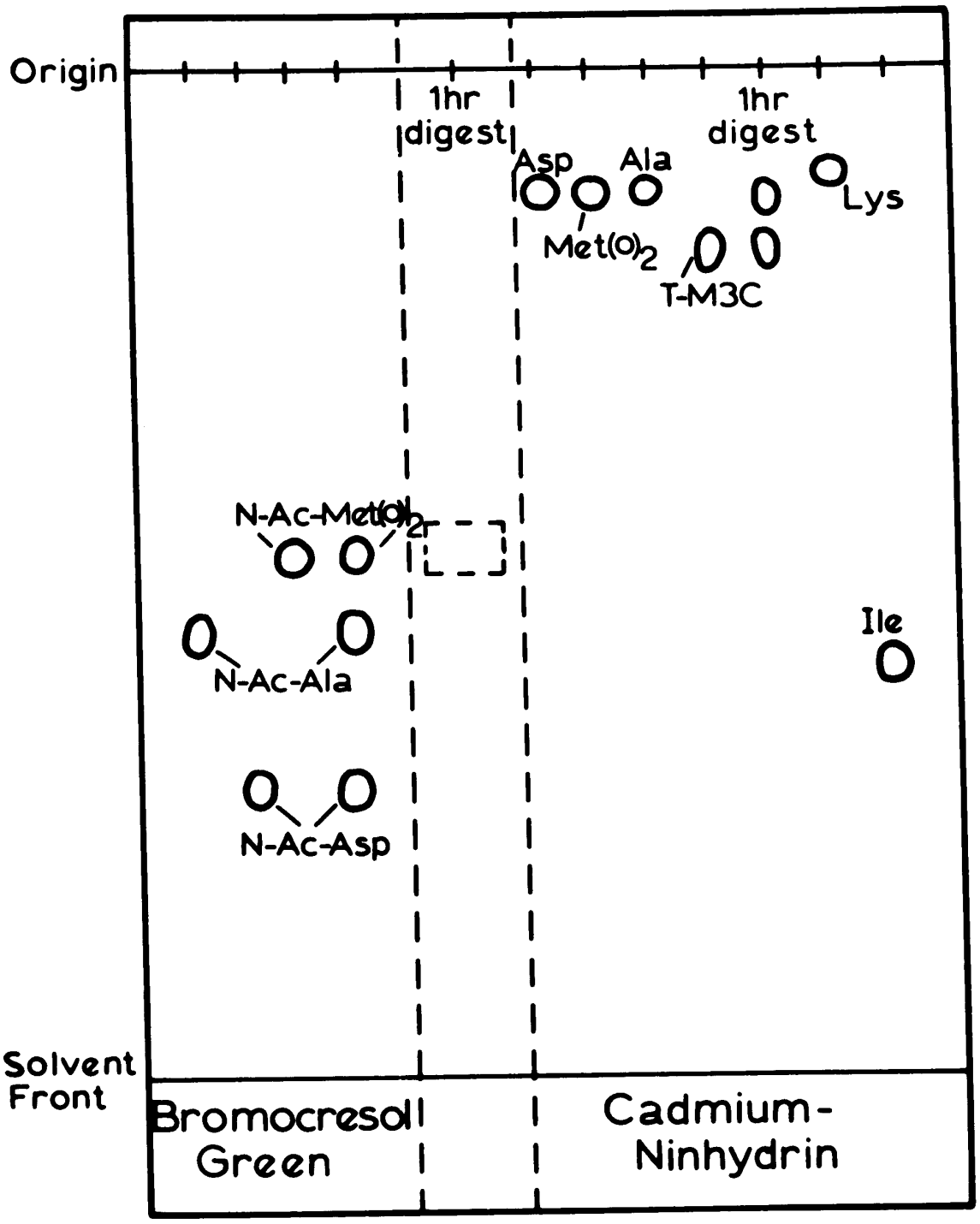


Fig. 24: Separation of a partial acid hydrolysate of peptide T-M3C on descending paper chromatography for the identification of N-acetyl methionine sulfone. The dotted square indicates the area of chromatogram eluted for acid hydrolysis and amino acid analysis. The conditions and nomenclature are as described in the text.

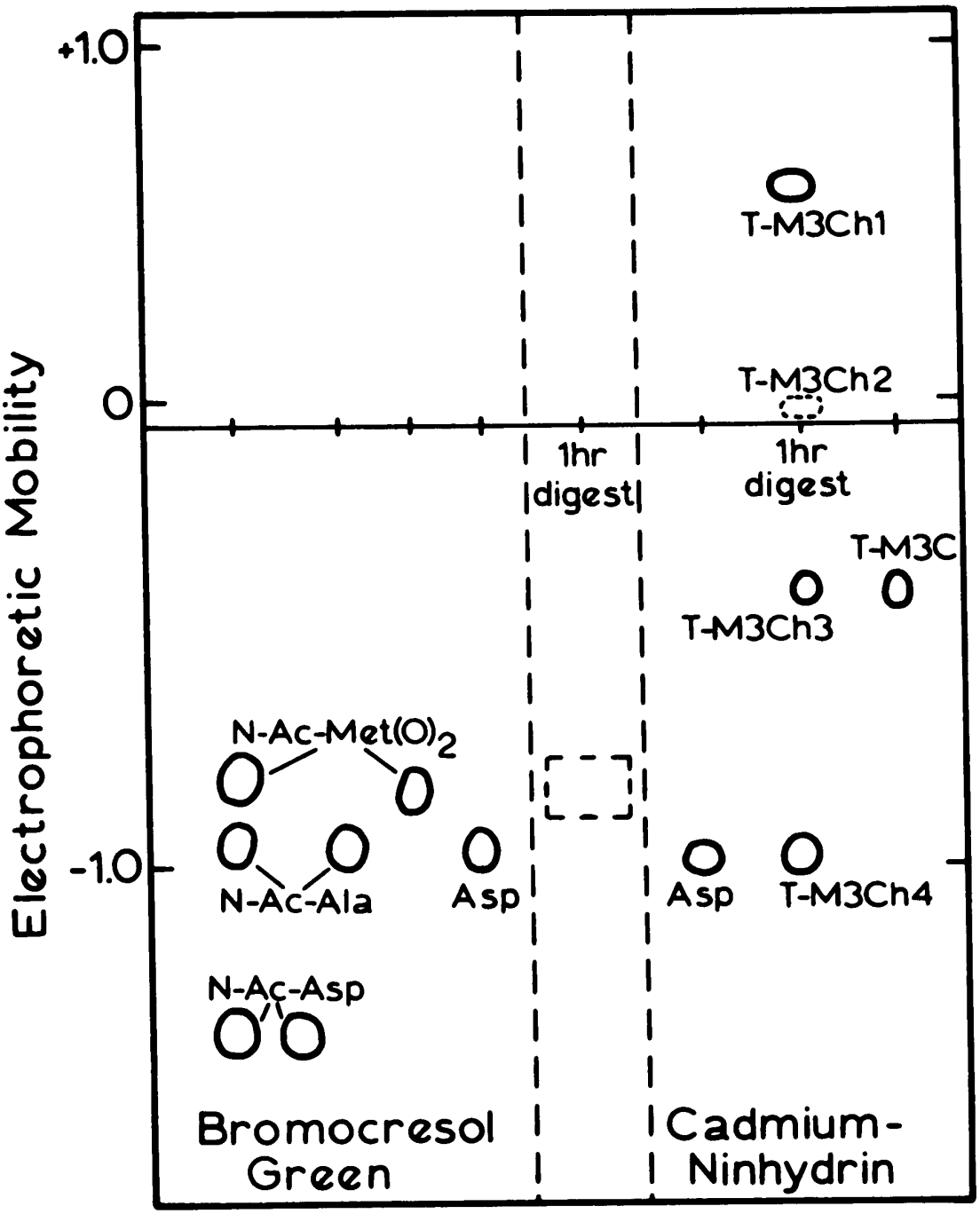


Fig. 25: Separation of a partial acid hydrolysate of peptide T-M3C on paper electrophoresis at pH 6.5 for the identification of N-acetyl methionine sulfone. The dotted square indicates the area of the electropherogram eluted for acid hydrolysis and amino acid analyses. The conditions and nomenclature are as described in the text.

Acid hydrolysis is occurring at the sites indicated by the arrows giving free aspartic acid, methionine sulfone, and the tripeptide.

A time study was carried out to determine the conditions of partial acid hydrolyses for preferential cleavage at Site 2 and leaving the majority of the N-acetyl bond unaffected (Site 1). T-M3C was incubated in 0.03 N HCl at 110°C for various times and the digests were subjected to electrophoresis at pH 1.8. At a partial acid hydrolysis time of 1 hour there was still complete hydrolysis at site 2 and 3 but little hydrolysis at site 1 indicated by the almost complete disappearance of methionine sulfone (Table XVII). The digest was separated on descending chromatography using 1-butanol: formic acid:ethyl butyrate:water (400:60:50:100 v/v) for 12 hours (Figure 24) and pH 6.5 electrophoresis (60 v/cm; 35 min) as shown in Figure 25. The area corresponding to the N-acetyl methionine sulfone standard was eluted and methionine sulfone identified on the amino acid analyzer after acid hydrolysis. The sequence of the N-terminal peptide may be written as follows:

$$\underrightarrow{\text{N-Ac-Met(O)}_2}\text{-}\underrightarrow{\text{Asp}}\text{-}\underrightarrow{\text{Ala}}\text{-}\underrightarrow{\text{Ile}}\text{-}\underrightarrow{\text{Lys}}.$$

4. DISCUSSION

The amino acid sequences of the two unique histidyl and 5 unique methionyl peptides isolated in this work are presented in Table XVIII. Interestingly, both N- and C-terminal peptides of tropomyosin were isolated. The sequence of peptide T-3C confirms the

TABLE XVIII

AMINO ACID SEQUENCES OF HISTIDINE AND METHIONINE PEPTIDES

Peptide	Amino Acid Sequence
T-1	Gly-Met-Lys
T-2	Met-Gln-Met-Leu-Lys
T-3C	N-Ac-Met-Asp-Ala-Ile-Lys
T-4A	His-Ile-Ala-Glu-Asp-Ala-Asp-Arg
T-4B1	Ala-Ile-Ser-Glu-Glu-Leu-Asp-His-Ala-Leu-Asn-Asp-Met-Thr-Ser-Ile
T-4B2	Ala-Ile-Ser-Glu-Glu-Leu-Asp-His-Ala-Leu-Asn-Asp-Met-Thr

presence of the N-acetylated tripeptide previously reported and is consistent with its amino acid composition (Alving et al., 1966). PeptidesT-M4B1 and T-M4B2, isolated from a tryptic digest of tropomyosin, contain no basic C-terminal residues and are believed to represent two varieties of the C-terminus of tropomyosin preparations. These peptides were isolated in approximately equal quantities. Fraction T-M4B contained approximately 53% and 47% of T-M4B1 and T-M4B2 respectively, as indicated by the recoveries of these peptides from the pH 6.5 electrophoretic separation (Table XI). The α-lytic protease digest of the mixture T-M4B showed this same ratio between the recoveries of peptides containing the Ser-Ile sequence and the total recoveries of threonine containing peptides (Table XI). This calculation gave 56% for Ser-Ile containing peptides which is in close agreement with the 53% of peptide T-M4B1.

The sequence of T-M4B1 is consistent with the previously described carboxypeptidase digests of the intact protein in which isoleucine and serine were liberated (Locker, 1954) and agrees with the first four C-terminal residues suggested by Locker (1954). However the presence of a C-terminal threonine residue had not previously been suspected. Threonine was released in the early minutes of carboxypeptidase digestion in lower quantities than serine and isoleucine (Kominz et al., 1957), however due to the variation in rates of liberation of different amino acids from the C-terminal position of a peptide it would be most difficult to distinguish between the following two possibilities:

1) Threonine as the third C-terminal residue

2) The mixture of peptides T-M4B1 and T-M4B2 where threonine is C-terminal on peptide T-M4B2 and the third residue in T-M4B1.

It seems highly unlikely that T-M4B2 could have arisen from T-M4B1 from a chymotryptic or other cleavage of the Thr-Ser bond during the tryptic digestion.

The finding of two unique histidine and five unique methionine peptides in tropomyosin is consistent with the presence of identical subunits. However the finding that the C-terminal end of the tropomyosin chains may differ by two residues in length is inconsistent with identity of polypeptide chains. The possibility remains that tropomyosin preparations contain more than one form of the protein with each form composed of two highly homologous or identical chains.

CHAPTER V

THE NUMBER AND AMINO ACID SEQUENCES OF CYSTEINE PEPTIDES FROM RABBIT SKELETAL TROPOMYOSIN

1. INTRODUCTION

Carboxymethylation of all free -SH groups of tropomyosin does not prevent reassociation of the subunits (Olander et al., 1967). Tropomyosin has been shown to influence the rate of polymerization of both ATP-G-actin and ADP-G-actin at 0°, and the acceleration of polymerization appears to be greatest when tropomyosin contains its full complement of -SH groups (Pragay and Gergely, 1968). However, modification of the -SH groups of tropomyosin whether by oxidation or blocking with -SH reagents neither prevents binding of the troponin-tropomyosin complex to actomyosin nor affects ability of this complex to confer calcium sensitivity (Yasui et al., 1968; Stewart and Levy, 1970).

The isolation of cysteine peptides of tropomyosin was carried out to ascertain the number of unique cysteine sequences and to relate this information to the subunit structure of the molecule. These peptides were isolated both as cysteic acid and ^{14}C-CMC peptides. Cysteic acid containing peptides were isolated from tryptic, peptic, and thermolytic digests of SH-tropomyosin using the diagonal electrophoretic procedure of Brown and Hartley (1963; 1966). This method relies on the change of electrophoretic mobility when cysteine peptides are oxidized by performic acid to cysteic acid peptides which are found off the diagonal. The ^{14}C-CMC peptides were isolated

from tryptic and peptic digests of ^{14}C-CM-tropomyosin using Dowex-50 ion-exchange chromatography for the initial separation and high voltage electrophoresis for final purifications. The isolated peptides were sequenced by the Dansyl-Edman procedure as previously described in Chapter II.

The results of this study have been reported briefly elsewhere (Hodges and Smillie, 1970) and the experimental evidence for these findings is presented in this chapter.

2. MATERIALS AND METHODS

A. Materials

Diethanoldisulfide (2-hydroxyethyldisulfide, dithiodiglycol), was purchased from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin, U. S. A. All other chemicals and enzymes have been described in this thesis (Chapter II) or were reagent grade and were used without further purification.

B. Methods

(1) Disulfide Interchange Reaction

a. Diethanol disulfide

Tryptic digests of SH-tropomyosin were carried out with a simultaneous disulfide interchange reaction to prevent oxidation and random disulfide interchange during proteolysis. The interchange was performed with excess diethanoldisulfide in the presence of β-mercaptoethanol (Smithies, 1965). The protein (1 μmole) was dissolved in a volume of 0.05 M N-ethylmorpholine-acetate buffer, pH 8.0

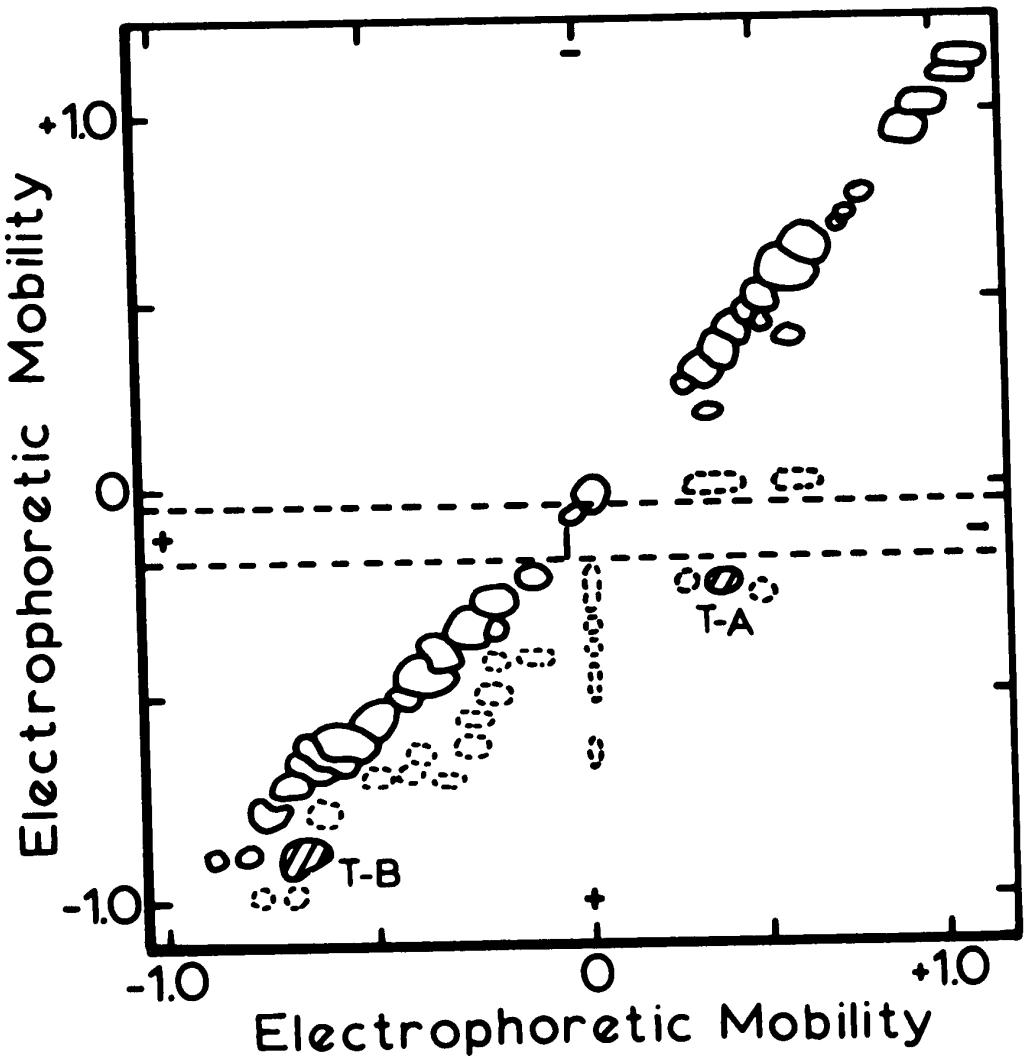


Fig. 26: A pH 6.5/pH 6.5 diagonal peptide "map" of a tryptic digest of SH-tropomyosin with precautions taken to eliminate disulfide bridge formation. The major cysteic acid peptides are hatched. The conditions and nomenclature are as described in the text.

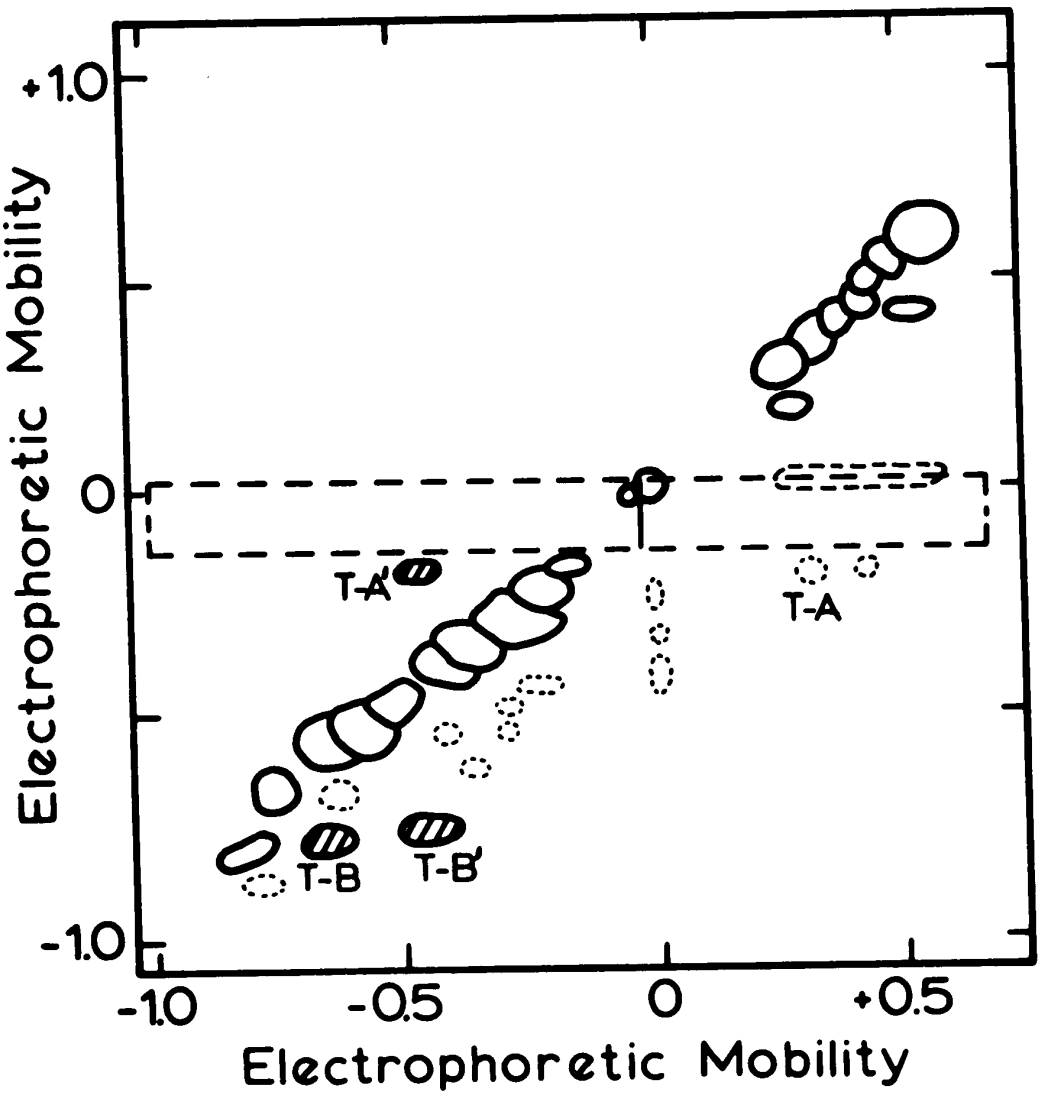


Fig. 27: A pH 6.5/pH 6.5 diagonal peptide "map" of a tryptic digest of SH-tropomyosin without precautions taken to eliminate disulfide bridge formation. The hatched spots indicate major cysteic acid peptides. The conditions and nomenclature are as described in the text.

containing diethanoldisulfide (210 μmole) and β-mercaptoethanol (70 μmole) such that the final concentration after the addition of the trypsin solution was 10 mg per ml. Freshly dissolved trypsin in 0.05 M N-ethylmorpholine-acetate buffer, pH 8.0 was added (ratio of trypsin to protein, 1:50 w/w) and the protein digested at 37°C for 5 hours. The identical pH 6.5/pH 6.5 diagonal electrophoretic peptide "map" to the "map" of the interchanged digest could be obtained by the following procedure. SH-tropomyosin was dialyzed exhaustively against 0.05 M β-mercaptoethanol adjusted to pH 8.0 with 6 N NH_4OH (2 days, 3 changes of dialysate) and then for 4-5 hours against water to remove excess β-mercaptoethanol. After freeze-drying SH-tropomyosin was digested with trypsin in the normal manner (Chapter II). An example of such a diagonal is shown in Figure 26. For comparative purposes a diagonal "map" of a tryptic digest of SH-tropomyosin without either precautionary step to eliminate disulfide bridge formation is shown in Figure 27. This diagonal clearly shows the disappearance of peptide T-A and the presence of a disulfide bridge which on performic acid oxidation gave peptides T-A' and T-B'. These peptides are identical to T-A and T-B which migrated as the cysteine peptides in the first dimension. The disulfide bridge formation could have arisen during storage of SH-tropomyosin or during the tryptic digestion. Nevertheless, precautionary measures are necessary. Tryptic cysteic acid peptides were isolated using either method of -SH group protection whereas in the case of thermolysin digests only the dialysis of SH-tropomyosin against mercaptoethanol was used.

b. Cystine-exchange

Cystine-exchanged tropomyosin was prepared as described by Weeds and Hartley (1967). Peptic digestion of both cystine-exchanged tropomyosin and SH-tropomyosin was carried out as described in this thesis (Chapter II). Comparison of the pH 6.5/pH 6.5 diagonal electrophoretic "map" of each digest indicated that the cystine-exchange reaction was unnecessary. This is probably the result of the peptic digest being performed under acidic conditions which reduces the incidence of disulfide interchange and oxidation of sulfhydryl residues.

(2) The Diagonal Purification Procedure

The diagonal electrophoretic procedure described by Brown and Hartley (1963; 1966) was employed for the purification of cysteine peptides from tryptic, peptic, and thermolytic digests of tropomyosin.

The pH 6.5/pH 6.5 diagonal electrophoretic peptide "maps" of cysteic acid peptides were prepared as follows: a 3 cm band from the preparative sheet was cut parallel to the direction of electrophoresis at pH 6.5, the strip was oxidized and electrophoresis at pH 6.5 at right angles to the original direction was carried out.

After location of the cysteic acid peptides on such a diagonal peptide "map" the corresponding bands from the original electropherogram were cut out, oxidized and the strip stitched to a new sheet of paper for electrophoresis at pH 6.5. Further purification of the bands of cysteic acid peptides was frequently necessary, because of diagonal contaminants or because of contaminating peptides with similar

mobilities. This was achieved by electrophoresis at pH 1.8, 3.5, and pH 6.5. The pure peptides were then eluted from the paper with water or volatile buffers.

a. Performic acid oxidation of the paper strips

The paper strips were placed in a dessicator over a mixture of 20.0 ml of 98 per cent (v/v) formic acid and 1.0 ml of 30 per cent (v/v) hydrogen peroxide. The dessicator was evacuated for 5 min and the strip left exposed for two hours to the performic acid vapours generated in the dessicator. After oxidation the Petri dish containing the performic acid was replaced by NaOH pellets, and the strip was allowed (under vacuo) to dry for an additional two hours.

(3) Isolation of ^{14}C-CMC Peptides

The ^{14}C-CMC peptides were obtained from tryptic and peptic digests of ^{14}C-CM-tropomyosin. The digests were separated on Chromobead Type P resin (Dowex-50) and radioactive peptides located by removing aliquots of the effluent fractions to scintillation vials for radioactivity measurements. All methods for digestion, column procedures and measurement of column effluent are described in this thesis Chapter II.

3. RESULTS

A. Peptide Nomenclature

The sequence of purification steps for the isolation and characterization of unique cysteine peptides were the following:

(1) The capital letters T, P, and L designate tryptic, peptic and thermolytic digests of tropomyosin.

(2) The Diagonal Purification Procedure

(a) When digests were submitted to the diagonal purification procedure capital letters are added to these designations in alphabetical order to indicate their relative mobility on pH 6.5 electrophoresis in the first dimension. Band T-A is basic and band T-B is acidic. Band P-A is neutral and band P-B is acidic.

(b) When more than one cysteic acid peptide was revealed on further electrophoretic purification of a given band an additional Arabic number was assigned in consecutive order according to its relative mobility at pH 6.5, pH 1.8, or pH 3.5. The most basic peptide was given the lowest number.

(3) Ion-exchange Chromatography

(a) When the digests were applied to ion-exchange columns for purification of ^{14}C-CMC peptides the radioactive fractions were assigned Roman numerals, I to V which were added to the enzymatic digest notation.

(b) If on further purification of the column fractions on electrophoresis or paper chromatography more than one CMC peptide appeared from a given fraction a capital letter was added to these designations in alphabetical order to indicate their relative mobility on electrophoresis at pH 6.5, pH 1.8 or pH 3.5 with the most basic peptide having the letter A.

(4) The products of peptides subsequently digested with trypsin or thermolysin have been designated by the letter T and L respectively. An Arabic number is assigned to each of the products to indicate the relative mobility at pH 6.5, the most basic peptide of the digest having the lowest number. On further purification of the pH 6.5 bands more than one peptide is obtained small letters in alphabetical order are added to the designations to indicate their relative mobility at pH 1.8 or pH 3.5 electrophoresis with the most basic peptide having the letter "a".

An example illustrates the system: peptide T-IA-L2b was obtained from an initial tryptic digest of the protein (T) as Fraction I on the ion-exchange chromatography and was the most basic radioactive peptide (A) on electrophoretic purification. After thermolysin digestion (L) of this fraction, it was recovered as the second most basic component(2) upon electrophoresis at pH 6.5 and the second most basic component on further electrophoretic purification (b).

B. Purification, Isolation, and Characterization of the Unique Cysteine Peptides

(1) Tryptic Cysteic Acid Peptides

a. Diagonal Peptide "Map"

The tryptic digest of SH-tropomyosin with simultaneous disulfide interchange, or mercaptoethanol dialysis of SH-tropomyosin prior to digestion, was submitted to the diagonal fingerprint procedure outlined in the Methods (page 86). The pH 6.5/pH 6.5 peptide "map" is shown in Figure 26. Each of the unique cysteine peptides of the original protein was found off the diagonal as a cysteic acid peptide (hatched). Each cysteic acid peptide thus located was isolated from the preparative sheets and was further purified by electrophoresis at pH 1.8 or pH 3.5.

b. Peptide T-A

1. Purification

The cysteine peptide band T-A from the original preparative pH 6.5 electrophoresis (60 v/cm; 40 min) was cut out, oxidized and re-run at pH 6.5 (60 v/cm; 2.5 hours). The peptide was still impure at this

TABLE XIX

AMINO ACID COMPOSITION OF CYSTEINE TRYPTIC PEPTIDES

(Values are Expressed as Mole Ratios)

Amino Acid	Peptide									
	T-A	T-B1	T-B2	T-IA	T-IB	T-IIIA	T-IIIB	T-IV-A	T-IVB	T-V
Lysine	1.01	0.96	0.84	1.22	0.88	1.12	1.11	2.01	2.07	1.08
Cysteic Acid	0.99	0.93	0.71							
Carboxymethylcysteine				1.04	0.92	1.04	0.91	1.10	0.99	0.92
(radioactivity										
		4.25	3.26	3.91	3.12	4.00	3.01	4.00	3.36	
Glutamic Acid			1.04		0.99		1.02		0.86	
Aspartic Acid			1.00		1.04		1.14		0.91	
Glycine		0.85		0.94		1.04		1.05		
Alanine		2.02	1.88	1.93	1.98	1.83	1.73	1.84	1.81	
Leucine										
Number of Residues	2	9	9	9	9	9	9	10	10	2
Mobility at pH 6.5(m)	-0.23	-0.86	-0.86	-0.87	-0.90	-0.87	-0.90	-0.66	-0.66	-0.10
N-terminal	Cya	Cya	Cya	CMC	CMC	CMC	CMC	CMC	CMC	CMC
Percentage Yield*	7.4	27.6	9.4	4.5	2.5	3.0	0.8	3.0	0.7	7.5

* Percentage yields of cysteine peptides calculated per 70,000 g; radioactive peptides as percentage total radioactivity.

stage of purification. It was finally isolated by eluting the band from pH 6.5 electrophoresis and re-running on Whatman No. 1 paper at pH 1.8 (60 v/cm; 45 min). The amino acid composition of this peptide is shown in Table XIX.

ii. Sequence determination

The yellow-orange colour produced with cadmium-ninhydrin stain is consistent with N-terminal cysteic acid as determined by the "dansyl" method for a lysine containing peptide. The N-terminal cysteic acid gives a yellow color with the cadmium-ninhydrin reagent. However, the ε-amino group of lysine produces a red color thereby giving a final orange appearance to the peptide. The dipeptide has the sequence:

$$\underset{\longrightarrow}{\text{Cya}}\text{-}\underset{\longrightarrow}{\text{Lys}}$$

The low basic mobility of this dipeptide on the first dimension of the pH 6.5 electropherogram is explained by the N-terminal cysteine residue where interaction of the -SH and α-amino group has lowered the pK's (Edsall and Wyman, 1958).

c. Peptides of Band T-B

i. Purification of peptides

The cysteine peptides of band T-B from the original pH 6.5 electrophoresis (60 v/cm; 40 min) were further purified by cutting out band T-B, oxidizing with performic acid, and re-running at pH 6.5 (60 v/cm; 90 min; origin 20 cm from cathode). Band T-B was cut out and run at pH 3.5 (60 v/cm; 40 min). Two cysteic acid peptides T-B1 and T-B2 were obtained. The amino acid composition of these peptides is shown in Table XIX.

ii. Sequence determinations

Peptide T-B1 and T-B2

The yellow-orange colour produced with cadmium-ninhydrin is consistent with an N-terminal cysteic acid as determined by the "dansyl" method. Their electrophoretic mobilities at pH 6.5 (m = -0.86) strongly suggest that there are no amidated residues. The sequence from the Dansyl-Edman procedure gives the following structure for peptide T-B1:

Cya-Ala-Glu-Leu-Glu-Glu-Glu-Leu-Lys.
→ → → → → → → → →

The low quantity of peptide T-B2 isolated, allowed the identification of only four residues by the Dansyl-Edman procedure and the following structure may be written for T-B2:

Cya-Gly-Asp-Leu-(Glu,Glu,Glu,Leu,Lys).
→ → → →

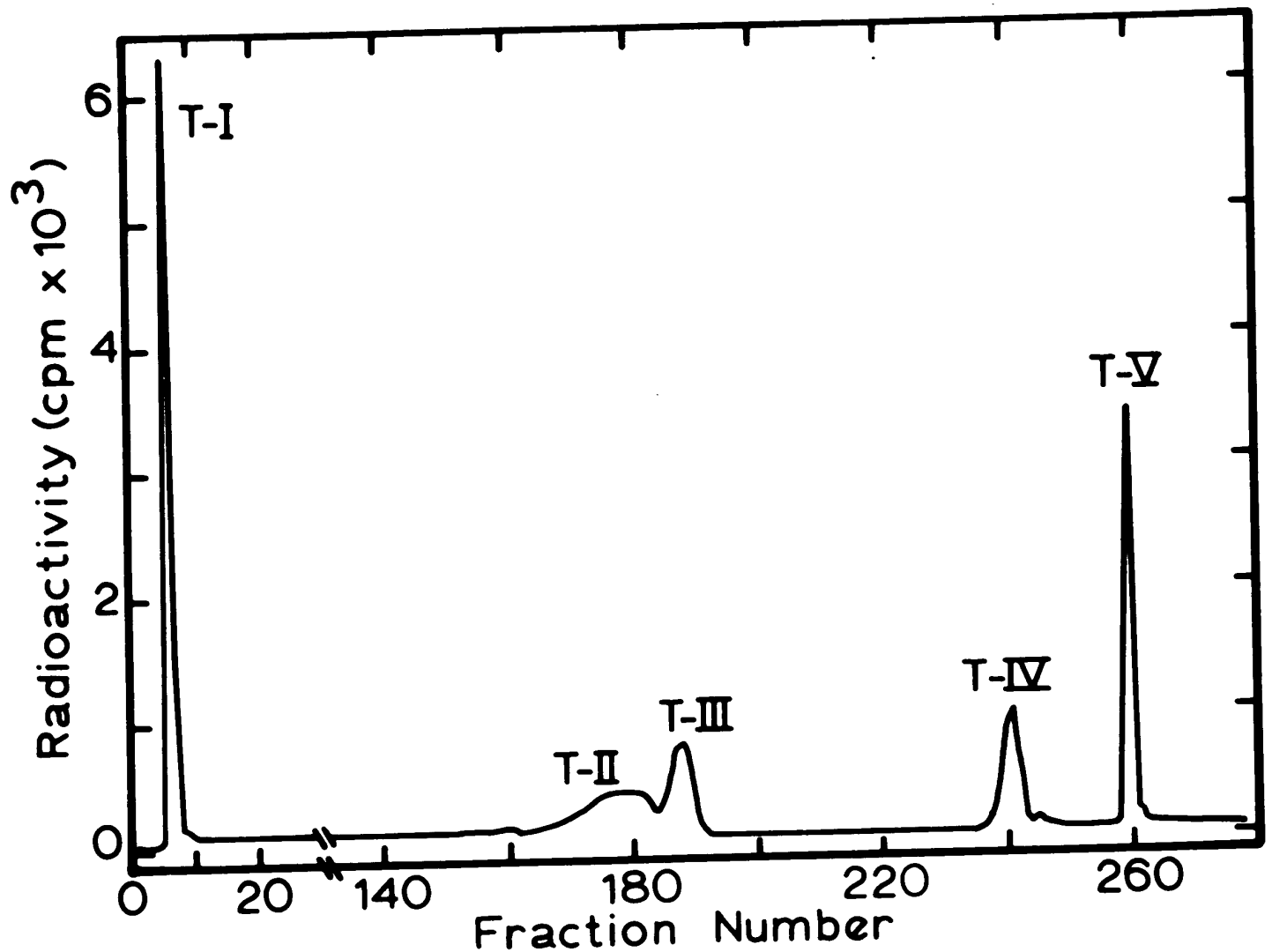


Fig. 28: Chromatography of a tryptic digest of ^{14}C-CM-tropomyosin on chromobead type P column. The effluent was monitored for radioactivity by taking 20 µl aliquots from 3.0 ml fractions for counting.

TABLE XX

QUANTITATIVE RECOVERIES OF S-CARBOXYMETHYLATED

TRYPTIC PEPTIDES BY CHROMOBEAD TYPE P CHROMATOGRAPHY

Radioactive Fractions	Percentage Recovery	Relative Per Cent Recovery
T-I (6,7)	22.0	35.0
T-II (172-184)	9.0	14.4
T-III (185-191)	8.9	14.2
T-IV (240-242)	9.0	14.4
T-V (259,260)	13.8	22.0
Total	62.7	100.0

(2) Tryptic ^{14}C-CMC Peptides

The cysteic acid diagonal procedure showed 3 unique cysteine sequences, two of which were highly homologous peptides (T-B1 and T-B2). Since this information was to be used to answer the question of subunit identity a more quantitative determination of the relative amounts of each unique cysteine sequence present in tropomyosin was necessary. A tryptic digest of ^{14}C-CM-tropomyosin was carried out and separated on a Dowex 50 ion-exchange column. The column chromatographic procedure was expected to provide proportionally higher yields of peptides than paper electrophoretic methods. The radioactive marker was used to ensure the location and easy quantitation of all cysteine containing peptides.

The tryptic digestion of ^{14}C-CM-tropomyosin (5.93×10^6 c.p.m. or 4.51 μmole of CMC) was subjected to a preliminary fractionation on a Chromobead Type P resin (Dowex 50) as described earlier (Chapter II) to give the elution pattern shown in Figure 28. On the basis of radioactivity the effluent was divided into fractions, I to V, shown in Figure 28 and the recoveries calculated are shown in Table XX. Of these, only fractions T-I, T-III, T-IV, and T-V were recovered in amounts adequate for further purification. The peptides of fraction T-II were considered as minor varieties of the sequences represented in the other fractions and were ignored in this study.

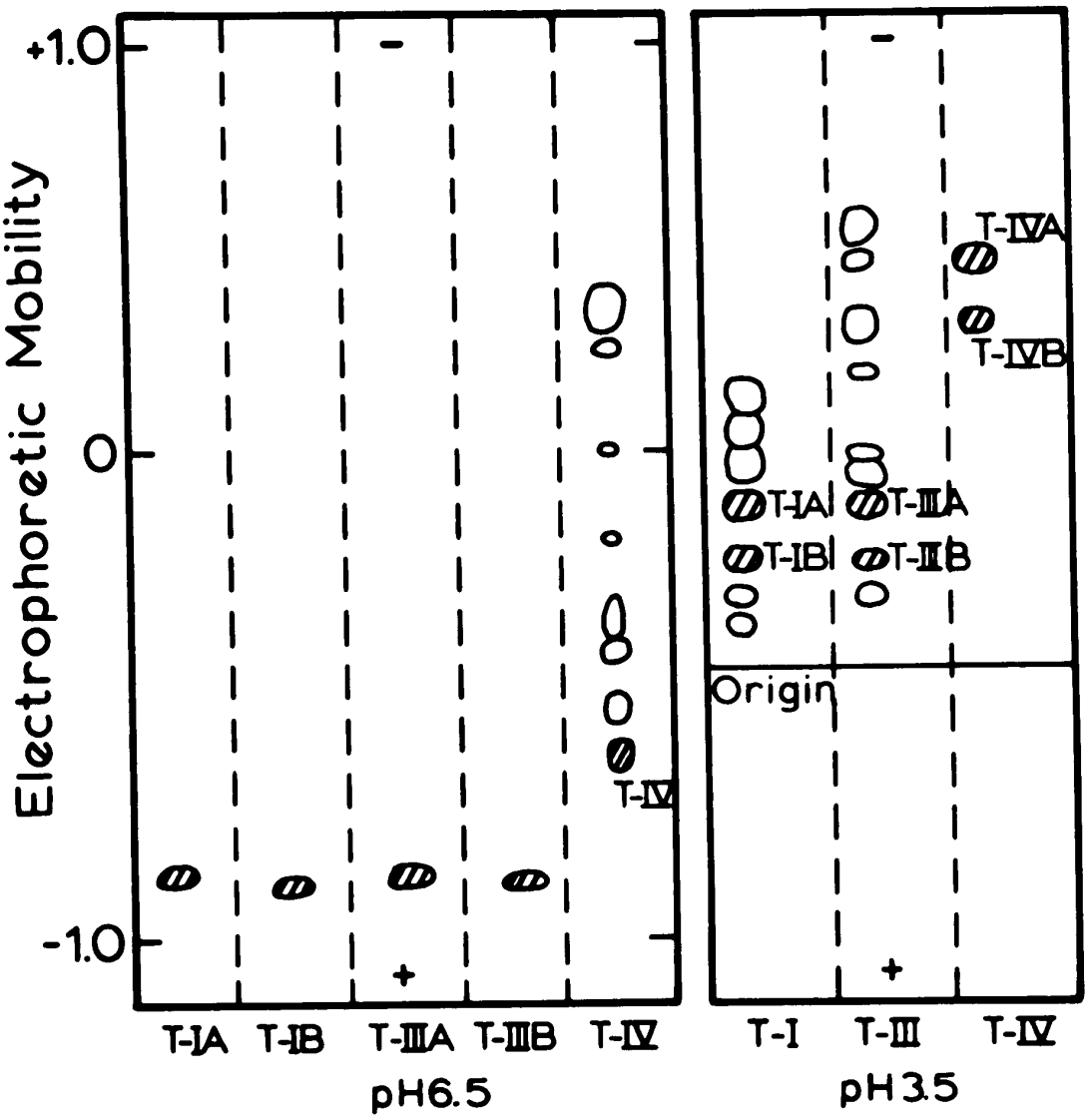


Fig. 29: Radioactive peptides from fractions T-I, T-III, and T-IV of Chromobead Type P chromatography obtained by electrophoresis at pH 6.5 and pH 3.5. Fractions T-I and T-III purified at pH 3.5 followed by pH 6.5 electrophoresis and fraction T-IV at pH 6.5 followed by pH 3.5 electrophoresis. The hatched spots indicate the radioactive peptides. The conditions and nomenclature are as described in the text.

Preliminary high-voltage paper electrophoresis at pH 6.5, pH 3.5, and pH 1.8 of each fraction tube of the radioactive peaks indicated the necessary paper purifications and the fraction tubes which should be pooled for each peak. The relative mobilities of the radioactive peptides arising from fractions T-I, T-III, and T-IV of the Chromobead Type P separation obtained by electrophoresis at pH 6.5 and pH 3.5 is shown in Figure 29.

a. Fraction T-I

This peak (fraction 6 and 7) was applied to Whatman No. 1 paper and electrophoresis at pH 3.5 (60 v/cm; 2 hours) was carried out. Two radioactive peptides were obtained T-IA and T-IB. These bands were cut out and rerun at pH 6.5 (60 v/cm; 45 min). These separations are shown in Figure 29. The final amino acid compositions of these peptides is shown in Table XIX.

i. <u>Peptide T-IA</u>

The N-terminal was found to be CMC as determined by the "dansyl" method. The peptide was digested with thermolysin (ratio of enzyme to peptide, 1:75) in 0.2 N ammonium-acetate buffer, 0.005 M $CaCl_2$, pH 8.5 for 2 hours at 37°C (Ambler and Meadway, 1968).

TABLE XXI

AMINO ACID COMPOSITION AND SEQUENCE OF THERMOLYTIC PEPTIDES FORMED BY THERMOLYTIC DIGESTION OF PEPTIDE T-IA

(Values are Expressed as Mole Ratios)

Peptide	Electrophoretic mobility at pH 6.5(m)	Amino Acid Composition and Sequence	Cadmium-Ninhydrin Colour
T-IA	-0.87	CMC-(Ala,Glu,Leu,Glu,Glu,Glu,Leu,Lys) 1.04 0.94 0.98 0.97 0.98 0.98 0.98 0.97 1.22 ⟶	yellow-orange
T-IA-L1	+0.50	Leu-Lys 0.99 1.01 ⟶ ⟶	orange
T-IA-L2a	-0.99	Leu(Glu,Glu,Glu) 0.97 1.01 1.01 1.01 ⟶	orange
T-IA-L2b	-0.99	CMC-(Ala,Glu) 1.09 0.86 1.06 ⟶	yellow

The digestion products were separated into three major cadmium-ninhydrin positive bands at pH 6.5 (60 v/cm; 30 min). Band T-IA-L3 was impure on amino acid analysis and most likely represented larger varieties of the other thermolytic peptides. Band T-IA-L2 was further purified at pH 3.5 (60 v/cm; 90 min) giving two peptides T-IA-L2a and T-IA-L2b. Peptide T-IA-L2a was finally purified at pH 1.8 (60 v/cm; 40 min). The results of the amino acid composition, sequence analysis by the Dansyl-Edman method and electrophoretic mobilities are shown in Table XXI. The electrophoretic mobilities of peptides T-IA-L2a (m = -0.99) and T-IA-L2b (m = -0.99) suggest a net negative charge of 3 and 2 respectively (Offord, 1966) indicating all acidic residues are charged. From the data presented the following sequence can be written for T-IA:

CMC-Ala-Glu-Leu-Glu-Glu-Glu-Leu-Lys.

ii. Peptide T-IB

CMC was identified as the N-terminal residue by the "dansyl" method. Since the 2 hour digestion with thermolysin of peptide T-IA suggested incomplete digestion, this peptide T-IB was digested with thermolysin (ratio of enzyme to peptide, 1:45) in 0.2 N ammonium-acetate buffer, 0.005 M $CaCl_2$, pH 8.5 for 12 hours at 37°C. The

TABLE XXII

AMINO ACID COMPOSITION AND SEQUENCE OF THERMOLYTIC PEPTIDES FORMED BY THERMOLYTIC DIGESTION OF PEPTIDE T-IB

(Values are Expressed as Mole Ratios)

Peptide	Electrophoretic mobility at pH 6.5(m)	Amino Acid Composition and Sequence	Cadmium-Ninhydrin Colour
T-IB	-0.90	CMC-(Gly,Asp,Leu,Glu,Glu,Glu,Leu,Lys) Q92 1.04 0.99 0.99 1.04 1.04 1.04 0.99 0.88 ⟶	yellow-orange
T-IB-L1	-	Leu-Lys 0.97 1.04 ⟶ ⟶	orange
T-IB-L2	-1.01	Leu(Glu,Glu,Glu) 0.86 1.05 1.05 1.05 ⟶	orange
T-IB-L3a	-1.02	CMC-(Gly,Asp) 0.90 1.05 1.05 ⟶	yellow

resulting thermolytic peptides were separated at pH 3.5 (60 v/cm; 75 min) into 3 major cadmium-ninhydrin positive bands. Peptide T-IB-L2 was further purified at pH 6.5 (60 v/cm; 30 min) and T-IB-L3 separated into two peptides T-IB-L3a and T-IB-L3b on pH 6.5 electrophoresis (60 v/cm; 30 min). Peptide T-IB-L3b was still impure on amino acid analysis and probably represents minor varieties of the thermolytic peptides already obtained. The results of the amino acid composition, electrophoretic mobilities and sequence by the Dansyl-Edman procedure are shown in Table XXII. The electrophoretic mobilities of T-IB-L2 and T-IB-L3a of m = -1.01 and m = -1.02 respectively suggest that these peptides contain no amidated residues (Offord, 1966). The sequence of T-IB is concluded to be:

CMC-Gly-Asp-Leu-Glu-Glu-Glu-Leu-Lys.

b. Fraction T-III

Tubes 185-191 inclusive were pooled to give fraction T-III. This fraction contained two radioactive peptides on further purification at pH 3.5 (60 v/cm; 2 hours). Final purification of peptides T-IIIA and T-IIIB was carried out at pH 6.5 (60 v/cm; 45 min). These purifications are shown in Figure 29. The amino acid compositions of these peptides are shown in Table XIX. Peptides T-IIIA and T-IA are equivalent. Similarly peptides T-IIIB and T-IB are equivalent. These pairs of peptides have identical amino acid compositions and the same electrophoretic mobilities at pH 6.5 (Table XIX). The apparent identity of these peptides by all criteria raises the question of why they separated as two well-defined peaks on Chromobead Type P chromatography

(Figure 28). Similar behavior for peptides of apparently identical sequence has previously been observed by Schroeder (1948) and Zarkadas (1969). One explanation is that the S-carboxymethylcysteine has been, in part, oxidized to the sulfoxide or sulfone during the preparative procedures which has lead to differences in the properties of the two varieties upon chromatography on Chromobead Type P resin. This modification would not be detected on the amino acid analyses nor would it be expected to affect their electrophoretic mobility, particularly with peptides such as these with a high proportion of charged residues. Peptides T-IIIA and T-IIIB were assigned the same sequence as T-IA and T-IB respectively.

c. Fraction T-IV

Tubes 240-242 inclusive were pooled to give fraction T-IV. Electrophoresis was carried out at pH 6.5 (60 v/cm; 45 min). One radioactive band was obtained and on further purification at pH 3.5 (60 v/cm; 2 hours) two radioactive peptides T-IVA and T-IVB were obtained (Figure 29). The amino acid compositions and N-terminal residues of these peptides is shown in Table XIX. The amino acid composition of these peptides differs from their corresponding peptides in fractions T-I and T-III only by the presence of an extra lysine residue.

TABLE XXIII

AMINO ACID COMPOSITION AND SEQUENCE OF THERMOLYTIC PEPTIDES

FORMED BY THERMOLYTIC DIGESTION OF PEPTIDE T-IVA

(Values are Expressed as Mole Ratios)

Peptide	Electrophoretic mobility at pH 6.5(m)	Amino Acid Composition and Sequence	Cadmium-Ninhydrin Colour
T-IVA	-0.66	CMC-(Ala,Glu,Leu,Glu,Glu,Glu,Leu,Lys,Lys) 1.10 1.05 1.00 0.92 1.00 1.00 1.00 0.92 1.00 1.00 ⟶	yellow-orange
T-IVA-L1	-	Leu(Lys,Lys) 1.03 0.98 0.98 ⟶	orange
T-IVA-L2	-1.00	Leu(Glu,Glu,Glu) 0.89 1.04 1.04 1.04 ⟶	orange
T-IVA-L3	-0.95	CMC-(Ala,Glu) 0.91 1.03 1.05 ⟶	yellow
T-IVA-L4	-1.17	CMC-(Ala,Glu) 0.86 1.04 1.10 ⟶	yellow

1. Peptide T-IVA

It is most likely that the position of the second lysine was C-terminal since this peptide was obtained from a tryptic digest. To obtain unequivocal proof of the position of this lysine residue in the sequence a thermolytic digest of this peptide was carried out (ratio of enzyme to peptide, 1:45) in 0.2 N ammonium acetate buffer, 0.005 M $CaCl_2$, pH 8.5 for 12 hours at 37°C. The digestion products were separated at pH 3.5 (60 v/cm; 60 min). Four cadmium-ninhydrin positive bands were obtained. Band T-IVA-L2 was further purified at pH 6.5 (60 v/cm; 30 min). The mobilities at pH 6.5 of peptides T-IVA-L3 and T-IVA-L4 were determined by running 1 cm wide strips of each band at pH 6.5 (60 v/cm; 30 min).

The results of the amino acid composition, electrophoretic mobilities and N-terminal analysis by the "dansyl" method are summarized in Table XXIII. The electrophoretic mobilities of peptides T-IVA-L2, T-IVA-L3, and T-IVA-L4 are consistent with no amidated residues. Peptide T-IVA-L4 has the same composition as T-IVA-L3 but an increased mobility at pH 6.5. This most likely results from a lowering of the pK of the α-amino group from oxidative modification of the N-terminal CMC residue. The electrophoretic mobility of peptide T-IVA-L1 (leu,lys,lys) at pH 3.5 (+0.98) is faster than the peptide T-IB-L1 (leu,lys) with a mobility at pH 3.5 of +0.82 and is consistent with their compositions. The mobilities were measured as the migration of the peptide from the origin relative to migration of lysine (+1.0). The results in Table XXIII are consistent with the

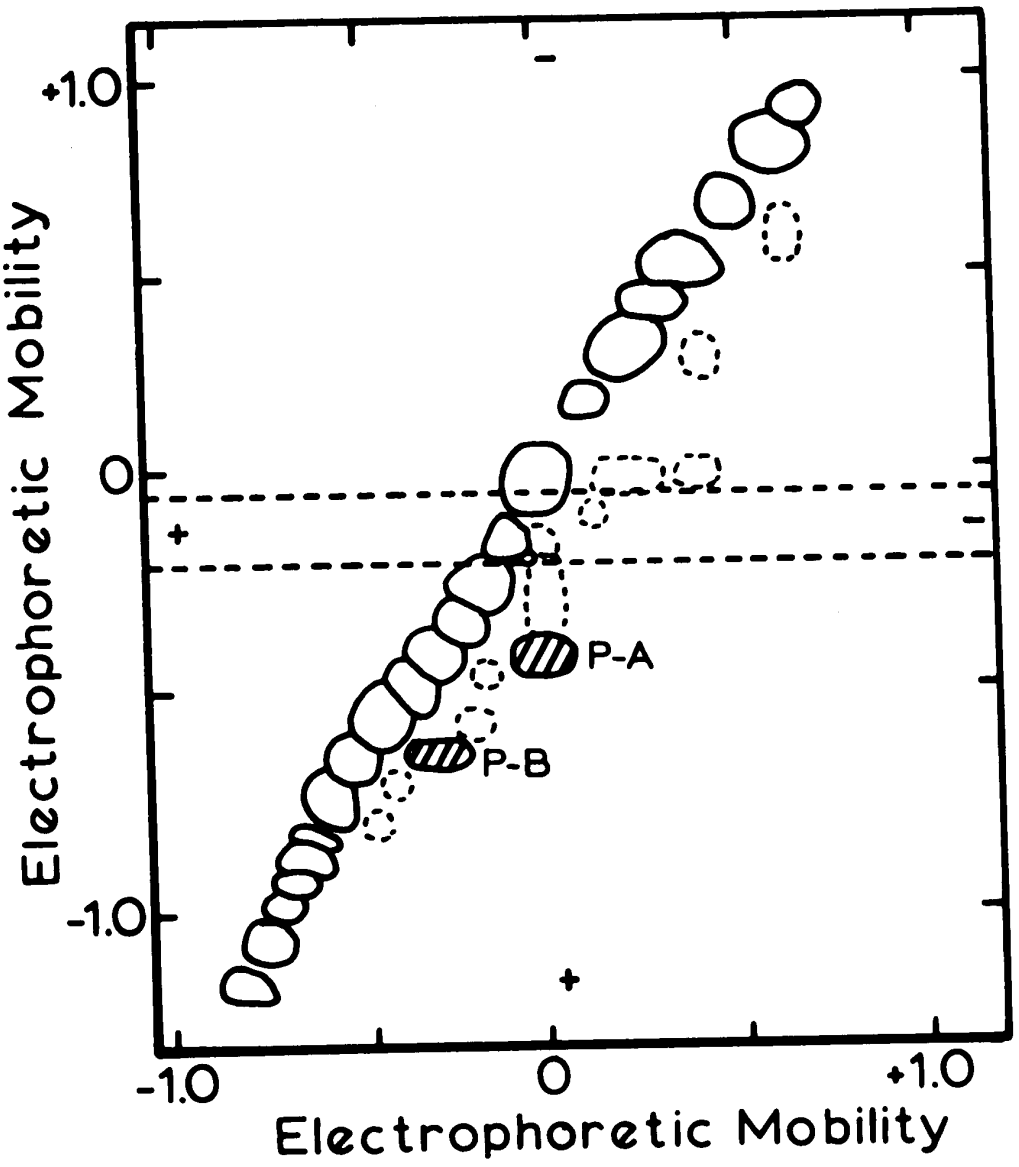


Fig. 30: A pH 6.5/pH 6.5 diagonal peptide "map" of a peptic digest of SH-tropomyosin. The major cysteic acid peptides are hatched. The conditions and nomenclature are as described in the text.

following sequence:

CMC-Ala-Glu-Leu-Glu-Glu-Glu-Leu-Lys-Lys.

ii. Peptide T-IVB

This peptide was isolated in insufficient quantities to digest with thermolysin to identify the position of the two lysine residues. However, the N-terminal, electrophoretic mobility and amino acid composition of this peptide compared to the other homologous peptides T-IVA and T-IB clearly indicates that it has the sequence:

CMC-Gly-Asp-Leu-Glu-Glu-Glu-Leu-Lys-Lys.

d. Fraction T-V

Tubes 259 and 260 of the Chromobead Type P fractionation were pooled and this fraction was purified at pH 6.5 (60 v/cm; 45 min) and pH 1.8 (60 v/cm; 45 min). The amino acid composition is shown in Table XIX. The sequence by the Dansyl-Edman procedure was

$\underrightarrow{\text{CMC-Lys}}$.

(3) Peptic Cysteic Acid Peptides

The objective of carrying out this digest was to extend the sequence obtained on the N-terminal side of the cysteine residues.

a. Diagonal Peptide "Map"

The peptic digest of SH-tropomyosin was subjected to the diagonal fingerprint procedure outlined in the Methods (page 86). The pH 6.5/pH 6.5 peptide "map" is shown in Figure 30. Two major cysteic acid peptides (hatched) were found off the diagonal. Each

TABLE XXIV

AMINO ACID COMPOSITION OF CYSTEINE PEPTIC PEPTIDES

(Values are Expressed as Mole Ratios)

Amino Acid	Peptide							
	P-A	P-B	P-I	P-II	P-IIIB1	P-IIIB2	P-228	P-230
Lysine	1.02	1.00	0.94	0.95	1.00	1.07	3.00	1.30
Cysteic Acid	1.05	1.02						
Carboxymethylcysteine			0.86	0.99	0.81	0.91	0.91	0.91
(radioactivity)							0.93	0.79
Arginine							5.18	1.91
Glutamic Acid	1.13	2.07	1.96	1.16	1.07	1.07	1.84	
Aspartic Acid						0.83		0.90
Serine	0.74	0.84	0.95	0.77				
Glycine	1.06	1.05	1.05	1.12		1.02		1.09
Alanine		1.03	1.04		0.97		1.11	1.04
Leucine			1.06		0.97	1.02	0.96	0.97
Number of Residues	5	7	8	5	5	6	14	9
Mobility at pH 6.5(m)	-0.43	-0.66	-0.56	-0.42	-0.36	-0.36	-0.30	-0.26
N-terminal	Ser	Ser	Ser	Ser	Lys	Ser		
Percentage Yield*	9.0	3.1	8.2	4.8	3.5	3.5	0.4	0.5

* Percentage Yield: For cysteic acid peptides calculated per 70,000 g.
For ^{14}C-CMC peptides calculated as percentage total radioactivity.

cysteic acid peptide thus located was purified preparatively as outlined below.

b. Peptide P-A

i. Purification

The cysteine peptide of band P-A from the original pH 6.5 electrophoresis (60 v/cm; 45 min) was cut out, oxidized and re-run at pH 6.5 (60 v/cm; origin 18 cm from cathode) for 2 hours. Peptide P-A was further purified at pH 1.8 (60 v/cm; 45 min). The amino acid composition of this peptide is shown in Table XXIV.

ii. Sequence determination

The orange colour produced with cadmium-ninhydrin is consistent with an N-terminal serine as determined by the "dansyl" method. Peptide P-A is neutral at pH 6.5 before oxidation with performic acid. Therefore, the acidic residue is charged. The mobility after oxidation at pH 6.5 (m = -0.43) of this cysteic acid peptide suggests that there was a net negative charge of 1 (Offord, 1966) which again indicates the presence of glutamic acid rather than glutamine. The sequence by the Dansyl-Edman procedure was shown to be:

$$\underset{\longrightarrow}{\text{Ser}}\text{-}\underset{\longrightarrow}{\text{Glu}}\text{-}\underset{\longrightarrow}{\text{Gly}}\text{-}\underset{\longrightarrow}{\text{Lys}}\text{-}\underset{\longrightarrow}{\text{Cya.}}$$

c. Peptide P-B

i. Purification

After oxidation of band P-B from the original pH 6.5 preparative electrophoresis (60 v/cm; 45 min) the second dimension pH 6.5 electrophoresis was carried out for 75 min (60 v/cm; origin 20 cm from

cathode). The cysteic acid peptide was purified at pH 1.8 (60 v/cm; 45 min) followed by pH 3.5 electrophoresis (60 v/cm; 60 min). This peptide was still impure at this stage and could not be purified by paper electrophoresis. Paper chromatography was performed on Whatman No. 4 paper using the solvent system 1-butanol:acetic acid: water (6.7:1:2.5) as described by Bailey (1967). Descending chromatography was carried out for approximately 7 hours. The separation in this solvent system is based mainly on the hydrophobicity of the peptides, the more hydrophobic peptide having the faster mobility. Peptide P-B had an R_f of 0.05 and the contaminating peptide 0.48. (Lys,Glu_3,Leu). This result emphasizes the value of this system in conjunction with paper electrophoresis. The cysteic acid peptide band was re-run at pH 1.8 (60 v/cm; 45 min) after paper chromatography. The amino acid composition is shown in Table XXIV.

ii. Sequence determination

The N-terminal residue of P-B was identified as serine using the "dansyl" method. Its electrophoretic mobility at pH 6.5 (m = -0.66) is consistent with no amidated residues. The composition, mobility, and N-terminus permit the assignment of the following structure to this peptide:

Ser-(Glu,Gly,Lys,Cya,Ala,Glu).
$\longrightarrow$

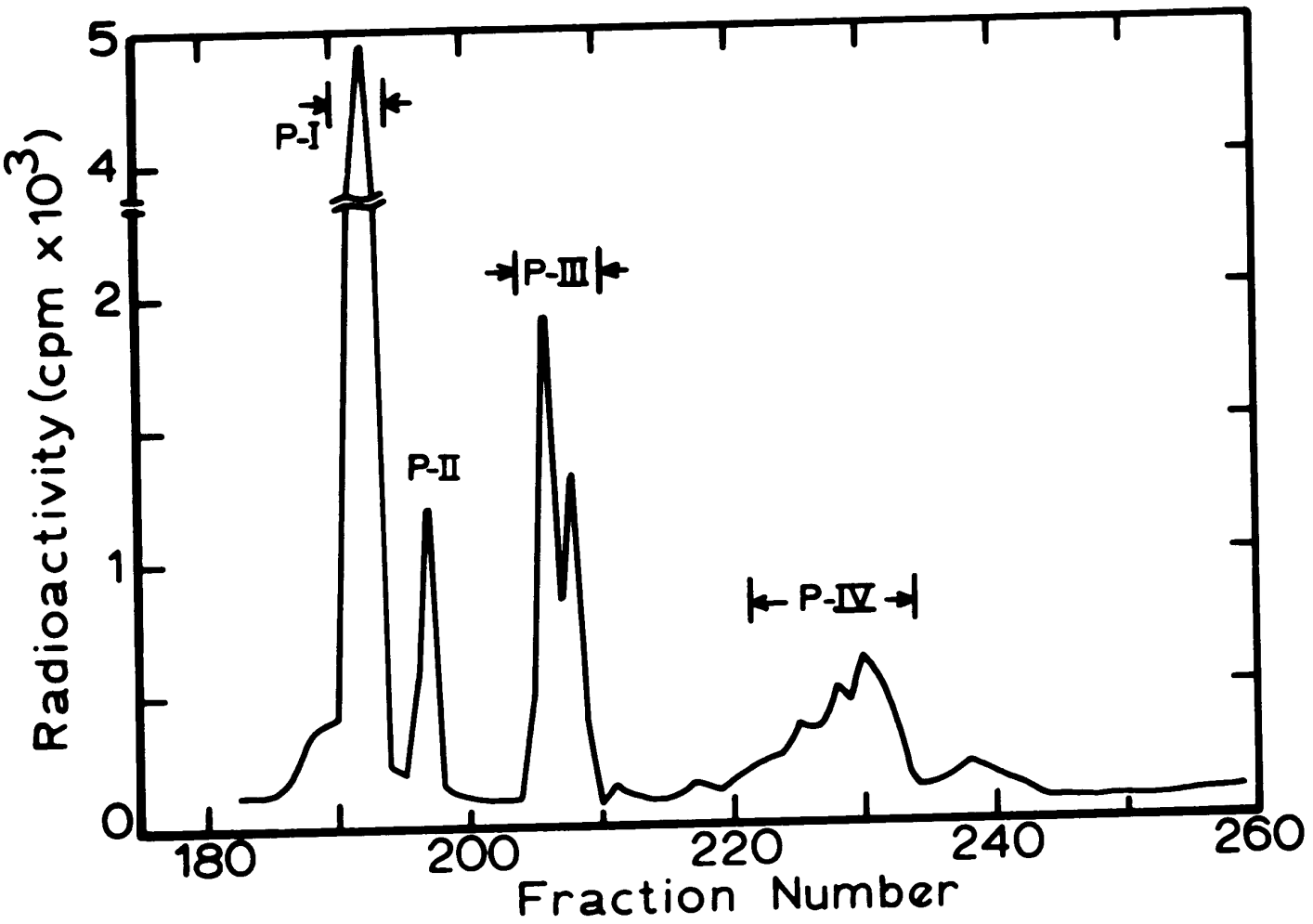


Fig. 31: Chromatography of a peptic digest of ^{14}C-CM-tropomyosin on a Chromobead Type P column. The effluent was monitored for radioactivity by taking 20 µℓ aliquots from 3.0 ml fractions for counting.

TABLE XXV

AMINO ACID COMPOSITION AND SEQUENCE OF TRYPTIC PEPTIDES

FORMED BY TRYPTIC DIGESTION OF PEPTIDE P-B

(Values are Expressed as Mole Ratios)

Peptide	Electrophoretic mobility at pH 6.5(m)	Amino Acid Composition and Sequence	Percentage Yield	Cadmium-Ninhydrin Colour	M.W.
P-B	-0.66	Ser-(Glu,Gly,Lys,Cya,Ala,Glu) 0.84 1.03 1.05 1.00 1.02 1.05 1.03 ⟶		orange	770.5
P-B-T1	-0.06	Ser- Glu-Gly-Lys 0.88 1.06 1.06 1.00 ⟶ ⟶ ⟶ ⟶	50.0	orange	419.3
P-B-T2	-0.67	Ser-(Glu,Gly,Lys,Cya,Ala,Glu) 0.98 1.01 1.27 0.94 0.75 1.02 1.01 ⟶	12.1	orange	
P-B-T3	-1.11	Cya-Ala-Glu 0.94 1.00 1.06 ⟶ ⟶ ⟶	50.8	yellow	369.2

The sequence of P-B was obtained from a tryptic digest. The digestion was carried out in 0.05 M N-ethylmorpholine-acetate buffer pH 8.0 for 5 hours at 37°C (molar ratio of enzyme to peptide 1:100). The digestion products were separated by pH 6.5 electrophoresis (60 v/cm; 40 min) on Whatman No. 1 paper. Three cadmium-ninhydrin positive bands were obtained. The results of the amino acid compositions, electrophoretic mobilities and Dansyl-Edman degradation of the tryptic peptides obtained from tryptic digestion of P-B are summarized in Table XXV. Peptide P-B-T2 is the original undigested peptide. From the data presented, the following sequence may be written for P-B:

Ser-Glu-Gly-Lys-Cya-Ala-Glu.

(4) Peptic ^{14}C-CMC Peptides

The results of the cysteic acid diagonal procedure of the peptic digest of SH-tropomyosin did not reveal the 3 unique cysteine sequences indicated by the tryptic digestion. To insure that no peptic cysteine peptides were missed on the diagonal purification procedure, the peptic digest was repeated on ^{14}C-CM-tropomyosin.

The peptic digest of ^{14}C-CM-tropomyosin (5.34×10^6 c.p.m. or 4.05 μmole of CMC) was separated on Chromobead Type P resin (Dowex-50) as described in this thesis (Chapter II) to give the elution profile shown in Figure 31. On the basis of radioactivity the effluent was divided into fractions I to IV shown in Figure 31 and the recoveries of each fraction were calculated and are shown in Table XXVI.

TABLE XXVI

QUANTITATIVE RECOVERIES OF S-CARBOXYMETHYLATED PEPTIC PEPTIDES BY CHROMOBEAD TYPE P CHROMATOGRAPHY

Radioactive Fractions	Percentage Recovery	Relative Per Cent Recovery
P-I (191-193)	27.1	43.7
P-II (196,197)	5.3	8.6
P-III (205-209)	15.4	24.8
P-IV (222-233)	14.2	22.9
Total	62.0	100.0

Preliminary high-voltage paper electrophoresis at pH 6.5, pH 3.5, and pH 1.8 of each fraction tube of peaks I to IV indicated the tubes which should be pooled for each peak and the necessary paper electrophoretic purifications.

a. Fraction P-I

Peak P-I (fraction tubes 192 and 193 pooled) was applied to Whatman No. 1 paper and electrophoresis at pH 6.5 (60 v/cm; 45 min) was performed. This separation is shown in Figure 32. The radioactive band was cut out and sewn on Whatman No. 4 paper for descending chromatography using the solvent system (Bailey, 1967) 1-butanol: acetic acid:water (6.7:1:2.5) for approximately 9 hours. The radioactive peptide had an R_f = 0.22. The final purification was pH 1.8 electrophoresis (60 v/cm; 45 min). The amino acid composition, percentage yield and electrophoretic mobility are shown in Table XXIV. The N-terminal as determined by the "dansyl" method was serine. The orange colour with cadmium-ninhydrin is consistent with this N-terminal residue. The electrophoretic mobility at pH 6.5 (m = -0.56) agrees with the conclusion that amidated residues are absent in this peptide. The amino acid composition of this peptide is in agreement with the sequence already obtained in previous studies. These results allow the following sequence to be written for P-I:

Ser-Glu-Gly-Lys-CMC-Ala-Glu-Leu.

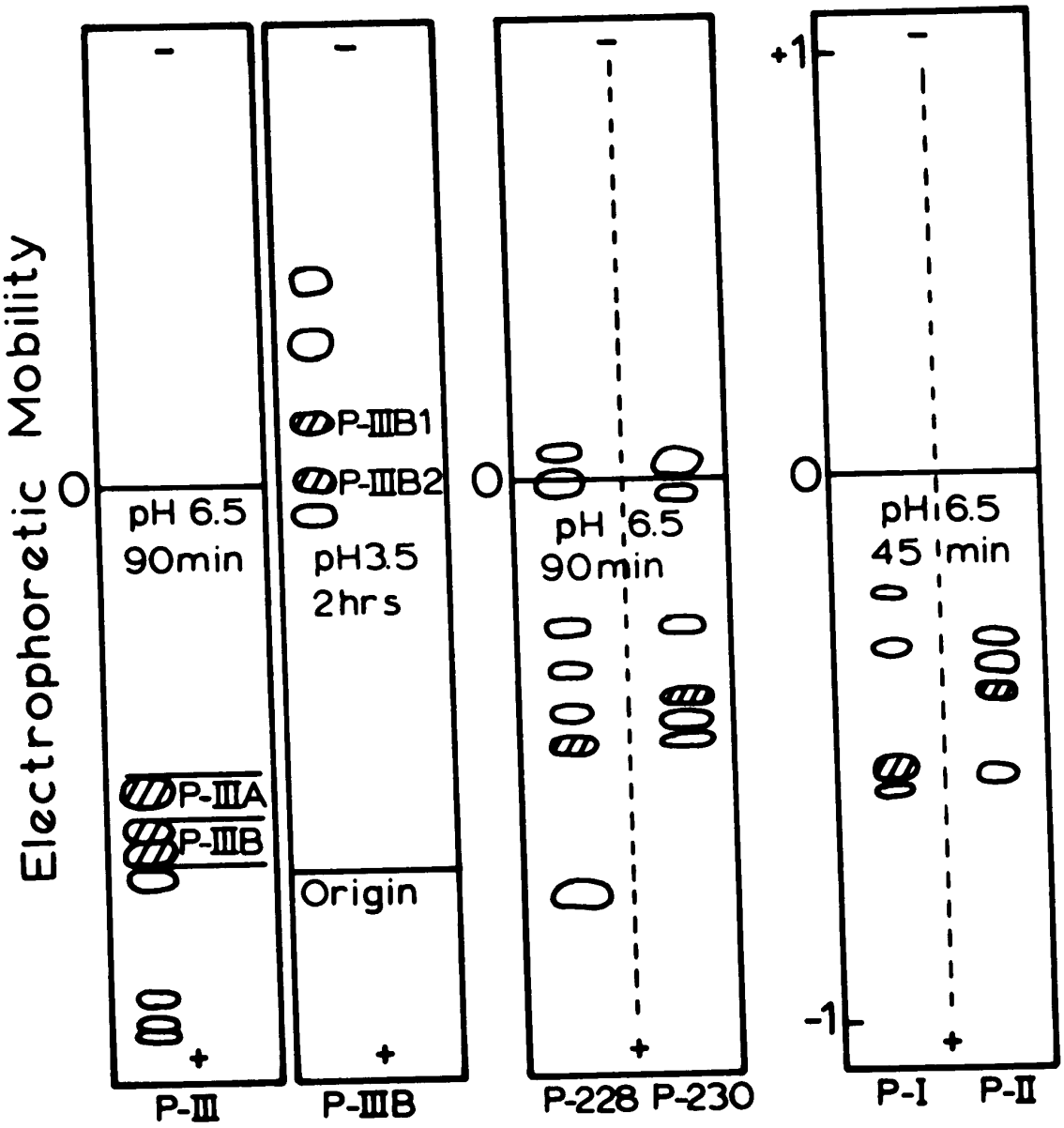


Fig. 32: Radioactive peptides obtained by electrophoresis at pH 6.5 and pH 3.5 of fractions from a Chromobead Type P separation of a peptic digest of ^{14}C-CM-tropomyosin.

b. Fraction P-II

Tubes 192 and 193 were pooled (P-II) and applied to Whatman No. 1 paper for electrophoresis at pH 6.5 (60 v/cm; 45 min). This separation is shown in Figure 32. The peptide was pure at this stage and the amino acid composition is shown in Table XXIV. This peptide gave an orange colour with cadmium-ninhydrin which is consistent with the N-terminal serine as determined by the "dansyl" method. The peptide P-II had identical color with cadmium-ninhydrin, electrophoretic mobility, N-terminal and amino acid composition as peptide P-A whose sequence had been determined. Therefore the sequence of this peptide may be written as:

Ser-Glu-Gly-Lys-CMC.

c. Fraction P-III

Two unresolved radioactive peaks were indicated on the elution profile of Chromobead Type P column (Figure 31). Trial paper electrophoretic separations indicated that these two radioactive peaks could be pooled together (P-III) and easily separated at pH 6.5 and pH 3.5 Fraction PIII (tubes 206-208) were applied to Whatman No. 1 paper and subjected to electrophoresis at pH 6.5 (60 v/cm; origin 20 cm from cathode) for 90 min. The radioactive band P-IIIB was further purified at pH 3.5 (60 v/cm; 2 hours) giving P-IIIB1 and P-IIIB2. These separations are shown in Figure 32. The amino acid compositions of these peptides are shown in Table XXIV.

i. Peptide P-IIIA

This peptide after pH 6.5 electrophoresis was purified by paper chromatography as described for peptide P-I. The strong ninhydrin color observed with band P-IIIA at pH 6.5 separated from the radioactive peak on paper chromatography. The radioactive band was purified at pH 1.8 (60 v/cm; 45 min). The yield of this peptide was low (0.8 per cent). The amino acid analysis indicated that this peptide was still impure and it was ignored in the present study.

ii. Peptide P-IIIB1

The red colour obtained with cadmium-ninhydrin is consistent with the N-terminal lysine obtained by the "dansyl" method. Comparison of the amino acid composition of this peptide to the sequence already obtained indicated the following sequence:

Lys-CMC-Ala-Glu-Leu.
⟶

iii. Peptide P-IIIB2

The electrophoretic mobility of this peptide at pH 6.5 is in agreement with the absence of amidated residues. The amino acid sequence as determined by the Dansyl-Edman procedure gave the following structure for P-IIIB2:

Ser-Lys-CMC-Gly-Asp-Leu.
⟶ ⟶ ⟶ ⟶ ⟶ ⟶

d. Fraction P-IV

Electrophoretic trials at pH 6.5 of the individual fractions of area P-IV (Figure 31) indicated that the radioactive peptides in tubes 225, 228, 230, and 232 were different. Tube 232 contained two

TABLE XXVII

AMINO ACID COMPOSITION OF CYSTEIC ACID

THERMOLYTIC PEPTIDES

(Values are Expressed as Mole Ratios)

Amino Acid	L-A1	Peptide	L-B
Lysine	1.00		0.96
Arginine	1.15		
Cysteic Acid	0.76		1.09
Glutamic Acid	3.02		1.13
Aspartic Acid	1.07		
Serine			1.00
Glycine			0.91
Alanine	1.01		
Leucine			0.91
Number of Residues	8		6
Mobility at pH 6.5(m)	-0.32		-0.39
N-terminal	Ala		Leu
Percentage Yield*	11.2		22.1

* Percentage yields of cysteic acid peptides calculated per 70,000 g.

radioactive peptide bands at pH 6.5. These results showed that these five peptides could be obtained without cross-contamination from each other. Therefore, each of the above tubes was applied separately to Whatman No. 1 paper and electrophoresis at pH 6.5 (60 v/cm; origin at 20 cm from cathode) for 90 min was carried out. The pH 6.5 separation of peptides P-230 and P-228 are shown in Figure 32. Each radioactive band after pH 6.5 electrophoresis was further purified at pH 3.5. Tube 225 was shown to contain two radioactive peptides after pH 3.5 electrophoresis. Fraction P-IV contains at least 6 different radioactive peptides. Each peptide was eluted from the pH 3.5 electropherograms and submitted to amino acid analysis. The amino acid compositions of two peptides P-230 and P-228 are shown in Table XXIV. The amino acid compositions of the other four radioactive peptides suggested that they were either larger varieties of P-230 and P-228 or still impure. The yields of these peptides were only adequate for amino acid analysis. The results of Fraction P-IV indicate multiple splitting has most likely occurred with pepsin about a single unique sequence (most likely the cysteine-lysine sequence) producing many peptides in low yields. This is further substantiated by the fact that the radioactive peptides isolated from PI, PII and P-III did not contain the cysteine-lysine sequence obtained from the tryptic digestions.

(5) Thermolytic Cysteic Acid Peptides

The thermolysin digest was carried out to extend the sequence about the cysteine-lysine sequence obtained from the tryptic digests of CM-tropomyosin and SH-tropomyosin.

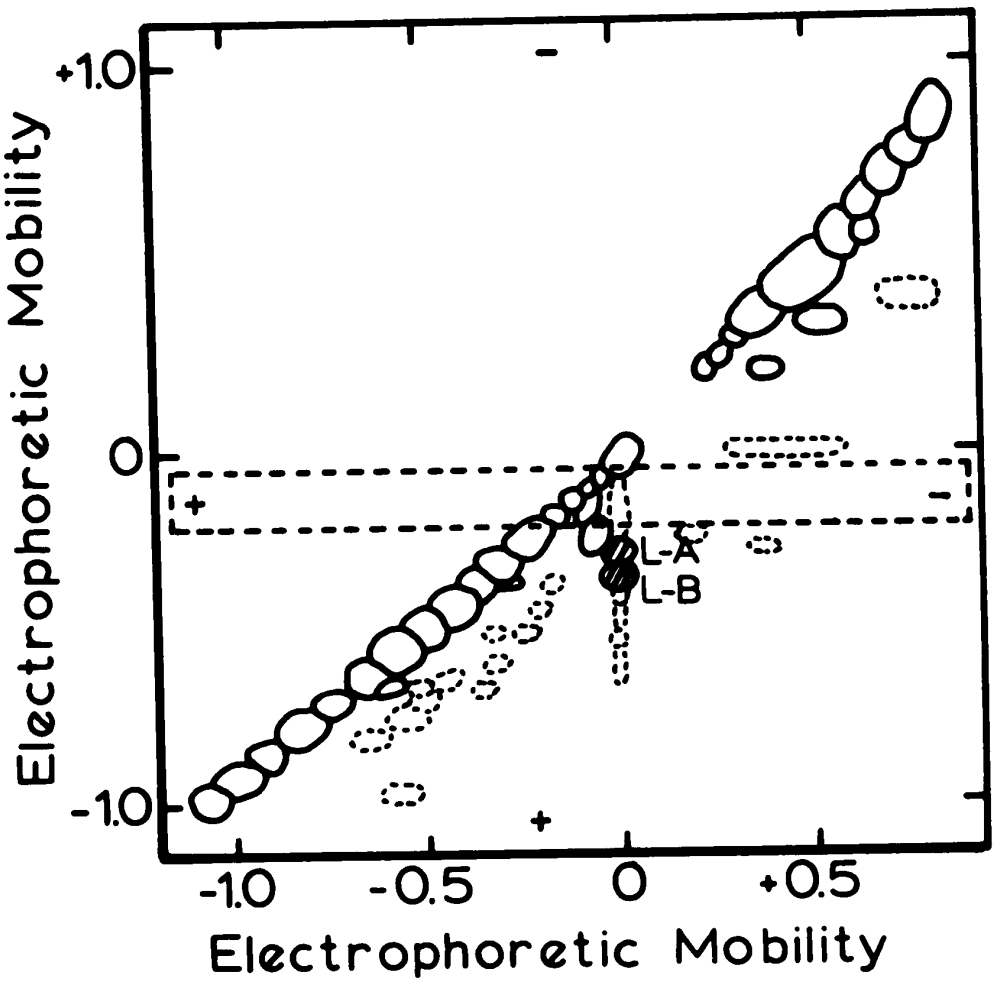


Fig. 33: A pH 6.5/pH 6.5 diagonal peptide "map" of a thermolytic digest of SH-tropomyosin. The major cysteic acid peptides are hatched. The conditions and nomenclature are as described in the text.

a. Diagonal Peptide "Map"

The thermolytic digest (Methods, Chapter II) of SH-tropomyosin with mercaptoethanol dialysis of SH-tropomyosin prior to digestion was submitted to the diagonal fingerprint procedure outlined in Methods (page 86). The pH 6.5/pH 6.5 peptide "map" is shown in Figure 33. The major cysteic acid peptides off the diagonal are hatched. Each cysteic acid peptide thus located was isolated from the preparative sheets and was further purified by electrophoresis at pH 1.8 or pH 3.5.

b. The Neutral Band

i. Purification

The neutral band on the original preparative pH 6.5 electrophoresis (60 v/cm; 40 min) was cut out, oxidized, and re-run at pH 6.5 (60 v/cm; origin 20 cm from cathode) for 1 hour. Two cysteic acid bands were obtained, L-A and L-B. Peptide band L-A was further purified at pH 3.5 (60 v/cm; 50 min) and gave two cysteic acid peptides L-A1 and L-A2. Peptide L-B was purified at pH 1.8 (60 v/cm; 45 min). The amino acid compositions of peptides L-A1 and L-B are shown in Table XXVII.

ii. Sequence determinations

Peptide L-A2 and L-B

Peptide L-A2 had the identical amino acid composition as peptide L-B. It was obtained in very low yields and was ignored in this study. Peptide L-B had a mobility with respect to aspartic acid m = -0.39 suggesting that there is a net negative charge of 1 (Offord, 1966) and

TABLE XXVIII

AMINO ACID COMPOSITION AND SEQUENCE OF TRYPTIC PEPTIDES

FORMED BY TRYPSIN DIGESTION OF PEPTIDE L-A1

(Values are Expressed as Mole Ratios)

Peptide	Electrophoretic mobility at pH 6.5(m)	Amino Acid Composition and Sequence	Percentage Yield	Cadmium-Ninhydrin Colour	M.W.
L-A1	-0.32	{Ala,Glu,Asp,Arg,Cya,Lys,Gln / Gln,Ala,Glu,Asp,Arg,Cya,Lys,Gln}	-	red	-
L-A1-T1	-0.24	Cya-Lys-Gln Q81 1.15 1.04 ⟶ ⟶ ⟶	16.0	yellow-orange	425.4
L-A1-T2	-0.32	Ala-Glu-Asp(Arg,Cya,Lys,Gln) 1.03 1.04 1.03 Q94 Q80 Q91 1.04 ⟶ ⟶ ⟶	13.3	red	896.7
L-A1-T3	-0.39	Gln(Ala,Glu,Asp,Arg) Q92 Q96 Q92 1.09 1.11 ⟶	7.8	red	616.6
L-A1-T4	-0.44	Ala-Glu-Asp,Arg 1.00 1.07 1.00 Q93 ⟶ ⟶ ⟶ ⟶	11.2	red	488.5

that glutamic was present. The sequence by the Dansyl-Edman procedure was shown to be:

Leu-Ser-Glu-Gly-Lys-Cya.
→ → → → → →

Peptide L-Al

The electrophoretic mobility of L-Al at pH 6.5 (m = -0.32) suggests that there is a net negative charge of 1 (Offord, 1966) and that two of the 4 acidic residues must be amidated. The results of two steps of the Dansyl-Edman procedure, were:

N-terminal	2nd residue
ala and glu	ala and glu

This result indicated that there was more than one variety of the peptide present. The sequence of L-Al was determined by digesting the thermolytic fragment with TPCK-trypsin at a 1:50 molar ratio of enzyme to peptide for 12 hours at 37°C in 0.05 M N-ethyl-morpholine-acetate buffer pH 8.0. The peptides produced were purified on Whatman No. 1 paper by electrophoresis at pH 6.5 (60 v/cm; 40 min). The band L-Al-T2 was further purified at pH 1.8 (60 v/cm; 45 min). Four major cadmium-ninhydrin positive bands were obtained. The amino acid compositions, electrophoretic mobilities, and sequence by the Dansyl-Edman procedure are shown in Table XXVIII. The C-terminal residue of peptide L-Al-T1 was identified after two Edman degradations as DNS-glutamine without acid hydrolysis and DNS-glutamic acid after acid hydrolysis. The negative mobility of this peptide at pH 6.5 is explained by a suppressed α-amino group with N-terminal cysteic acid (Offord, 1966). Peptide L-Al-T4 has a

TABLE XXIX

RELATIVE PERCENTAGE RECOVERIES OF CYSTEINE SEQUENCES OF TOTAL RADIOACTIVITY FROM CHROMOBEAD TYPE P COLUMN SEPARATION OF A TRYPTIC DIGEST

Peptide	Amino Acid Sequence	Relative Per Cent Recovery*	
T-IA	CMC-Ala-Glu-Leu-Glu-Glu-Gly-Leu-Lys	22.2	44.8
T-IIIA	CMC-Ala-Glu-Leu-Glu-Glu-Glu-Leu-Lys	11.1	
T-IVA	CMC-Ala-Glu-Leu-Glu-Glu-Glu-Leu-Lys-Lys	11.5	
T-IB	CMC-Gly-Asp-Leu-Glu-Glu-Glu-Leu-Lys	12.8	18.7
T-IIIB	CMC-Gly-Asp-Leu-Glu-Glu-Glu-Leu-Lys	3.0	
T-IVB	CMC-Gly-Asp-Leu-Glu-Glu-Glu-Leu-Lys-Lys	2.9	
T-V	CMC-Lys		22.0
T-II	Not Determined		14.4
Total			99.9

* The relative ratios of the radioactive peptides in each peak of the Chromobead Type P separation was determined from the yields of the pure peptides after electrophoretic purification and by radioactive strip scanning of radioactive peptides on the electropherograms and calculating the areas of each peak. Both methods were in close agreement.

TABLE XXX

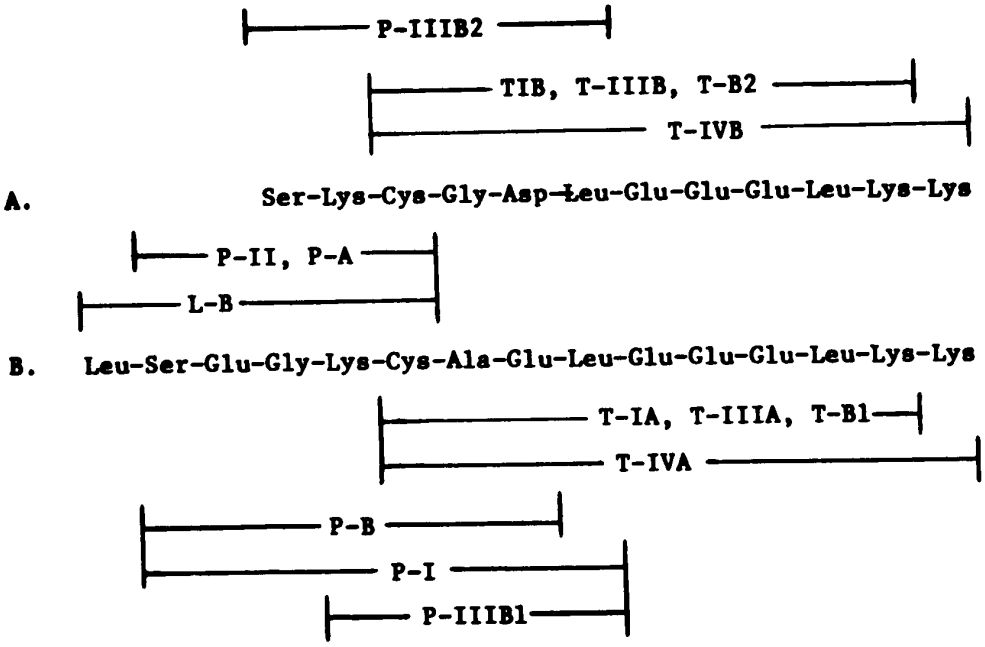
RELATIVE PERCENTAGE RECOVERIES OF CYSTEINE SEQUENCES OF TOTAL RADIOACTIVITY FROM CHROMOBEAD TYPE P COLUMN SEPARATION OF A PEPTIC DIGEST

Peptide	Amino Acid Sequence	Relative Per Cent Recovery[1]	
P-I	Ser-Glu-Gly-Lys-CMC-Ala-Glu-Leu	43.7	
P-IIIB1	Lys-CMC-Ala-Glu-Leu	11.1	57.4
P-IIIA[2]	Not Determined	2.6	
P-II[3]	Ser-Glu-Gly-Lys-CMC	8.6	
P-IIIB2	Ser-Lys-CMC-Gly-Asp-Leu	11.1	
P-IV	Not Determined	22.9	
Total		100.0	

[1] The relative ratios of the radioactive peptides in each peak of the chromobead Type P separation was determined from the yields of pure peptides after electrophoretic purification.

[2] Amino acid composition suggested that this peptide belongs to this unique sequence.

[3] This sequence could belong to either the CMC-ala-glu or CMC-gly-asp sequence.



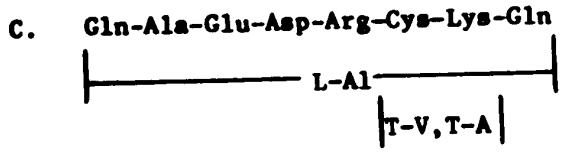


Fig. 34: Unique Amino Acid Sequences of Cysteine Peptides from Tropomyosin. T, L, and P represent tryptic, thermolytic and peptic peptides. In most cases peptic and tryptic peptides were isolated as both CMC and cysteic acid peptides. Thermolysin peptides were isolated as cysteic acid peptides.

mobility at pH 6.5 which is consistent with the presence of no amidated residues. These results establish the following sequence for peptide L-A1:

Gln-Ala-Glu-Asp-Arg-Cya-Lys-Gln

4. DISCUSSION

Chemically dissimilar polypeptide chains are indicated by the isolation and sequence determination of the three unique cysteine peptides shown in Figure 34. A summary of the ^{14}C-CMC peptides isolated from the tryptic digest of ^{14}C-CM-tropomyosin is shown in Table XXIX with their relative recoveries of total radioactivity from the Chromobead Type P column. Similarly the results of the peptic digest of ^{14}C-CM-tropomyosin is shown in Table XXX. The radioactivity from the column in P-IV is almost identical to that of T-V of 22.9 and 22.0 per cent respectively. T-V has been shown to contain the cysteine-lysine sequence. This close agreement along with the fact that this sequence was not found in the peptic digest would suggest that fraction P-IV contains this unique sequence. Considering the ^{14}C-results of the tryptic digest and ignoring fraction T-II the ratio of sequences A, B, and C of tropomyosin (Figure 34) would be approximately 1:2:1.

The possibility that the sequence represented by P-II or P-A and L-B (Figure 34) may occur with either unique sequence Cys-Gly-Asp or Cys-Ala-Glu cannot be ruled out.

Significantly, a high degree of homology exists between peptides A and B which differ only in three amino acid residues. These three substitutions are highly conservative in nature. All three changes can occur from a single-base mutation.

The results of this chapter clearly are incompatible with a homogeneous tropomyosin preparation of two chemically identical subunits.

CHAPTER VI

THE AMINO ACID SEQUENCES OF CNBr FRAGMENTS OF TROPOMYOSIN

1. INTRODUCTION

The sequence studies in this chapter were carried out to determine the sequence specificity required to maintain the coiled-coil structure of tropomyosin.

A detailed model for α-keratin based on the coiled-coils was suggested by Pauling and Corey (1953). They proposed a repeating sequence of residues which was necessary to explain the α-keratin X-ray diffraction pattern. Crick (1953) concluded that the α-pattern could be explained in terms of the α-helix, that is the α-helices tending to pack side-by-side in a "knobs-into-holes" manner. Thus a postulate of a repeating sequence of residues is not necessary. The basis of Crick's two-stranded coiled-coil model was that optimum side chain interactions will occur when the α-helices pack at an angle of 20° to each other. It was pointed out that the more awkward, less flexible nature of the valine, leucine, and isoleucine residues may make it easier for the α-helices to fall into a coiled-coil configuration. Because of the non-integral nature of the α-helix, that is 3.6 residues turn, the non-polar groups may occur every seventh residue so that they point to the inside of the coiled-coil. Cohen and Holmes (1963) suggested the possibility for paramyosin that the main hydrophobic bonding may be between alanine and leucine since 2/7 of all residues are alanine and leucine. If the bonding were regular, every seventh

residue would be leucine and each leucine would have an alanine three residues on either side of it. If the chains were anti-parallel, this sequence could be used to form a densely hydrophobic region between the two chains.

The segmented rope model with a repeating distribution of hydrophobic residues has been shown capable of accounting for the X-ray diffraction results (Parry, 1970). This model consists of short lengths of straight α-helix inclined to one another so that perfect knob-into-hole packing occurs at the cross-over point; that is, it approximates the coiled-coil over a short range. The inter-chain packing would deteriorate with increasing length of the inclined α-helices and it is necessary to postulate interruptions in the axes of the α-helices at intervals of 20 - 30 Å.

Although the present sequences are not of sufficient length to properly test these suggested sequence specificities, there is a good indication that a periodicity of non-polar residues does occur. Thus in peptides T-M4B (Table XVIII), isoleucine or leucine residues occur at positions 2, 6, and 10 while in the cysteine peptide B (Figure 34) leucine occurs at positions 1, 9, and 13. An extension of these sequences was required to test the validity of the apparent repeating hydrophobic pattern. To this end, a cyanogen bromide cleavage of ^{14}C-CM-tropomyosin was carried out. The major radioactive fragment was isolated and submitted to further sequence studies.

2. MATERIALS AND METHODS

A. Materials

All chemicals and enzymes have been described in this thesis (Chapter II) or were reagent grade and were used without further purification.

B. Methods

The methodology for sedimentation velocity, sedimentation equilibrium, disc-gel electrophoresis, and Dowex 1 and Dowex 50 chromatography are described in Chapter II of this thesis.

(1) Cyanogen Bromide Cleavage of ^{14}C-CM-Tropomyosin

The general procedure as described by Steers *et al*. (1965) was used. The lyophilized, reduced carboxymethylated protein was dissolved in 70% formic acid such that after the addition of the cyanogen bromide (100-fold excess of crystalline cyanogen bromide to theoretical methionine) dissolved in 70% formic acid, the protein concentration was 1%. The flask was stoppered and the reaction was allowed to proceed at room temperature (22-25°C) for 20 hours. After the cyanogen bromide cleavage, the reaction mixture was diluted with 10 volumes of deionized water and freeze-dried.

(2) G-75 Sephadex Chromatography of CNBr-Fragments

The column (250 cm x 5 cm) was prepared as described in Chapter III of this thesis. Approximately 470 mg of freeze-dried CNBr treated CM-tropomyosin was dissolved in 25 ml of 0.2 M KCl-8M urea - 0.025 M KH_2PO_4 - KOH buffer, pH 6.0, applied to the column and eluted with this buffer at a flow rate of approximately 40 ml per hour by upward flow. The eluate was collected in 14.0 ml fractions and monitored by absorbancy measurements at 280 mμ and 230 mμ.

(3) G-50 Sephadex Chromatography of CNBr-Fragments

The column (100 cm x 5 cm) was a Sephadex glass column, fitted with adaptors with nylon nets and bed support screens. Sephadex G-50 (20-80 μ particle size) was allowed to swell in 0.05 N acetic acid with several decantations to remove the fines. The gel slurry and the 0.05 N acetic acid solution were de-aerated before packing the column according to the procedure outlined in the Sephadex Manual. The column was operated at a flow rate of 2 ml/cm^2/hr or 40 ml/hr. The flow rate was maintained by the use of a constant volume pump.

A 25.0 ml sample containing approximately 500 mg of CNBr-fragments were dissolved in 0.05 N acetic acid and applied to the column. The eluate was collected in 20.0 ml fractions and monitored by absorbancy measurements at 230 mμ and radioactivity counting of 50 μl aliquots from each fraction. The small CNBr fragments were identified by the ninhydrin procedure after alkaline hydrolysis (Hirs et al., 1956; Moore, 1954).

Ninhydrin Assay

0.2 ml of sample (The sample volume is not critical).

0.5 ml of 2.5 N NaOH

Heat at 90° in open tubes for 2-1/2 hours

Cool

Add 0.5 ml of 30% acetic acid with vigorous shaking

Add 0.5 ml of ninhydrin reagent and shake

Heat for 15 min at 100°C

Cool

Read at 570 mμ (if too concentrated dilute with 50% ethanol)

Ninhydrin reagent

1 g ninhydrin

0.15 g hydrindantin

12.5 ml 4N NaAc buffer, pH 5.5

37.5 ml methyl cellosolve

Store in dark under nitrogen.

(4) QAE-Sephadex A-50 Chromatography of CNBr-Fragments X-A

A Sephadex glass column (100 cm x 5 cm) with adaptors with nylon nets and bed support screens was used. The resin was prepared as described in this thesis Chapter III.

The sample (CNBr fragments, peak X-A from G-50 Sephadex or G-75 Sephadex) was dissolved in starting buffer to give a 2% protein solution and applied to the column. After application a linear gradient elution was generated by using 2.5 litre volumes of equilibration buffer (0.10 M KCl-8M urea-0.05 M Tris-HCl buffer, pH 7.5) and 0.30 M KCl - 8M urea - 0.05 M Tris-HCl buffer, pH 7.5) contained in a two chambered apparatus of identical cross-section. The column was eluted at a flow rate of 35 ml per hour at room temperature and fractions of 20.0 ml were collected. The fractionation was monitored by radioactivity counting on 100 µl aliquots of the effluent fractions and by absorbancy measurements at 230 mµ.

(5) Isoelectric Focusing of CNBr-Fragments

Approximately 10 mg of the fragments were submitted to the two step isoelectric focusing procedure as described in Chapter III.

Fractions 35-75 inclusive were pooled (32 ml) from the first isoelectric focusing run (46 hr) and re-run in a second isoelectric focusing experiment (46 hr). The solutions for the second isoelectric focusing experiment were as follows:

Light gradient solution

Ampholyte fraction	8.0 ml
Urea solution (7M)	
- 23.1 g urea diluted	
to 55 ml with water	42.0 ml
	50.0 ml

Dense gradient solution

Ampholyte fraction	24.0 ml
Urea (7M) - sucrose solution	
- 21 g urea, 27.5 g	
sucrose to 50 ml with	
water	26.0 ml
	50.0 ml

(6) N-terminal Identification of CNBr-Fragment X-A2

Approximately 1 mg of X-A2 was dissolved in 5 ml of a solution containing 0.21 g $NaHCO_3$ and 2.4 g urea (0.5 M carbonate, 8 M urea). To this solution 5 ml of acetone containing 100 mg of DNS-Cl was added. This reaction mixture was incubated at room temperature overnight (19 hr). The mixture was then dialyzed exhaustively against water. The dialyzate was centrifuged on an International Clinical centrifuge and the supernatant discarded. The precipitate of the DNS-peptide was washed once with approximately 3.0 ml of deionized water. The sediment was dispersed in 500 µl of deionized water and aliquots taken for acid hydrolysis and identification of the N-terminal by the usual procedure (Chapter II).

(7) Limited Chymotryptic Digest of CNBr-Fragment X-A2

The digestion of X-A2 with chymotrypsin (1:100 molar ratio of enzyme to peptide) was followed on the pH stat by measuring the volume of base (0.15 N NaOH) required to maintain the pH at 8.0. The peptide (170 mg or 10 μmole) was dissolved in 0.01 M KCl solution brought to pH 8.0 with NaOH to give a protein concentration 1%. The reaction was carried out at room temperature (22-25°) and allowed to proceed for 40 min at which time the titration curve began to plateau. The reaction was terminated by freeze-drying.

(8) G-25 Sephadex Chromatography of a Chymotryptic Digest of X-A2

a. Termination of Chymotryptic Activity

The freeze-dried digest was dissolved in 3.0 ml of 0.05 N-ethylmorpholine-acetate buffer, pH 8.0. Diisopropylfluorophosphate (DFP) was added to give a final concentration of 0.01 M. A 10 mM ATEE solution (N-acetyl-L-tyrosine ethyl ester) was used for the assay of chymotryptic activity. To 3.0 ml of ATEE solution (10 mM Ca^{++}, 0.1 M KCl) was added 5 μl aliquots of the digest dissolved in N-ethylmorpholine buffer before DFP treatment and 15 min after the addition of DFP. The chymotryptic activity was followed on the pH stat at 25° at pH 8.0 by titration with 0.04 N NaOH. Complete loss of activity was observed on the 15 min sample.

b. Column Preparation

A Sephadex glass column with adaptors (2.5 cm x 100 cm) was used. Sephadex G-25 fine (20-80 μ) was allowed to swell in 0.05 M N-ethyl-morpholine-acetate buffer, pH 8.0, the fines were removed, the gel slurry

and solutions were de-aerated and then packed and equilibrated with the same solution by the recommended Sephadex procedure. A 3.0 ml sample of the chymotryptic digest of the peptides was applied to the column, and was then eluted with 0.05 M N-ethylmorpholine acetate buffer, pH 8.0 at a flow rate of 12 ml per hour (2.5 ml/cm^2/hr) and at a temperature of 4°C. The flow rate was maintained by the use of a constant volume pump. The eluate was collected in 3.0 ml fractions. The fractionation was monitored by radioactive counting on 25 µl aliquots of the effluent fractions and by absorbancy measurements at 230 mµ and 280 mµ. The appropriate fractions were combined and recovered by lyophilization.

(9) G-50 Sephadex Chromatography of Chymotryptic Fragments X-A2-C1

Sephadex G-50 fine (20-80µ) was allowed to swell in 0.05 M N-ethylmorpholine-acetate buffer, pH 8.0, the very fine particles were removed, the gel slurry and solutions were de-aerated and then packed into a Sephadex glass column with adaptors (2.5 cm x 100 cm) by the recommended Sephadex procedure. The column was equilibrated with this buffer before sample application. The fraction X-A2-C1 from the G-25 Sephadex separation of the chymotryptic digest was dissolved in 3.0 ml of 0.05 M N-ethylmorpholine-acetate buffer, pH 8.0 and applied to the column. The column was eluted with this buffer at a flow rate of 12 ml per hour and at a temperature of 4°C. The flow rate was maintained by the use of a constant volume pump. The eluate was collected in 3.0 ml fractions. The fractionation was monitored by absorbancy measurements at 280 mµ and 230 mµ and by radioactivity

counting on 25 µℓ aliquots of the effluent fractions. The appropriate fractions were pooled and freeze-dried.

(10) Optical Rotatory Dispersion (ORD) and Circular Dichroism (CD) Measurements

ORD and CD measurements were carried out in the laboratory of Dr. C.M. Kay by Mr. K. Oikawa whose skilled assistance is gratefully acknowledged.

A Cary Model 60 recording spectropolarimeter equipped with a Cary Model 6001 CD attachment and water cooled lamp housing, was used for these measurements in accordance with methodology described by Kay and Oikawa (1966) and Oikawa *et al.* (1968). The α-helical content was determined from the ORD data in the visible region using the Moffit equation (Moffit and Yang, 1956) and in the ultraviolet region by the amplitude of the conformational Cotton trough at 233 mµ (Davidson *et al.*, 1966).

The Moffit equation is represented as follows:

$$[m'] = \frac{a_o \lambda_o^2}{\lambda^2 - \lambda_o^2} + \frac{b_o \lambda_o^4}{(\lambda^2 - \lambda_o^2)^2}$$

where $[m'] = \frac{3}{n^2+2} \cdot \frac{m}{100} [\alpha]$ is equal to the reduced mean residue rotation.

$[\alpha]$ is the specific rotation in c.c. deg/g dm,

λ is the wavelength at which the rotation is measured,

m is the average residue weight

n is the refractive index of the solvent.

The Moffit equation was plotted as $-[m']\lambda^2-\lambda_o{}^2/\lambda_o{}^2$ versus $\lambda_o{}^2/\lambda^2-\lambda_o{}^2$ from which the slope corresponded to b_o and the intercept to a_o. The value of λ_o was taken as 212 mμ. The percentage α-helix was calculated from the b_o (helix constant) value on the assumption that a figure of -640 degrees characterizes a fully coiled, right handed α-helix (Jirgensons, 1969).

The mean residue rotation values $[m']_{233}$, of -16,000° and -2,000° for a completely α-helical and random coil polypeptide were used for the calculation of percent α-helix at 233 mμ.

For CD measurements the mean residue molecular ellipticity obtained was given by the following relation:

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

where m is the mean residue molecular weight,

θ is the observed ellipticity in degrees,

ℓ is the path length of the cell in dm,

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

The units of [θ] are degrees cm^2 per dmole.

The reference values (Cassim and Yang, 1967) used for determination of percent α-helix from $[\theta]_{220}$ and $[\theta]_{210}$ are as follows:

	0% α-helix	100% α-helix
$[\theta]_{220}$	+4,400	-39,700
$[\theta]_{210}$	-5,800	-37,000

3. RESULTS

A. Peptide Nomenclature

The sequence of purification steps for the isolation and characterization of the cyanogen bromide fragments of tropomyosin was the following:

1) The cyanogen bromide cleavage of CM-tropomyosin was denoted by the letter X.
2) The cyanogen bromide cleavage products were separated on G-50 Sephadex. The two peaks were designated X-A and X-B where A denotes the first peak in the elution profile.
3) The small cyanogen bromide fragments of fraction X-B were selectively pooled into fractions XB1 and XB2. These fractions were further purified by high voltage electrophoresis. Each peptide in fraction X-B1 and XB2 was assigned a small letter in alphabetical order, according to its relative mobility at pH 6.5 or 1.8, the most basic peptide having the letter "a".

4) The large cyanogen bromide fragments of fraction X-A were further purified by column chromatography on QAE-Sephadex. The two fractions X-A1 and X-A2 represent the first and second peaks off the column, respectively.

5) Chymotryptic digestion of fragment X-A2 was designated by the letter C. The digest was separated on G-25 Sephadex and the first peak off this column was denoted X-A2-C1.

6) Separation of fraction X-A2-C1 on Sephadex G-50 resulted in two column fractions designated X-A2-C1a and X-A2-C1b where "a" is assigned to the first peak in the elution profile.

7) Separation of fraction X-A2-C1b on Dowex 1 gave eleven fractions which were assigned Roman numerals I to XI. Several of the Dowex 1 fractions were purified by Dowex 50 chromatography. Capital letters were added to these designations in alphabetical order to indicate their elution order from the Dowex 50 column, the lowest letter being assigned to the first peak in the elution profile.

8) The products of peptides subsequently digested with trypsin have been designated by the letter T. An Arabic numeral is assigned to each of the products to indicate the relative mobility of the peptide on pH 6.5 electrophoresis, the most basic peptide of the digest having the lowest number. If further purification of a pH 6.5 band produced more than one peptide at pH 3.5 or pH 1.8 small letters were

TABLE XXXI

AMINO ACID COMPOSITION OF CNBr-TREATED

CM-TROPOMYOSIN AND CNBr FRAGMENT X-A2

(All Values are for Hydrolysates Obtained after 22 Hours at 110°C)

Amino Acid	CM-tropomyosin[a]	CNBr-treated CM-tropomyosin[a]	X-A2[b]	
Lysine	40.8	40.9	15.8	16
Histidine	2.0	2.1	1.8	2
Arginine	14.8	14.7	5.6	6
Aspartic acid	30.1	30.3	13.4	13
Threonine	7.8	7.7	4.4	5
Serine	12.1	11.6	7.5	8
Glutamic acid	77.2	76.6	37.6	38
Proline	0.5	0.4	-	
Glycine	4.0	4.0	1.8	2
Alanine	36.4	37.1	15.7	16
Valine	9.7	9.6	7.8	8
Methionine	6.3	0.4	-	
Isoleucine	10.8	11.1	7.3	7
Leucine	33.3[c]	33.3[c]	17.0[d]	17
Tyrosine	5.9	5.6	4.6	5
Phenylalanine	1.4	1.4	1.0	1
CM-cysteine	1.3	1.2	0.9,1.0[e]	1
Homoserine	-	5.7	1.0	1
			Total Residues	146

[a] Analyses converted to residues per 35,000 g.

[b] Amino acid analysis of X-A2 from preparative isolation.

[c] Leucine = 95.0 residues per 100,000 g.

[d] Leucine = 17.0 residues per mole of fragment.

[e] Value obtained by radioactive incorporation.

added to the designations in alphabetical order with the letter "a" assigned to the most basic peptide.

An example illustrates the system: peptide X-A2-C1b-VIIA-T1b was obtained from the first fraction (A) on the Sephadex G-50 gel-filtration step of cyanogen bromide treated CM-tropomyosin (X), and the second fraction on QAE-Sephadex chromatography (2). After chymotryptic digestion (C) of this fraction, it was recovered as the first fraction on Sephadex G-25, the second fraction on G-50 Sephadex, the seventh fraction on Dowex 1 chromatography, and the first fraction on Dowex 50 chromatography. After tryptic digestion of this fraction, it was recovered as the most basic component on pH 6.5 electrophoresis and the second most basic component on further purification at pH 3.5.

B. Cyanogen Bromide Cleavage of CM-Tropomyosin

The amino acid analysis of CM-tropomyosin and the CNBr cleavage mixture before column fractionation have been converted to residues per 35,000 g for comparison to the CNBr fragment X-A2 (Table XXXI). Essentially quantitative conversion of methionine to homoserine has occurred during the CNBr cleavage. Comparison of the amino acid analyses of CM-tropomyosin before reaction with CNBr and the CNBr cleavage mixture indicate a 94% disappearance of methionine and a 91% conversion of methionine to homoserine (Table XXXI). No correction for destructive losses of homoserine during acid hydrolysis have been made. The data also indicate that cyanogen bromide, under the conditions used, causes no significant destruction or conversion of other amino acid

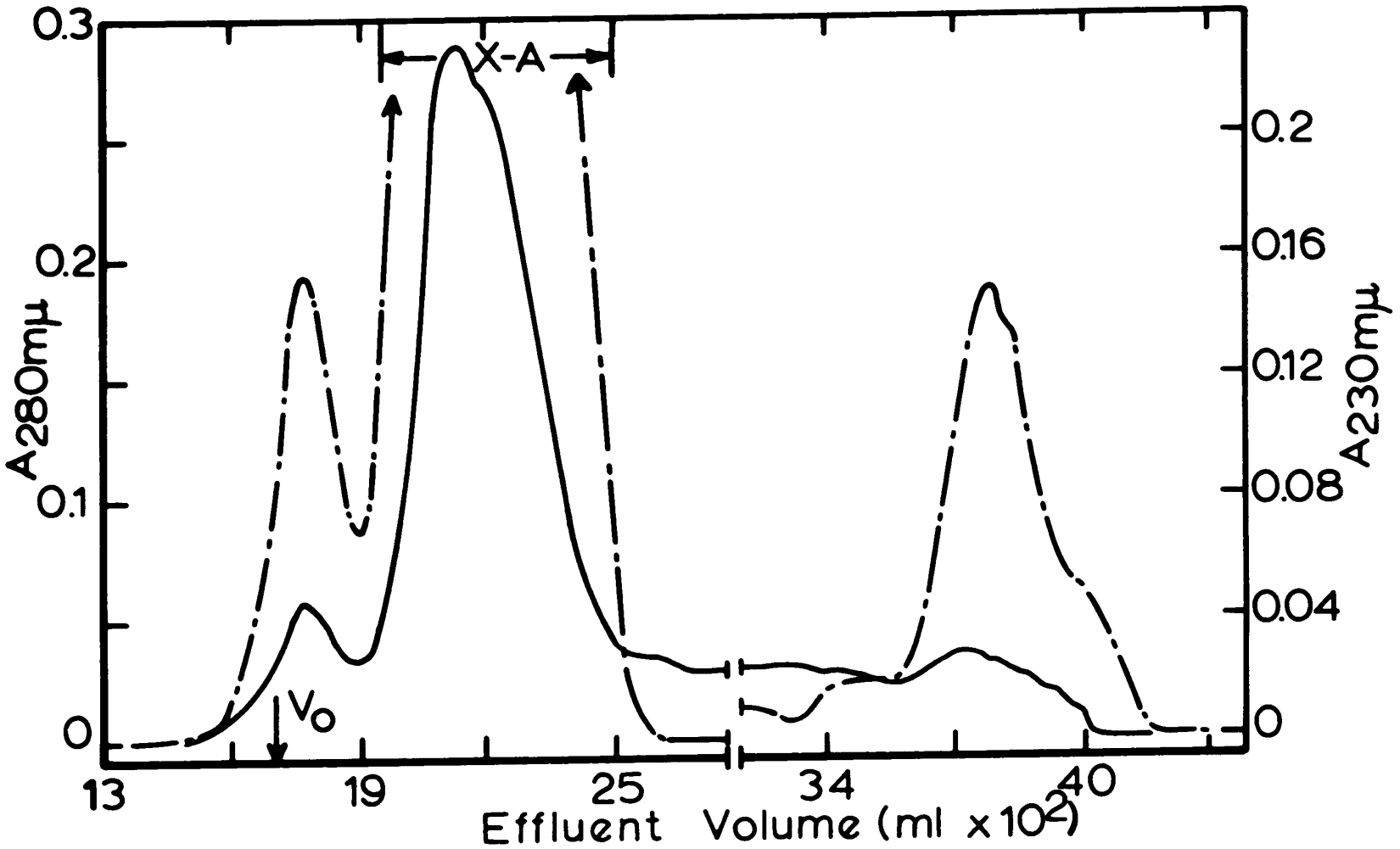


Fig. 35: Gel-filtration of CNBr cleavage products of CM-tropomyosin on a 250 cm x 5 cm Sephadex G-75 column in 0.2 M KCl - 8 M urea 0.025 M phosphate buffer, pH 6.0. The effluent was monitored for absorbancy at 280 mμ (————) and 230 mμ (—— - ——).

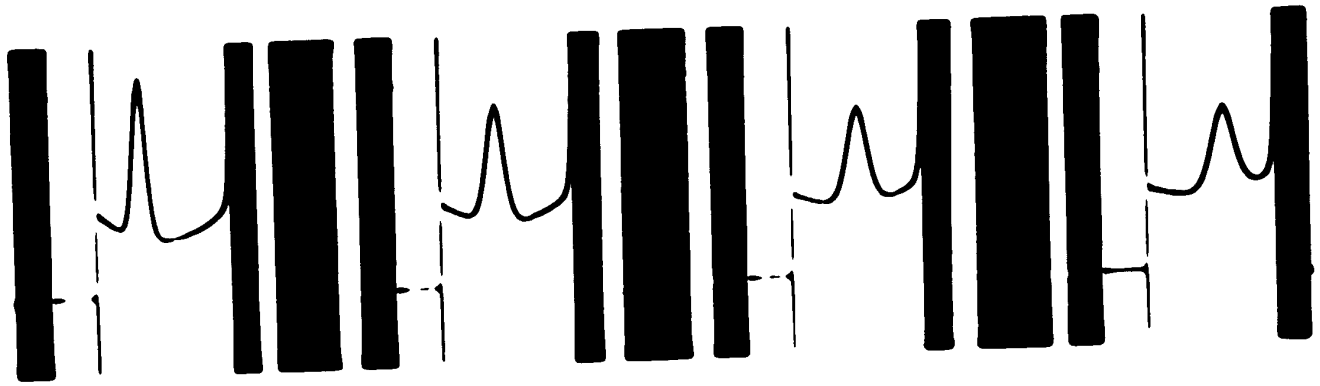


Fig. 36: Sedimentation velocity patterns of CNBr fragments from G-75 Sephadex chromatrography at a concentration of 6.6 mg/ml (0.66%) in 1.1 M KCl - 0.025 M phosphate buffer, pH 7.0 at 60,000 r.p.m. Photographs were taken at 16 min intervals after top speed was reached. The intervals shown are at 32, 64, 96, and 128 minutes. The direction of sedimentation is toward the right.

residues in the protein derivative as reflected by the unchanged values for the other amino acid residues.

C. Fractionation of the CNBr Cleavage Fragments on Sephadex G-75

The G-75 Sephadex chromatography of the CNBr fragments was carried out in a strong denaturing media (8 M urea) to prevent the possible formation of "coiled-coils" among the CNBr fragments. Because of the likelihood of CNBr fragments being obtained lacking tyrosine residues the absorbancy measurements of the column effluent were performed at 230 mμ as well as 280 mμ.

The elution profile on the cleavage products of CM-tropomyosin is shown in Figure 35. The small peak eluting from the column just after the void volume has the identical position of CM-tropomyosin and most likely represents uncleaved material. The chromatogram shows only one major peak, X-A. The position of this peak indicates that large CNBr fragments of similar size have been obtained. A small 230 mμ peak was found late in the chromatogram. The low 280 mμ absorption in this region signifies the absence of tyrosine residues. This peak probably contains very small CNBr fragments. Due to the difficulty in isolating small peptides from 8 M urea solutions no further studies were carried out on these peptides. Fraction X-A was pooled, dialyzed exhaustively against water and freeze-dried. To determine the approximate size and number of CNBr fragments contained in fraction X-A, disc-gel electrophoresis, sedimentation velocity, and sedimentation equilibrium studies were carried out.

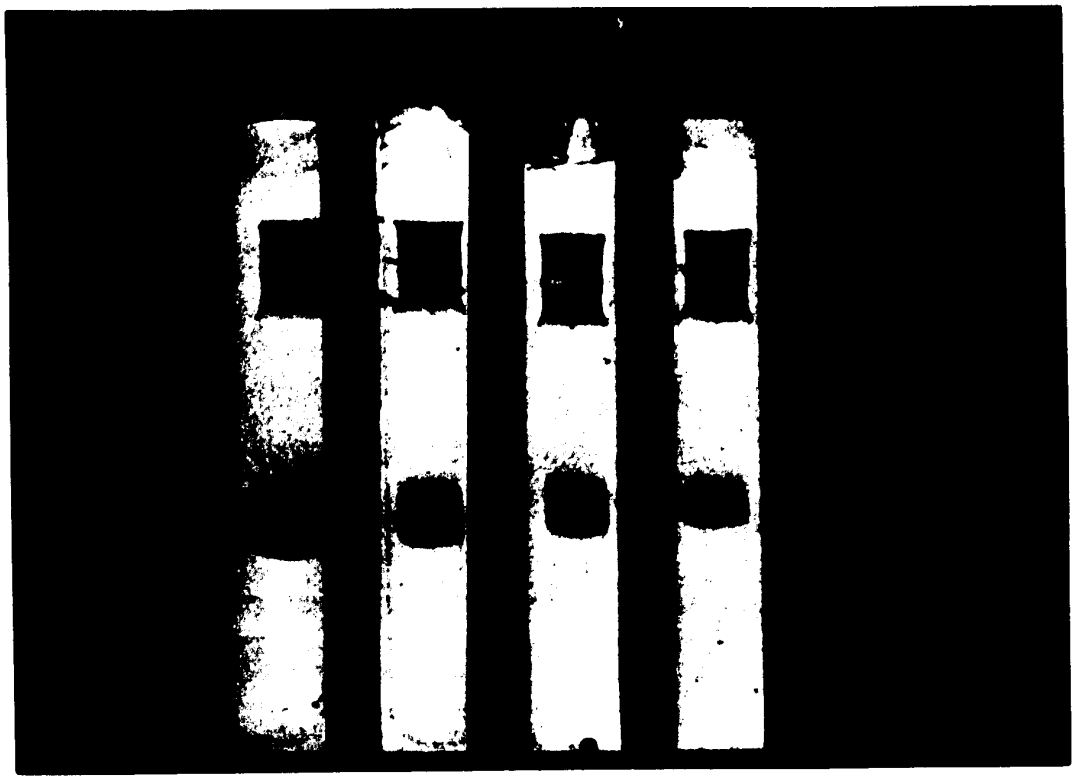


Fig. 37: Disc electrophoresis of CNBr fragments from G-75 Sephadex chromatography at pH 9.5 - 7% gel in 8 M urea at four different protein concentrations. The concentrations from left to right were 200 µg, 100 µg, 40 µg, and 20 µg.

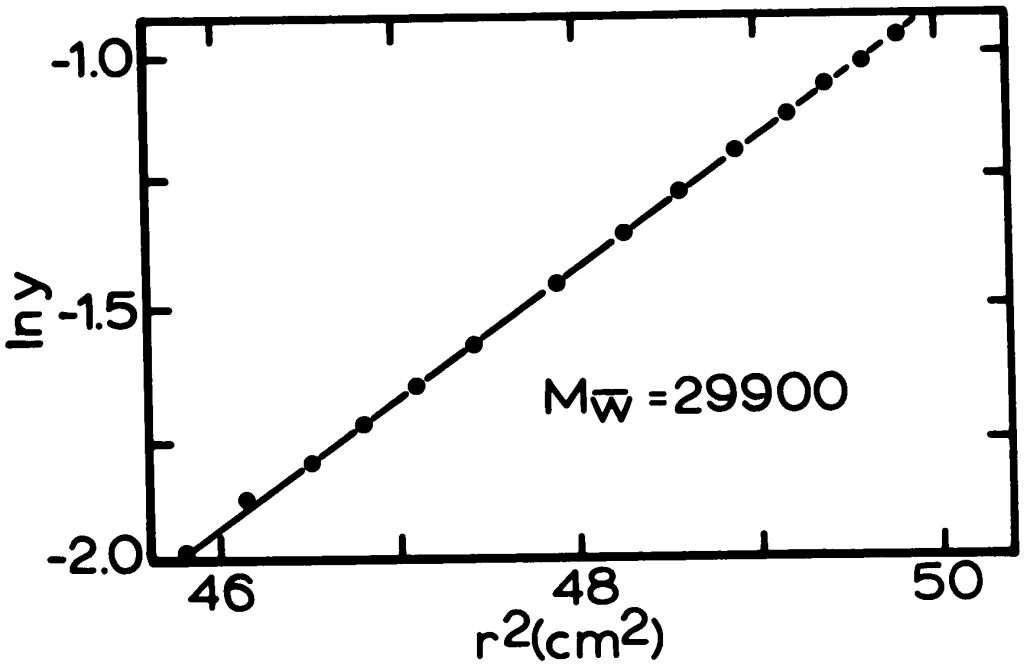


Fig. 38: Plot of the natural log of the concentration (fringe displacement) as a function of the square of the distance from the axis of rotation. A low speed equilibrium centrifugation experiment with 0.22% X-A in 1.1 M KCl - 0.025 M phosphate buffer, pH 7.0. The data was calculated after 48 hours of centrifugation at 12,964 r.p.m. using $\bar{v}$ = 0.733 ml/g.

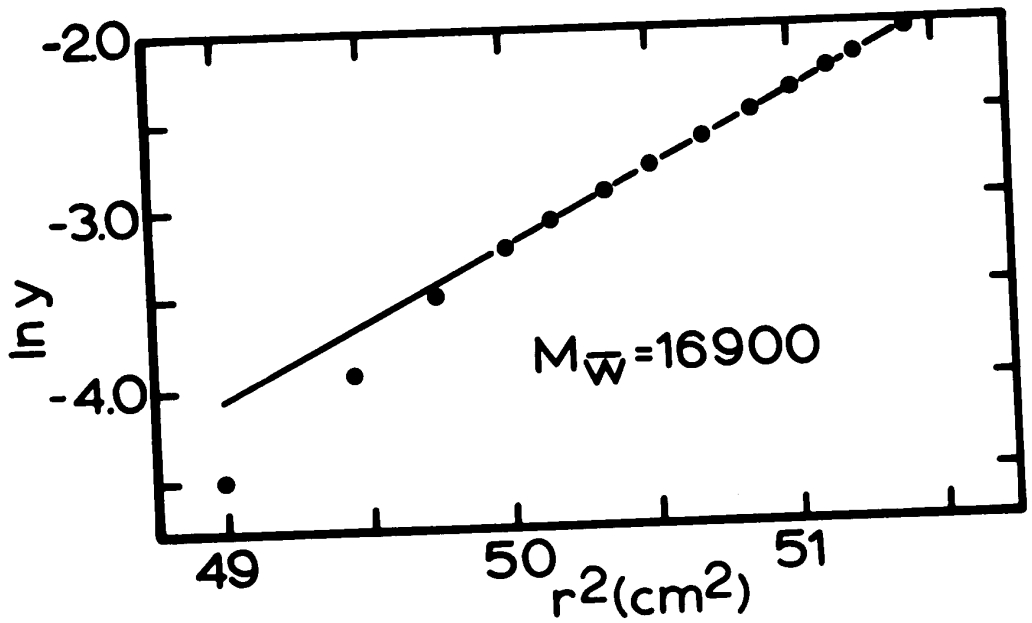


Fig. 39: Plot of the natural log of the concentration (fringe displacement) as a function of the square of the distance from the axis of rotation. A high speed sedimentation equilibrium experiment with 0.125% X-A in 0.2 M KCl - 8 M urea - 0.025 M phosphate buffer, pH 7.0. Centrifugation speed was 36,000 r.p.m. A value $\overline{v}$ = 0.728 ml/g was used for molecular weight calculation.

(1) Sedimentation Velocity

The sedimentation velocity pattern in Figure 36 displays only a single symmetrical peak at a protein concentration of 6.6 mg/ml for fraction X-A. Sedimentation patterns by themselves cannot be used as an absolute criterion for homogeneity; however the pattern in Figure 36 does indicate that if more than one CNBr fragment is present, they are of similar size.

(2) Disc-Gel Electrophoresis

Disc-gel electrophoresis of fraction X-A in 8 M urea at pH 9.5 at four different protein concentrations is shown in Figure 37. A high similarity of the CNBr fragments is indicated. Two poorly separated bands can be seen at low protein concentrations which are not discerned in the photographic reproduction.

(3) Sedimentation Equilibrium

A low speed sedimentation equilibrium run was carried out to determine the approximate molecular weight of the CNBr fragments in fraction X-A in benign media (1.1 M KCl - 0.025 M phosphate buffer, pH 7.0) and a high speed sedimentation equilibrium run in denaturing media (8 M urea - 0.20 M KCl - 0.025 M phosphate buffer, pH 7.0).

Figures 38 and 39 show ℓn y versus r^2 plots of the data obtained by this technique. These plots do not indicate the heterogeneity of fraction X-A observed with the disc-gel electrophoresis. This would suggest a comparable molecular weight of the CNBr fragments contained in fraction X-A. It is possible for a heterogeneous sample exhibiting

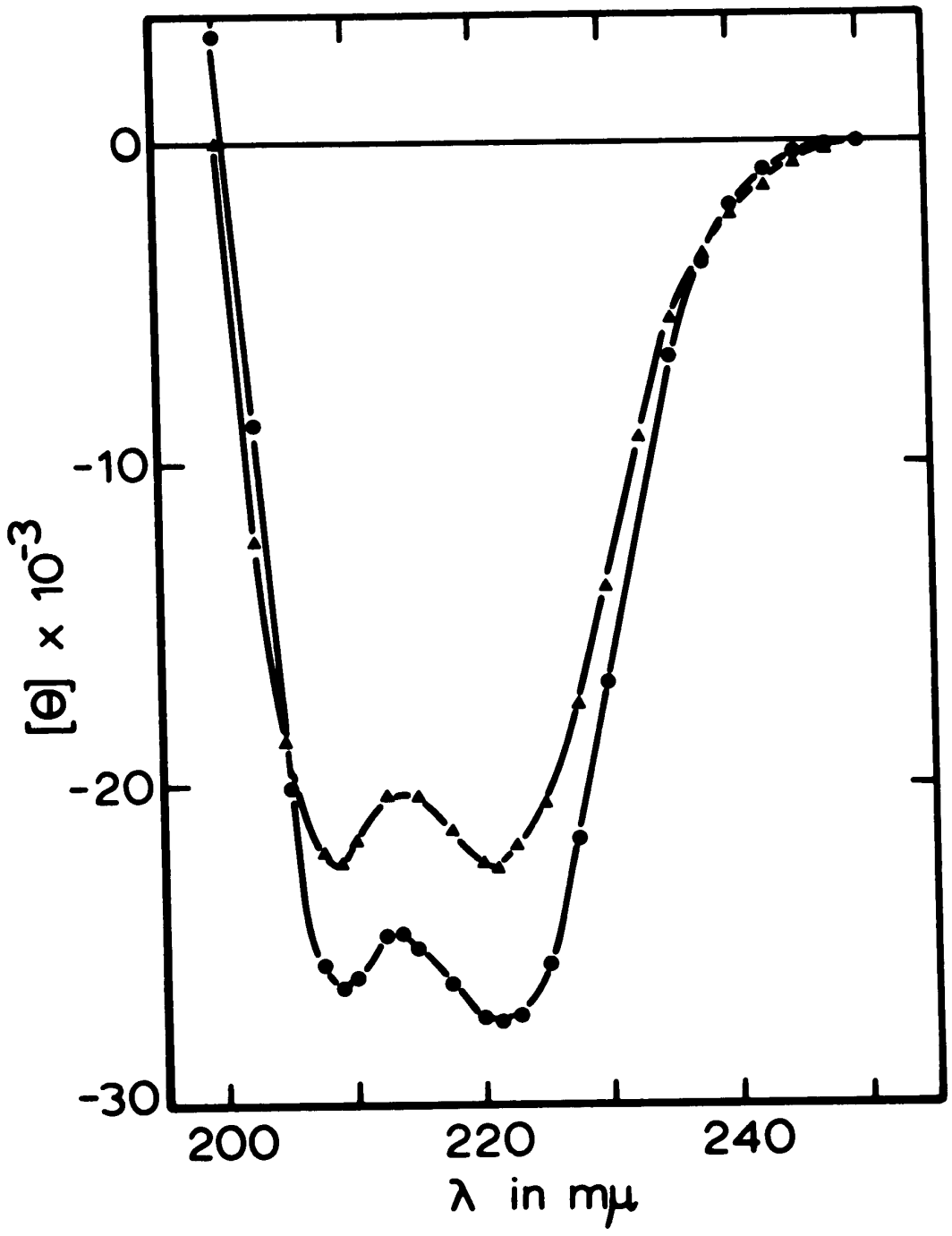


Fig. 40: A circular dichroism spectrum of cyanogen bromide fragments X-A (●—●—●) and X-A2 (▲—▲—▲).

TABLE XXXII

ORD AND CD PARAMETERS FOR CNBr-FRAGMENTS X-A AND X-A2

Sample	Solvent	a_o°	b_o°	% H[a]	$[m']^\circ_{233\ m\mu}$	% H[a]	$[\theta]^\circ_{221\ m\mu}$	% H[b]	$[\theta]^\circ_{209\ m\mu}$	% H[b]
X-A	1.1 M KCl - 0.025 M phosphate, pH 7.0	-42	-450	70	-9,280	52	-27,360	72	-26,260	66
X-A2	0.6 M KCl - 0.05 M phosphate, pH 7.0						-22,506	61	-22,440	53
Rabbit Skeletal Tropomyosin	0.6 M KCl - 0.01 M phosphate						-38,940[d]	99	-36,940[d]	100
	1.1 M NaCl - 0.025 M phosphate, pH 7.0	+ 5	-615[c]	96	-15,800[c]	99				

[a] Standard values for b_o and $[m']_{233\ m\mu}$ for calculation of % α-helix (Jirgensons, 1969).

[b] Standard values for $[\theta]_{221\ m\mu}$ for calculation of % α-helix (Cassim and Yang, 1967).

[c] Woods (1969).

[d] Oikawa et al. (1968).

a concentration dependence of molecular weight to give a straight line plot due to the cancellation of one effect by the other. The molecular weight of the mixture was found to be approximately 30,000 in benign media and about 17,000 in denaturing media. These results suggest the subunit (35,000) is not necessary for "coiled-coil" formation and that a species less than one-half the subunit molecular weight may be capable of forming a "coiled-coil". To further substantiate this conclusion the mixture X-A was submitted to ORD and CD measurements.

(4) Optical Properties of X-A

A CD spectrum of CNBr fragments X-A in 1.1 M KCl - 0.025 M phosphate, pH 7.0 is presented in Figure 40. These fragments exhibit the normal pattern observed for α-helical containing proteins, with two negative dichroic peaks located at 209 mμ and 221 mμ. The ORD and CD results are summarized in Table XXXII. Rabbit skeletal tropomyosin has been included in this table for comparative purposes. The percent α-helix has been calculated using the standard values for 100% α-helix discussed in the methods of this Chapter. These calculations estimate the % α-helix for X-A to correspond to approximately 70% and rabbit skeletal tropomyosin to 100% α-helix.

The results of sedimentation velocity, sedimentation equilibrium disc-gel, and G-75 Sephadex chromatography taken together clearly indicate that cyanogen bromide cleavage of the 35,000 molecular weight subunit has given two large fragments of approximately 15,000 M.W. and the remaining cleavage products as very small peptides. The large fragments

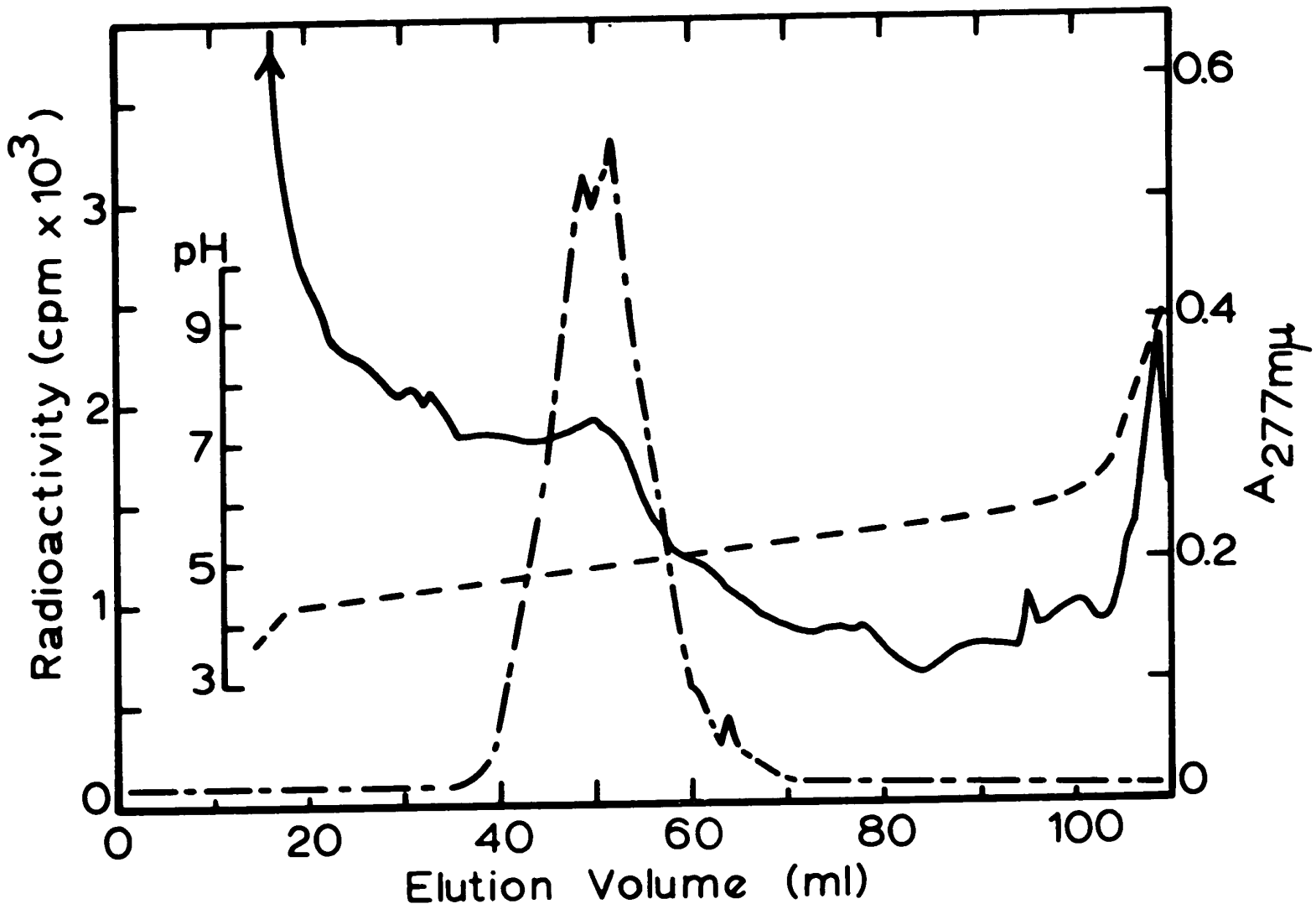


Fig. 41: The second run of a two-step isoelectric focusing experiment of cyanogen bromide fragments of ^{14}C-CM-tropomyosin in 7 M urea. The effluent was monitored for radioactivity (——·——) and absorbancy at 277 mμ (————). The pH gradient is indicated by the broken line.

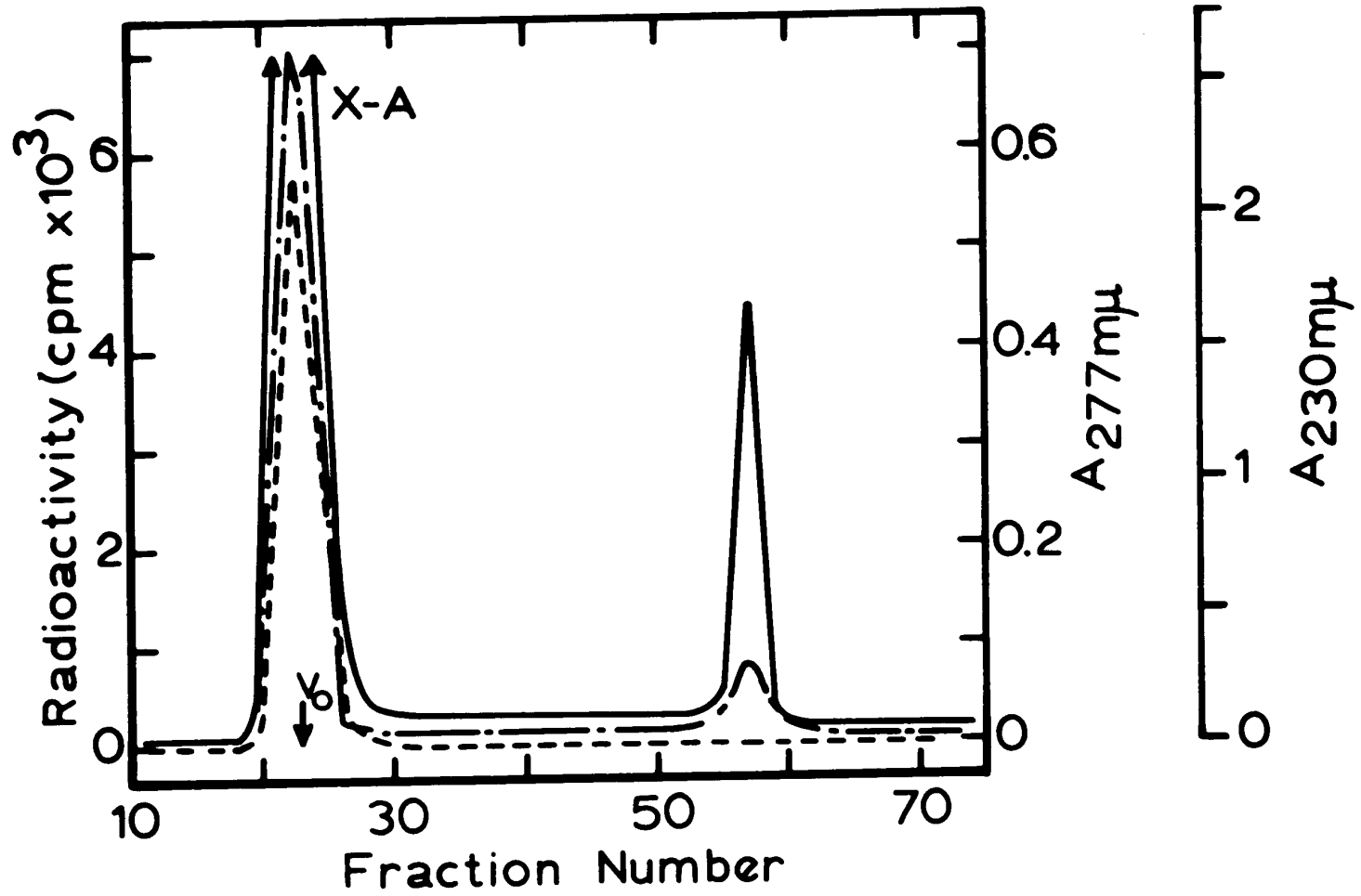


Fig. 42A: Fractionation of CNBr fragments of ^{14}C-CM-tropomyosin by gel-filtration on Sephadex G-50 fine in 0.2 M KCl - 8M urea - 0.025 M phosphate buffer, pH 6.0. Sample concentration 2.0 mg per ml. Flow rate 2.0 ml per cm^2 per hour. The effluent was monitored for absorbancy at 277 mμ (——·——) and at 230 mμ (————) and by radioactive counting (---------).

possess considerable α-helix and in benign media double their molecular weight strongly suggesting the presence of a coiled-coil. Definitive proof of the existence of the coiled-coil with these fragments would require a thorough physicochemical study of the pure CNBr fragments.

D. Further Purification of CNBr Fragments X-A

Preliminary studies on the purification of the large CNBr fragments on QAE-Sephadex showed that the absorption at 280 mμ of one of the fragments was extremely low. Therefore, as an aid in the purification and subsequent sequence studies of the fragments, labelled tropomyosin (^{14}C-CM-tropomyosin) was used for the preparation of the CNBr fragments.

(1) Isoelectric Focusing of CNBr Fragments

The results of a two step isoelectric focusing experiment on the radioactive labelled CNBr fragments are shown in Figure 41. Although the separation of the fragments was unsuccessful this experiment further substantiated the conclusion of the high similarity of the CNBr fragments in mass and charge.

(2) G-50 Sephadex Chromatography of CNBr Fragments in 8 M Urea

Since as described above the Sephadex G-75 chromatography did not separate the large CNBr fragments, in subsequent work the purification of fraction X-A from the small CNBr peptides was carried out on G-50 Sephadex where the large fragments were excluded from the column and eluted in a smaller volume. Figure 42A shows a G-50 Sephadex column separation of the ^{14}C-CNBr fragments in 0.2 M KCl - 8 M urea - 0.025 M phosphate buffer, pH 6.0. Fraction X-A was dialyzed exhaustively against water and freeze-dried.

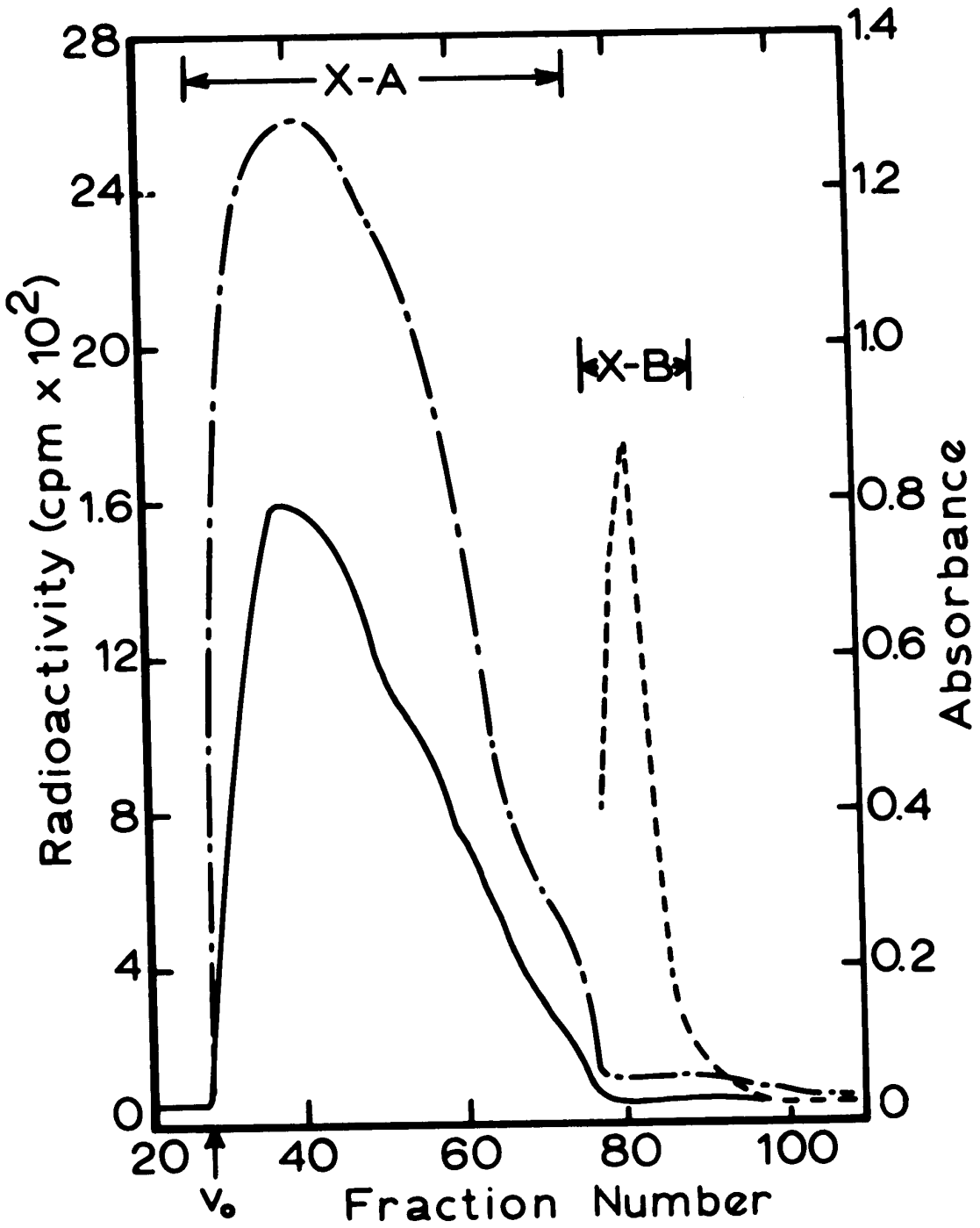


Fig. 42B: Fractionation of CNBr fragments of ^{14}C-CM-tropomyosin by gel-filtration on Sephadex G-50 fine in 0.05 N acetic acid Sample concentration 20 mg/ml. Flow rate 2.0 ml/cm^2/hr. The effluent was monitored for absorbancy at 230 mμ (——·——) and 570 mμ (-------) by manual ninhydrin assays and by radioactivity counting (————).

(3) G-50 Sephadex Chromatography of ^{14}C-CNBr Fragments in 0.05 N Acetic Acid

To isolate the small cyanogen bromide peptides the G-50 Sephadex separation was carried out in 0.05 N acetic acid instead of 8 M urea. This fractionation is shown in Figure 42B. A considerable change in the exclusion limit of the G-50 Sephadex in 8 M urea is indicated by the complete exclusion of the large CNBr fragments in 8 M urea compared to the partial inclusion of the large CNBr fragments in 0.05 N acetic acid. The elution profile of fraction X-A in the acetic acid solvent is most likely due to an aggregating system. Fraction X-A was combined and freeze-dried for further purification of the large CNBr fragments.

The small CNBr peptides were located by manual ninhydrin assays (100 - 200 μℓ aliquots of individual fractions). Aliquots of the individual fractions of peak X-B were spotted on paper for electrophoresis at pH 1.8 and pH 6.5. The paper electrophoresis results enabled the selective pooling of tubes to give fractions X-B1 (76-79) and X-B2 (80-87) for a preliminary study of the isolation of these peptides by paper electrophoretic methods.

This study suggested that to obtain a more quantitative result on the numbers and relative yields of the small cyanogen bromide peptides that fraction X-B be separated on Dowex 50 ion-exchange chromatography. Also to simplify the mixture, fraction X-B after lypholization should be treated with anhydrous trifluoroacetic acid at 20° for 1 hour (Ambler, 1965) to convert all homoserine containing

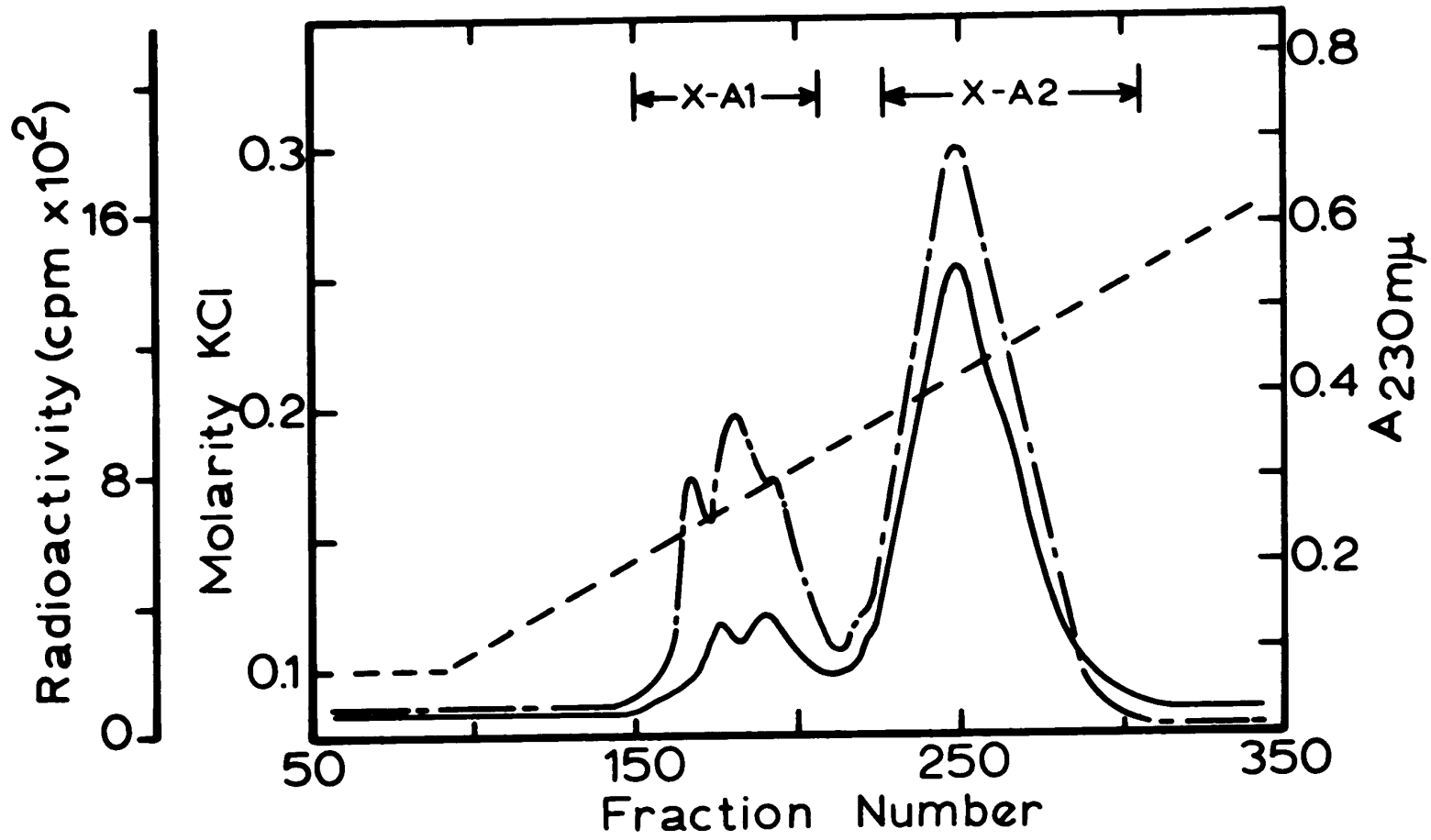


Fig. 43: QAE-Sephadex A-50 chromatography of CNBr fragments X-A obtained from G-50 Sephadex fractionation. The column effluent was monitored for absorbancy at 230 mμ (——·——) and radioactivity (————). The salt gradient is indicated by the broken line. Fractions 1 and 2 were taken as indicated by the arrows.

TABLE XXXIII

AMINO ACID COMPOSITION AND SEQUENCE OF CNBr FRAGMENTS OF FRACTION X-B FROM G-50 SEPHADEX CHROMATOGRAPHY

(Values are Expressed as Mole Ratios)

Peptide	Method of Purification[1]	Electrophoretic Mobility at pH 6.5 (m)	Amino Acid Composition and Sequence	Percentage Yield	M.W.	Net Charge[2]
X-B1a	pH 6.5, pH 3.5	+0.89	(Asx,Ala,Ile,Lys,Lys,Lys,HSer) 092 1.08 094 1.04 1.04 1.04 094			
X-B1b{1,2}	pH 6.5, pH 3.5	+0.60	*			
X-B1c	pH 6.5, pH 3.5	+0.25	Lys(Lys,Lys,Arg,Asx,Ser,Glx,Glx,Glx,Glx,Ala,Val,Ile,HSer) 1.02 1.02 1.02 1.05 1.14 089 1.02 1.02 1.02 1.02 1.04 096 084 093 ⟶			
X-B1d	pH 6.5, pH 3.5	0	Lys-Asp-Glu-Glu-Lys-HSer 1.00 1.06 1.04 1.04 1.00 086 ⟶ ⟶ ⟶ ⟶ ⟶ ⟶	2.1	749	0
X-B1e	pH 6.5	-0.32	Lys-Asp-Glu-Glu-Lys-HSer 1.10 090 1.01 1.01 1.10 090 ⟶ ⟶ ⟶ ⟶ ⟶ ⟶	1.2	749	-1
X-B1f	pH 6.5	-0.41	Glx-Leu-Glx-Glx-HSer 1.00 1.00 1.09 1.00 099 ⟶ ⟶ ⟶ ⟶ ⟶	6.6	620	-1
X-B1g	pH 6.5	-0.72	Glx-Leu-Glx-Glx-HSer 1.01 093 1.01 1.01 1.04 ⟶ ⟶ ⟶ ⟶ ⟶	2.3	620	-2
X-B2a	pH 1.8		Glx-HSer 098 1.02 ⟶ ⟶	8.0		
X-B2b	pH 1.8		Glx-HSer 1.04 096 ⟶ ⟶	3.0		

* Two peptides which could not be obtained pure by paper electrophoretic methods.

[1] Paper electrophoresis carried out at 60 v/cm for 45 min on Whatman No. 1 paper.

[2] Offord (1966).

peptides to the lactone form. The preparative scale isolation of these cyanogen bromide peptides is now in progress in this laboratory and only the results of the preliminary investigation by paper electrophoresis will be discussed in this thesis.

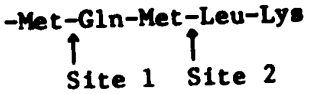
The amino acid compositions, electrophoretic mobilities, and sequence by the Dansyl-Edman procedure of the small CNBr peptides isolated in the preliminary investigation are shown in Table XXXIII. Only the amino acid composition of peptide X-B1a was obtained. The two homoserine peptides of band X-B1b could not be obtained pure by paper electrophoretic methods. Three of the unique cyanogen bromide peptides were isolated in two forms with identical amino acid composition and sequence but different electrophoretic mobilities. Comparison of net charge at pH 6.5 (Offord, 1966) between peptides X-B1c and X-B1d and X-B1e and X-B1f show a difference of one net negative charge between the two forms. This result can be explained by the more basic peptide of the pair containing a C-terminal homoserine lactone residue. The electrophoretic mobilities of X-B1c and X-B1d are consistent with the absence of amidated residues.

(4) QAE-Sephadex A-50 Chromatography of CNBr Fragments X-A

The large CNBr fragments in fraction X-A from G-50 Sephadex chromatography in 0.05 N acetic acid were separated on QAE-Sephadex. The elution profile is shown in Figure 43. Fraction X-A1 was very complex and no further work was carried out on this fraction. Fraction X-A2, containing the majority of the radioactivity, was collected, dialyzed exhaustively against deionized water and freeze-dried.

Fraction X-A2 was judged pure from its amino acid composition shown in Table XXXI. The minimum molecular weight of X-A2 calculated from the amino acid composition was approximately 17,000. This fragment contains the two histidine sequences of tropomyosin and is therefore most likely the C-terminal half of the molecule. X-A2 contains nearly all the aromatic residue content of tropomyosin (5 tyrosine and 1 phenylalanine residue per 17,000 g).

N-terminal analysis of X-A2 showed glutamic acid or glutamine and leucine as the N-terminal residues. This result could be explained by incomplete cleavage at the methionine residues. For example, if peptide



represented the point of cleavage for the N-terminal of X-A2 incomplete cleavage at site 2 and complete cleavage at site 1 would give two peptides varying in length by only two residues, one with gln N-terminal, the other with leucine as the N-terminal residue. Fraction X-A2 was assumed to be homogeneous with respect to contamination of other CNBr fragments from other regions of the tropomyosin molecule. Since this fragment possessed the majority of the radioactivity it was assumed to contain the two homologous forms of the cysteine sequences previously isolated and in this respect is heterogeneous.

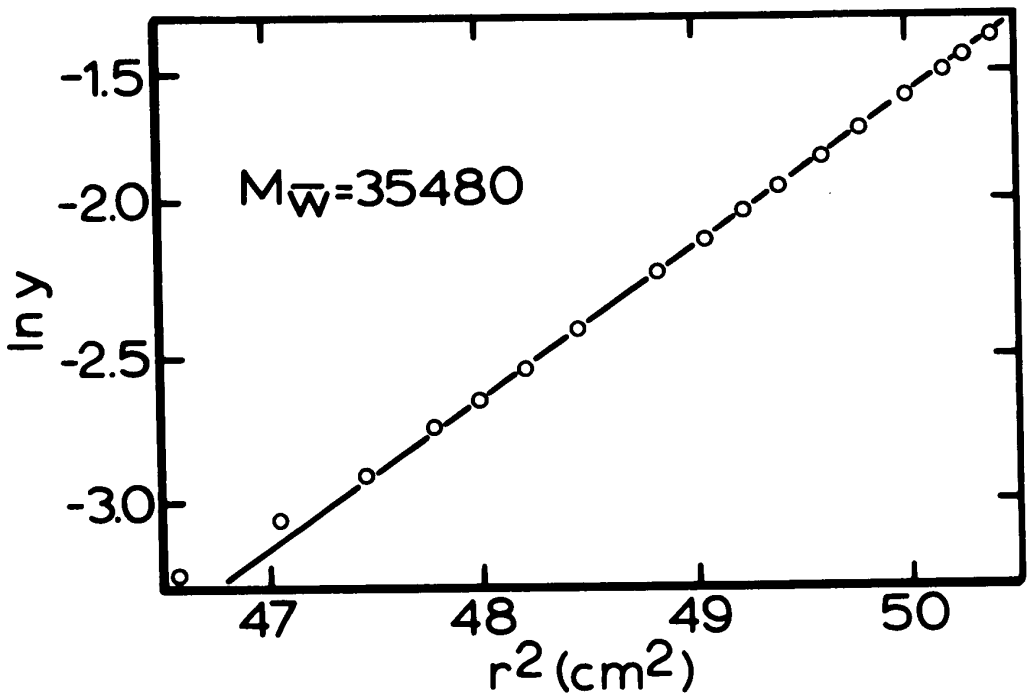


Fig. 44: Plot of natural log of the concentration (fringe displacement) as a function of the square of the distance from the axis of rotation. A low speed sedimentation equilibrium run with 0.092% X-A2 in 0.6 M KCl - 0.05 M phosphate buffer, pH 7.0 at 16,000 r.p.m.

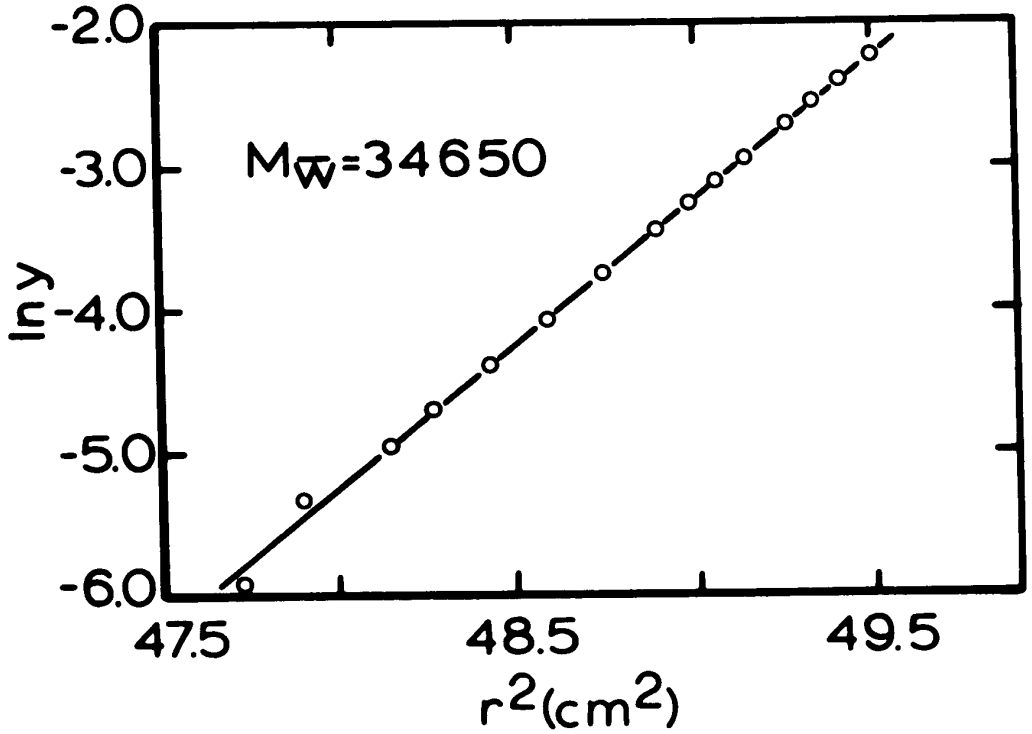


Fig. 45: Plot of the natural log of the concentration (fringe displacement) as a function of the square of the distance from the axis of rotation. A high speed sedimentation equilibrium experiment with 0.092% X-A2 in 0.6 M KCl - 0.05 M phosphate buffer, pH 7.0 at 32,000 r.p.m.

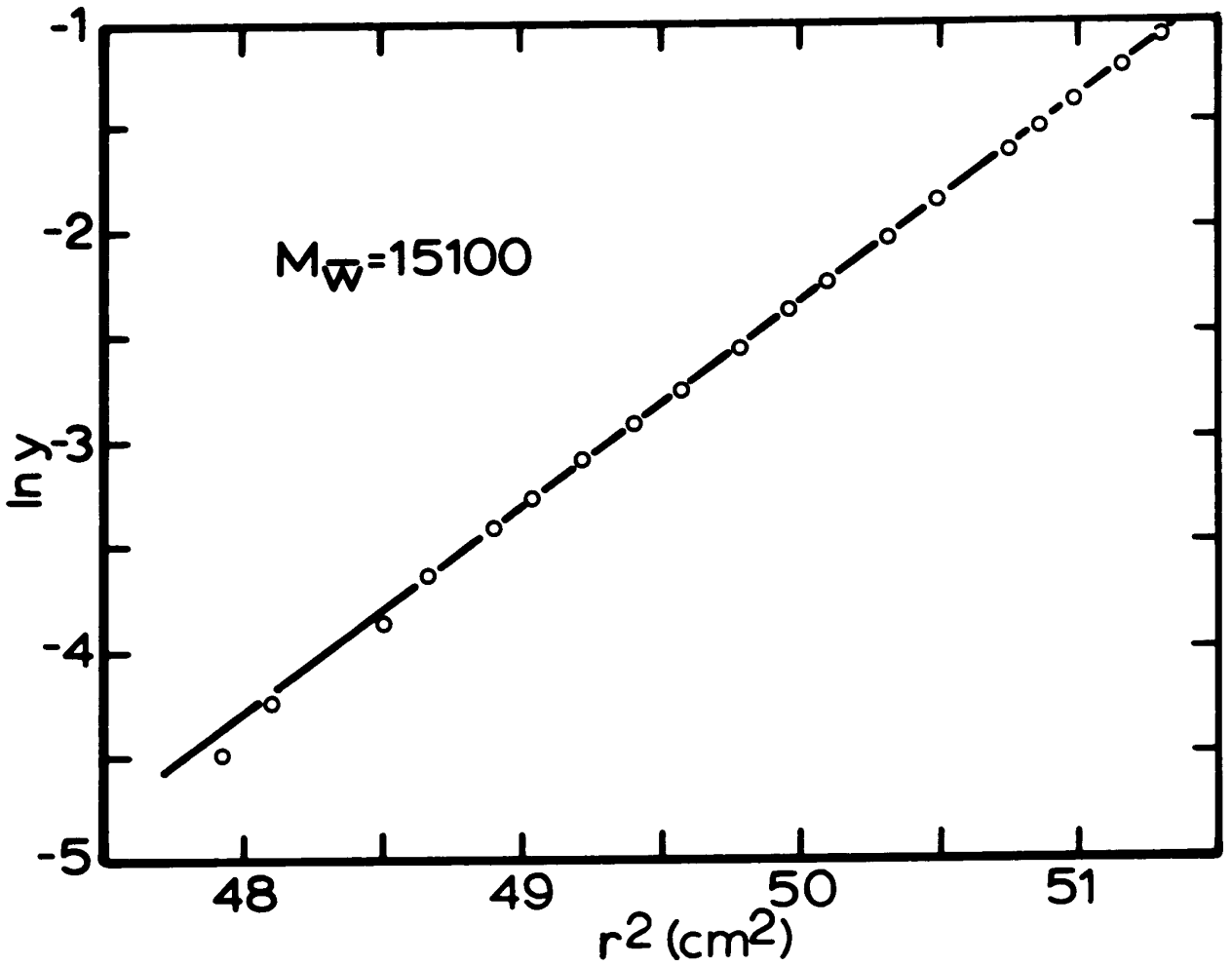


Fig. 46 : A plot of ℓn y (fringe displacement) as a function of the square of the distance from the axis of rotation. A high speed sedimentation equilibrium experiment with approximately 0.1% X-A2 in 0.2 M KCl - 8 M urea - 0.05 M phosphate buffer, pH 7.0 at 40,000 r.p.m.

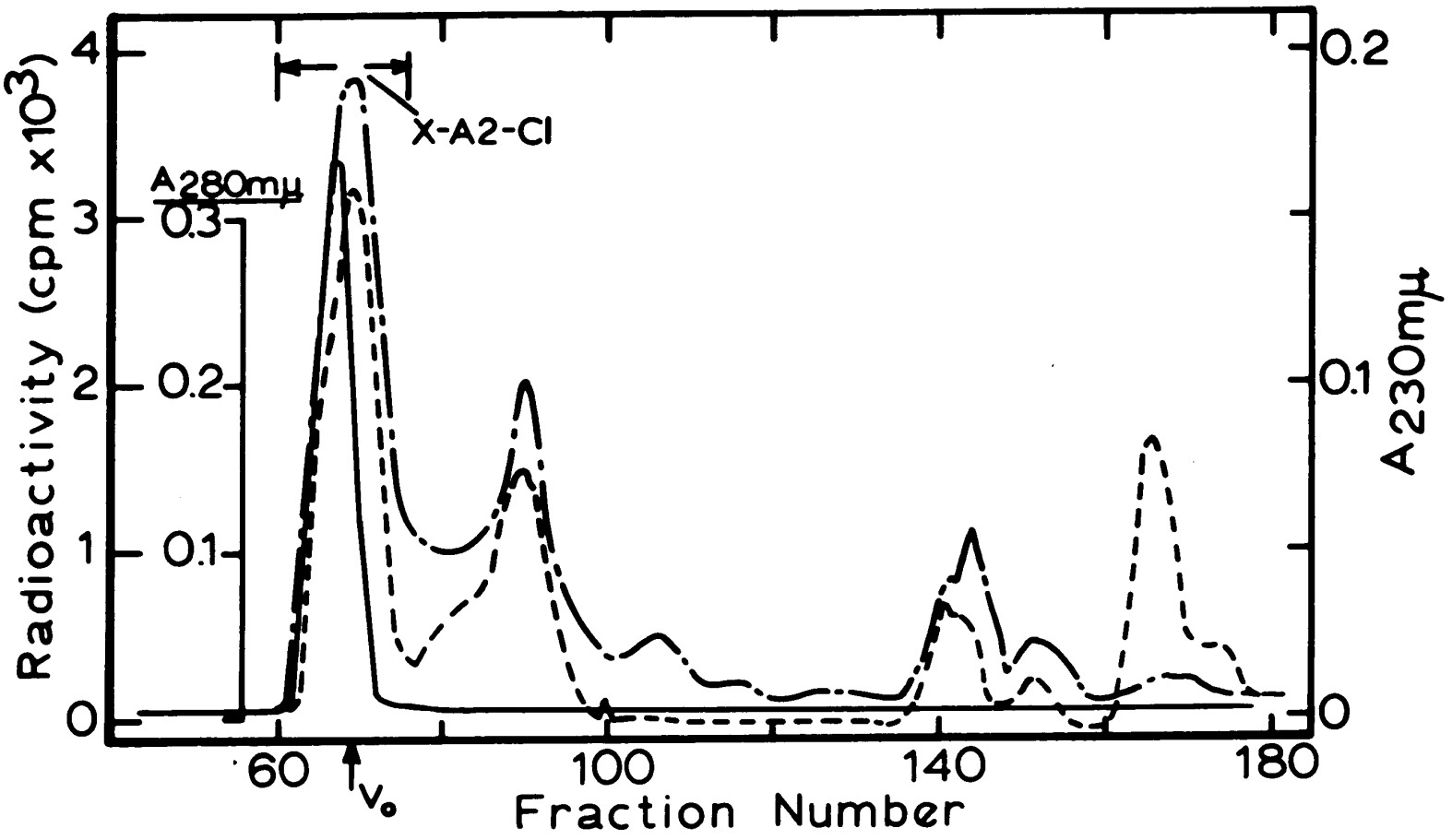


Fig. 47: Gel-filtration on Sephadex G-25 of the chymotryptic digest of CNBr fragment X-A2 in 0.05 M N-ethylmorpholine-acetate buffer, pH 8.0. Absorbancy measurements of the column effluent at 280 mμ (- - - - - - -) and 230 mμ (——— - ———) were carried out. Radioactivity (———————) was determined by counting 25 μℓ aliquots from the 3.0 ml fractions.

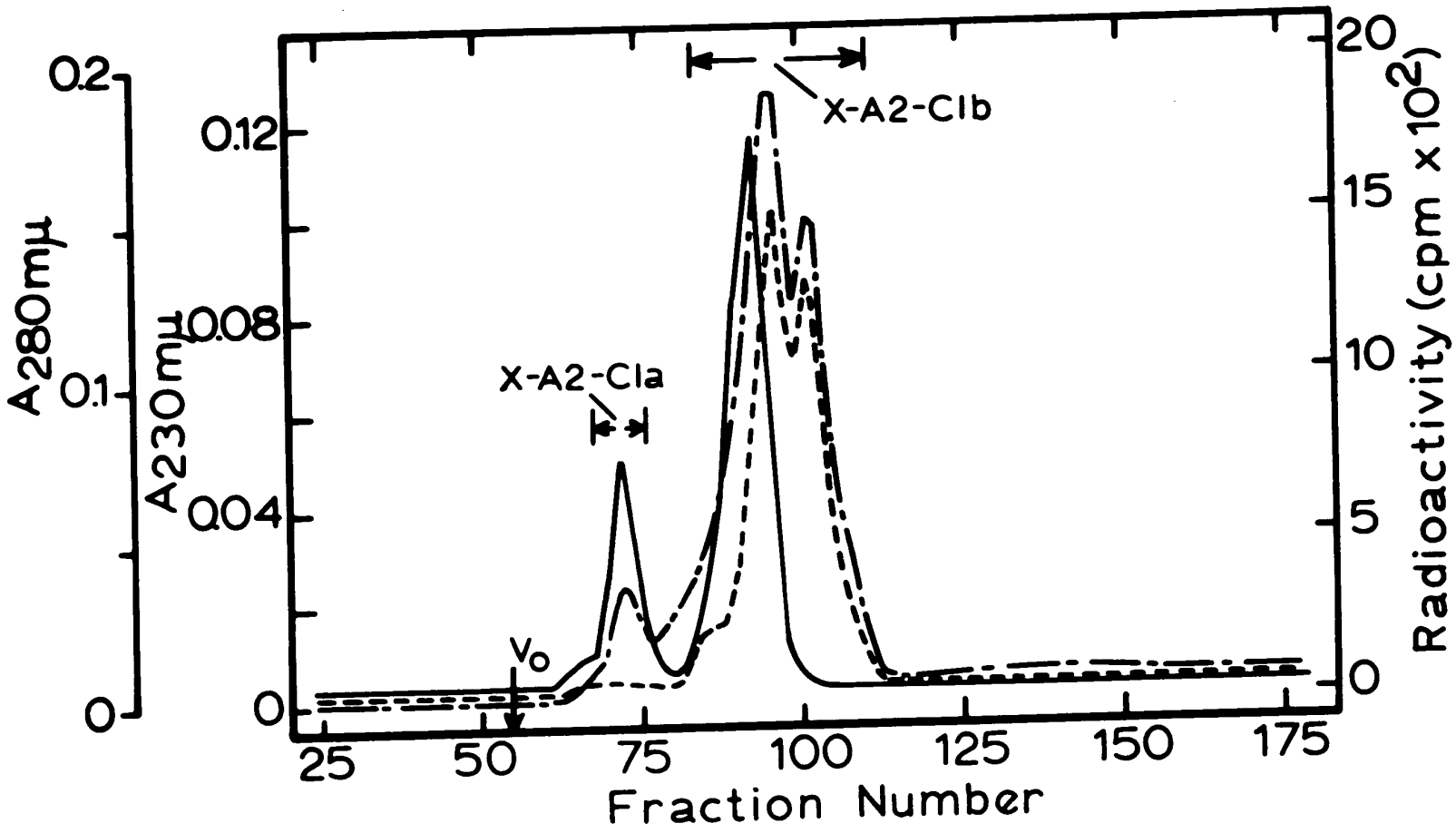


Fig. 48: Gel-filtration on Sephadex G-50 of fraction X-A2-C1 in 0.05 M N-ethylmorpholine-acetate buffer, pH 8.0. The effluent was monitored for absorbancy at 280 mμ (- - - - -) and at 230 mμ (——- ——). Radioactivity (————————) was determined by counting 25 μℓ aliquots from the 3.0 ml fractions. Fractions "a" and "b" were taken as indicated by the arrows.

The molecular weight of X-A2 was determined in benign media (0.6 M KCl - 0.05 M phosphate buffer, pH 7.0) by both high and low speed sedimentation equilibrium. The molecular weight obtained from the slope of ℓn y vs r^2 plots is shown in Figures 44 and 45. The molecular weight value obtained is approximately 35,000 by either method. The molecular weight by high speed sedimentation equilibrium in denaturing media (0.2 M KCl - 8 M urea - 0.05 M phosphate buffer, pH 7.0) of X-A2 was found to be approximately 15,000. The plot of ℓn y vs r^2 is shown in Figure 46. This value is in close agreement with M.W. value of 17,000 by amino acid analysis. The finding that the cyanogen bromide fragment doubles its molecular weight in benign media is indicative of the formation of a coiled-coil. The results of circular dichroism studies showed a helical content of approximately 60% (Table XXXII). The CD spectrum of X-A2 is seen in Figure 40. This result adds further support that fragment X-A2 is still of sufficient length to form a coiled-coil.

(5) Chymotryptic Digestion of CNBr Fragment X-A2

The limited chymotryptic digestion of X-A2 and separation of the digest on Sephadex G-25 are described in the methods of this chapter. Figure 47 shows the elution profile of the digest on G-25 Sephadex. The void volume peak X-A2-C1 was pooled and freeze-dried. The remaining peptides on the G-25 Sephadex column have been isolated and sequenced in this laboratory by Dr. J. Sodek. Fraction X-A2-C1 was further purified on Sephadex G-50. The fractionation is shown in Figure 48. Fractions X-A2C1a (68-76) and X-A2C1b (84-111) were pooled and freeze-dried.

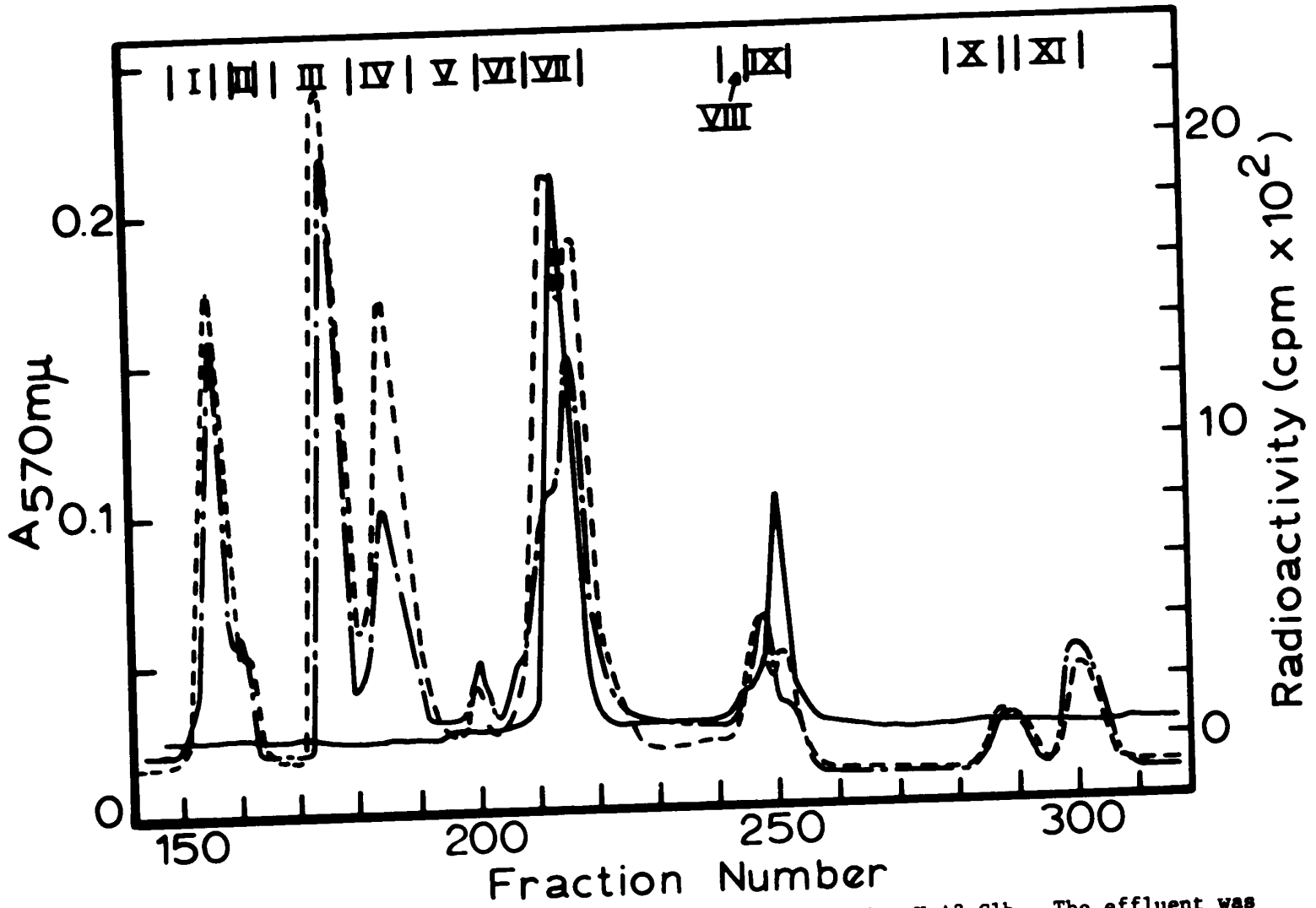


Fig. 49: Dowex 1 chromatography of the chymotryptic peptides of fraction X-A2-C1b. The effluent was monitored by automatic ninhydrin analysis before (—— - ——) and after (---------) alkaline hydrolysis at 570 mμ. Aliquots of 25 μl were taken for radioactivity measurements (————) from each 3.0 ml fraction. Fractions I to XI were taken as shown by the vertical.

TABLE XXXIV

CHYMOTRYPTIC PEPTIDE FRACTIONS POOLED FROM DOWEX 1 CHROMATOGRAPHY OF FRACTION X-A2-C1b

Fraction No.	Fractions pooled	Subsequent Purification Step[1]
I	152-158	P
II	161-165	Dowex 50
III	168-181	Dowex 50
IV	182-191	Dowex 50
V	192-202	Dowex 50
VI	203-210	Dowex 50
VII	211-218*	Dowex 50
VIII	243-247	Dowex 50
IX	248-254*	pH 6.5 electrophoresis
X	280-290	Dowex 50
XI	292-303	P

* Radioactive fractions

[1] Symbol P denotes that the fraction was pure from amino acid composition and no further purification was carried out.

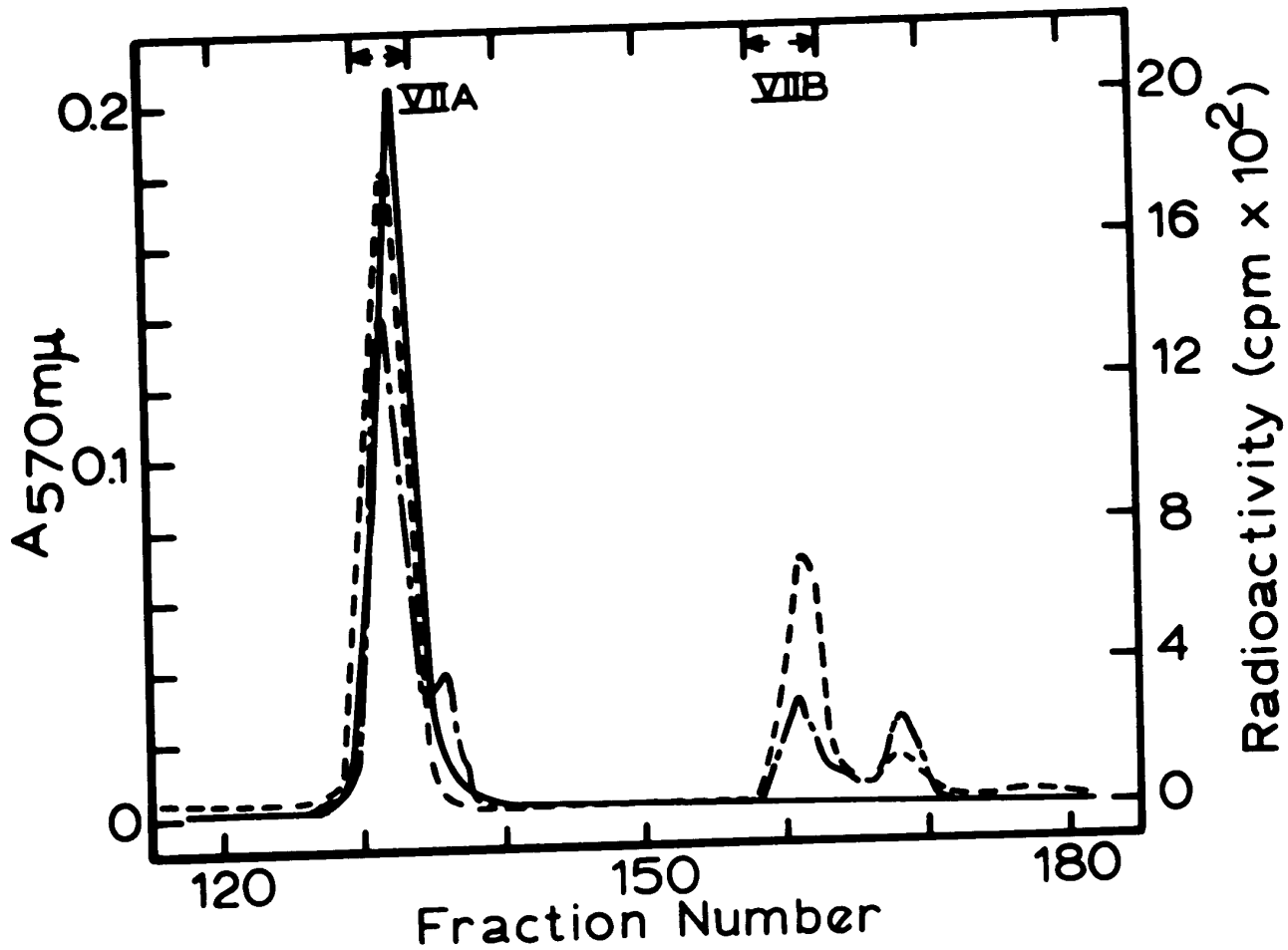


Fig. 50: Chromatography of radioactive peptide fraction X-A2-C1bVII on Chromobead Type P column. The effluent was monitored for radioactivity (————) and by automatic ninhydrin analysis at 570 mμ before (——–––——) and after alkaline hydrolysis (--------). The fractions taken are indicated by arrows.

a. Separation of Fraction X-A2C1b on Dowex 1 Chromatography

The Dowex 1 ion exchange chromatography of X-A2C1b is shown in Figure 49. Each individual fraction in the peaks (50-100 µℓ) was spotted on paper for electrophoresis at pH 6.5. The results indicated the fractions to be pooled and the type of purification. Table XXXIV lists the fractions I to XI that were pooled from the Dowex 1 column and the subsequent purification step. Only the two radioactive containing fractions will be discussed in this thesis. The remaining peptides from the Dowex 1 column are being sequenced in this laboratory with the aim of obtaining the total amino acid sequence of the CNBr fragment X-A2.

i. Fraction IX (X-A2C1b-IX)

This peptide was judged pure from its amino acid composition after pH 6.5 electrophoresis (Table XXXV). The electrophoretic mobility at pH 6.5, m = -0.86, is consistent with the absence of amidated residues. The sequence of this peptide from the Dansyl-Edman procedure and homology to previously isolated peptides may be written as:

Ser-Glu-Gly-Lys-CMC-Ala-Glu-Leu-Glu-Glu-Glu-Leu.
→ → → → →

ii. Fraction VII

Fraction VII from Dowex 1 chromatography was further purified by chromatography on Dowex 50 (Chromobead Type P). This separation is shown in Figure 50. Aliquots (40-60 µℓ) of the individual fractions of the peaks indicated by automated ninhydrin analysis and radioactivity

TABLE XXXV

AMINO ACID COMPOSITION AND SEQUENCE OF THE CHYMOTRYPTIC PEPTIDES ABOUT THE CYSTEINE RESIDUES OF S-A2

(Values are Expressed as Whole Ratios)

Peptide	Method of Purification[1]	Electrophoretic Mobility at pH 6.5(m)	Sequence and Amino Acid Composition	Percentage Yield[2]	M.W.	Net Charge[3]
S-A2-CM6T1B	Bands 1, Bands 10	-0.66	Val-Ile-Ile-Glu-Ser-Asn-Leu-Glu-Arg-Ala-Glu-Glu-Arg-Ala-Glu-Leu 671 673 673 103 688 107 100 105 695 695 105 105 695 695 105 100	7.4	1856	-3
S-A2-CM6T1A	Bands 1, Bands 10	-0.62	Ser-Glu-Gly-Lys-CMC-Ala-Glu-Leu-Glu-Glu-Glu-Leu-Lys-Thr-Val-Thr-Asn 695 104 100 100 694 688 104 698 104 104 104 698 100 694 693 694 102	11.2	1935	-4
- T1a	pH 6.5, pH 3.5 (50)	-0.88	Ser-Glu-Gly-Lys 699 105 101 104	46.0	-	0
- T1	pH 6.5	-0.98	CMC-Ala-Glu-Leu-Glu-Glu-Glu-Leu-Lys 107 696 101 698 101 101 101 698 699	20.0	1119	-4
- T1b	pH 6.5, pH 3.5 (50)	-0.88	Thr-Val-Thr-Asn 695 104 695 106	51.5	-	0
S-A2-CM6I2	Bands 1, pH 6.5	-0.86	Ser-Glu-Gly-Lys-CMC-Ala-Glu-Leu-Glu-Glu-Glu-Leu 670 100 106 110 696 699 100 110 100 100 100 110	3.4	1302	-5
S-A2-C1a			Val-Ile-Leu-Glu-Gly-Glu-Leu(——— Amino Acid Composition in Table XXXVI ———)	10.5	-	-
- T3	pH 6.5, pH 3.5 (90)	-0.40	Val-Ile-Leu-Glu-Gly-Glu-Leu-Glu-Arg 690 694 699 101 695 101 699 101 104	20.0	1067	-2
- T4b	pH 6.5, pH 3.5 (30)	-0.44	Ser 696 Ala 697 Glu-Glu-Arg 101 101 101	50.4	504	-1
- T4a		-0.41				
- T3	pH 6.5, pH 3.5 (50)	-0.36	Ala-Glu-Val-Ala-Glu-Ser-Lys 694 102 698 696 102 100 100	44.8	723	-1
- T4	pH 6.5, pH 3.5 (90)	-0.55	Ala-Glu-Leu 696 100 104	5.9	331	-1
- T2	pH 6.5, pH 3.5 (70)	-0.65	Ser-Glu-Gly-Lys 696 101 112 699	5.9	-	0
- T2a	pH 6.5, pH 3.5 (90)	-0.91	CMC-Ala-Glu-Leu-Glu-Glu-Glu-Leu-Lys 692 103 101 699 101 101 101 699 695	6.0	1119	-4
- T2b	pH 6.5, pH 3.5 (90)	-0.91	CMC-Gly-Asp-Leu-Glu-Glu-Glu-Leu-Lys 697 100 695 109 697 697 697 109 695	11.2	1091	-4
- T0	pH 6.5, pH 3.5 (75)	-1.10	CMC-Gly-Asp-Leu-Glu-Glu-Glu-Leu 670 106 697 100 699 699 699 100	10.0	963	-5
- T1	pH 6.5, pH 3.5 (70)	0	Ile-Val-Thr-Asn 690 699 667 111	9.2	-	0

[*] Acid hydrolysis was carried out for 22 hours on all peptides.

[1] Purification methods subsequent to G-30 Sephadex chromatography; tryptic digestion products of chymotryptic peptides S-A2-CM6T1A and S-A2-C1a were purified by paper electrophoresis on Whatman No. 1 paper at 60 v/cm for 45 min except where indicated.

[2] Chymotryptic peptides calculated as percentage yield of starting material S-A2 taken for chymotryptic digestion; tryptic digestion products as percentage yield of the amount of chymotryptic peptide taken for digestion.

[3] Net charge determinations from Offord (1966).

measurements were spotted on paper for pH 6.5 electrophoresis. The paper electrophoresis suggested the pooling of fractions 130-134 (VII-A) and 158-163 (VII-B). These peptides were pure as judged from their amino acid compositions (Table XXXV).

Peptide X-A2-ClbVIIA

The sequence of this peptide shown in Table XXXV was obtained by the identification of the first five residues by the Dansyl-Edman procedure, homology to peptides previously sequenced, and trvptic digestion. The peptide was digested with TPCK-trypsin at an enzyme to peptide ratio of 1:100 for 2.5 hours at 37°C in 0.05 M N-ethyl-morpholine acetate buffer, pH 8.0. The digest was separated by high voltage paper electrophoresis at pH 6.5. The results of the electrophoretic mobilities, amino acid composition, and sequence of the tryptic peptides so obtained are summarized in Table XXXV. The tryptic peptide -T1b gave a yellow colour with cadmium-ninhydrin stain, which is consistent with threonine as the N-terminal residue found by the "dansyl" method. The very low anionic mobility (m = -0.08) at pH 6.5 is attributable to the low pK for the α-amino group of the N-terminal normally observed with such a peptide. The essentially zero electrophoretic mobility suggested that peptide -T1b had an asparagine residue present rather than the corresponding acid. After three Edman degradations asparagine was identified as the N-terminal residue without acid hydrolysis and as aspartic acid after acid hydrolysis. The sequence of this peptide may be written as:

Thr-Val-Thr-Asn.
→ → → →

TABLE XXXVI

AMINO ACID COMPOSITION OF CHYMOTRYPTIC FRAGMENT X-A2-Cla

(Values expressed as Mole Ratios)

Lysine	2.3
Arginine	1.8
Aspartic acid	2.4
Glutamic acid	10.5
Threonine	0.6
Serine	1.9
Glycine	1.7
Alanine	2.8
Leucine	4.5
Isoleucine	1.4
Valine	2.3
CM Cysteine	0.9
	33.1

Hydrolysis time of 22 hours.
Mole ratios calculated on 33 residues/mole.

The mobilities of peptide X-A2-ClbVIIA and its tryptic digestion products are in agreement with the presence of only one amidated residue.

Peptide X-A2-ClbVII B

The electrophoretic mobility with respect to aspartic acid was m = -0.46, suggesting that there was a net negative charge of 3 (Offord, 1966), and that there was only one amidated residue present. The amino acid composition and sequence of this peptide is shown in Table XXXV.

b. Sequence Determinations of X-A2-Cla

This peptide fraction was obtained from G-50 Sephadex chromatography. The amino acid composition is shown in Table XXXVI. A single N-terminal valine residue was obtained by the "dansyl" method. After the seventh Edman step of peptide X-A2-Cla, difficulties were encountered in determining unequivocally the N-terminal residue. The partial sequence of this peptide may be written as:

Val-Ile-Leu-Glx-Gly-Glx-Leu-
→ → → → → → →

Evidence for the sequence of this peptide was obtained from tryptic digestion. The peptide was digested with TPCK-trypsin (ratio of trypsin to peptide, 1:100) for 5 hours at 37°C in 0.05 M N-ethyl-morpholine acetate buffer, pH 8.0. The digestion products were separated by electrophoresis at pH 6.5. The results of the amino acid composition, electrophoretic mobilities and sequence of the tryptic peptides are shown in Table XXXV.

The tryptic fragments were arranged in the correct order by homology to peptides X-A2-ClbVIIA and X-A2-ClbVIIB.

Peptide -T4a and -T4b

These two peptides were only partially separated at pH 6.5 and could not be separated at pH 1.8, pH 3.5, or by paper chromatography (n-butanol-acetic acid-water, 67:10:25). Peptide -T4a and -T4b were red and yellow respectively with cadmium-ninhydrin. Their electrophoretic mobilities were -0.41 and -0.44 for -T4a and -T4b respectively, and suggest the absence of amidated residues. These result together with the amino acid composition of the mixture suggested the presence of peptides Ser Glu_2 Arg and Ala Glu_2 Arg. Serine would be N-terminal on peptide -T4b to account for the yellow colour and increased negative mobility. The amino acid sequence of the mixture as determined by the Dansyl-Edman procedure was:

$$\begin{matrix}\text{Ser} \\ \longrightarrow \\ \text{Ala} \\ \longrightarrow\end{matrix} \text{-Glu-Glu-Arg.} \quad \longrightarrow\ \longrightarrow\ \longrightarrow$$

Peptide -T1

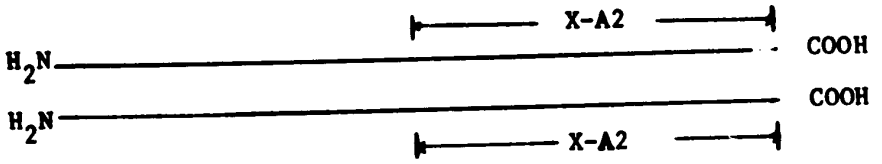
The electrophoretic mobility (m = 0) is consistent with the presence of asparagine rather than aspartic acid. The fourth residue was identified with and without acid hydrolysis as aspartic acid and asparagine respectively; therefore asparagine must be the C-terminal residue of peptide -T1. The sequence may be written as:

$$\text{Ile-Val-Thr-Asn.} \quad \longrightarrow\ \longrightarrow\ \longrightarrow\ \longrightarrow$$

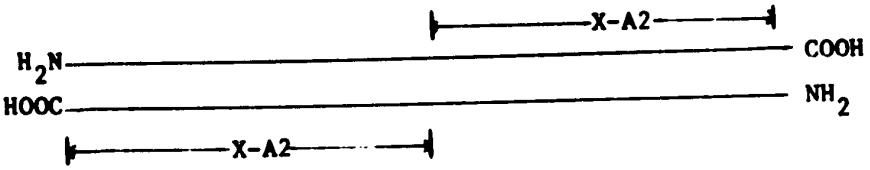
4. DISCUSSION

Of considerable importance in the relationship of primary sequence to the coiled-coil structure is the knowledge of whether the chains of tropomyosin are parallel or antiparallel. A schematic representation is shown below.

Parallel



Antiparallel



If the subunits of tropomyosin are identical or highly homologous and parallel, the formation of a coiled-coil in benign media of CNBr-fragment X-A2 would be expected. In the antiparallel case the formation of a coiled-coil would not be expected unless the complementary portion of the tropomyosin molecule was present. Fragment X-A2 contains the two histidine residues of tropomyosin. The tryptic peptide T-4B (Table VIII, page 81) which contains histidine was shown to be most likely the C-terminal sequence of tropomyosin. From this result the CNBr fragment X-A2 was assumed to be the C-terminal portion of the tropomyosin molecule less the tripeptide -Thr-Ser-Ile of T-4B1 and -Thr of T-4B2 (Table XVIII, page 81).

The molecular weight, ORD, and CD studies strongly suggest the formation of a coiled-coil in benign media among the CNBr-fragments in the mixture X-A and of the purified fragment X-A2. Fragment X-A2 was shown to contain approximately 60% α-helix and to double its molecular weight to 35,000 in benign media. A molecular weight of 17,000 would be expected for X-A2 if the chains of tropomyosin were antiparallel. This result is strongly indicative of parallel chains but does not rule out the possibility that the tropomyosin chains are antiparallel. For example, if there exists a repeating pattern of amino acid sequence throughout each of the entire polypeptide chains of tropomyosin, then it is conceivable that the fragment X-A2 would not be markedly dissimilar in structure to that portion (N-terminal half) to which it would be complimentary in the anti-parallel case. Thus if the native protein is anti-parallel in nature, fragment X-A2 would form a highly ordered structure with the N-terminal half of the molecule but may still form a partially ordered structure with itself.

In the isolation of the small cyanogen bromide fragments at least four new unique methionine sequences were found. This brings the total number of unique methionine sequences to at least eight which is inconsistent with two identical polypeptide chains.

The non-integral nature of the amino acid composition of X-A2-Cla (Table XXXVI) can be explained by the following three points. Firstly, the 22 hour acid hydrolysis is not sufficiently long enough to obtain complete cleavage of the Val-Ile bond shown to be present in this peptide. Secondly, at least two different lengths of this

peptide are present as indicated by the isolation of the tryptic peptides -T8, -T7a, -T7b, and -T1 (Table XXXV). This conclusion is further supported by the isolation of the chymotryptic peptide X-A2-C1bIX which corresponds to peptide -T8, and X-A2-C1bVIIA which corresponds to the peptides of the tryptic digestion represented by -T7a, -T7b, and -T1. A difference in length of 5 residues is indicated by these findings. Thirdly, the large number of amino acid replacements shown to exist in X-A2C1a at residue 10, 16, 17, 19, 22, and 23 (Table XXXV) would further complicate the molar ratios in the amino acid composition.

TABLE XXXVII

UNIQUE METHIONINE PEPTIDES OF TROPOMYOSIN

1. Met - Gln-Met-Leu-Lys

2. Gly - Met-Lys

3. N-Ac-Met-Asp-Ala-Ile-Lys

4. Lys-(Lys,Lys,Arg,Asx,Ser,Glx,Glx,Glx,Glx,Ala,Val,Ile,HSer)

5. (Lys,Lys,Lys,Asx,Ala,Ile,HSer)

6. Lys-Asp-Glu-Glu-Lys-HSer

7. Glx-Leu-Glx-Glx-HSer

8. { Ala-Ile-Ser-Glu-Glu-Leu-Asp-His-Ala-Leu-Asn-Asp-Met-Thr-Ser-Ile
   { Ala-Ile-Ser-Glu-Glu-Leu-Asp-His-Ala-Leu-Asn-Asp-Met-Thr

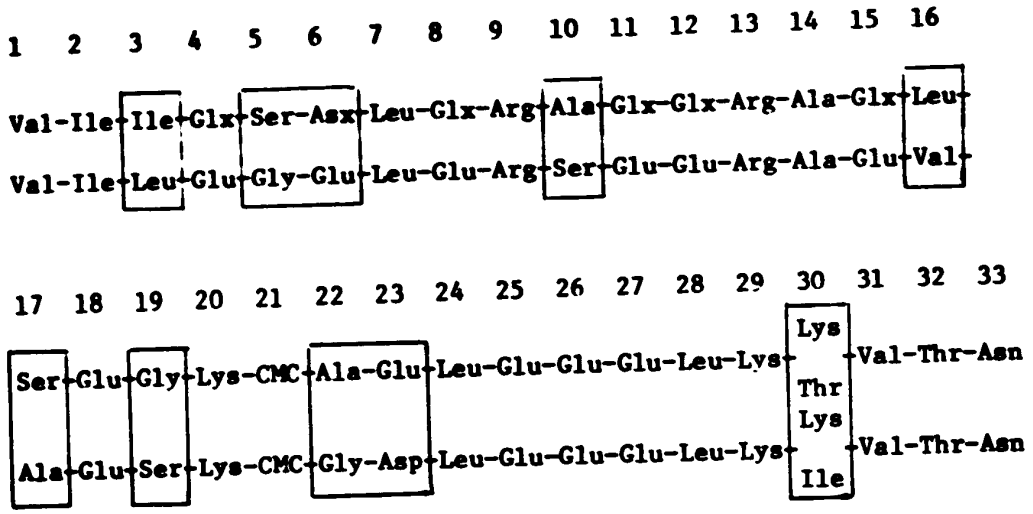


Fig. 51: The amino acid sequence about the cysteine residues in the C-terminal region of the tropomyosin molecule.

CHAPTER VII

DISCUSSION

The isolation and determination of the number of unique histidyl, methionyl, and cysteinyl peptides and their sequence in the tropomyosin molecule were designed to derive information on the identity or non-identity of the subunits of tropomyosin. The isolation of two unique histidyl peptides (Table XXXVI) is consistent with two identical polypeptide chains of molecular weight 35,000. Chemically dissimilar polypeptide chains are indicated by the isolation and sequence determination of at least 8 unique methionine peptides (Table XXXVII) and three unique cysteine peptides (Figure 34). The isolation of the C-terminal CNBr fragment and sequence of various chymotryptic peptides has given an extended sequence of 33 residues. The sequence in this region of tropomyosin molecule is summarized in Figure 51. It has not been possible to establish the number of different polypeptide chains represented by this sequence unequivocally. The experimental data strongly suggests the sequences as shown and indicates a minimum of four polypeptide chains. The lysine residue at position 30 was found to occur in both sequences (Figure 34) and is the only evidence for more than one amino acid replacement at one position in the highly homologous polypeptide chains represented in Figure 51. These two amino acid sequences vary in ten positions. The amino acid substitutions are all highly conservative in nature and can be explained by a single base mutation. These results are clearly incompatible with a homogeneous tropomyosin preparation of two

chemically identical subunits. At present it is impossible to distinguish between the situation of non-identical but similar subunits, or of more than one homologous form of tropomyosin, or both. Obviously, further attempts must be made to isolate chemically pure tropomyosin, and its constituent polypeptide chains.

A further point of interest in this work has been the relationship between the postulated coiled-coil structure of tropomyosin and its amino acid sequence. In studies of synthetic polyamino acids it has been suggested that branching on the β-carbon atom (such amino acids as valine, isoleucine, threonine, and tryptophan) would prevent α-helix formation (Mitchell et al., 1957; Blout, 1962; Bloom et al., 1962). However the existence of poly-L-valine in an α-helical form has now been experimentally verified (Epand and Scheraga, 1968; Ooi et al., 1966). Epand and Scheraga (1968) using ORD and CD found that about 50% of a short valine block of $(D,L\text{-lysine}\cdot HCl)_{18}$-$(L\text{-valine})_{15}$-$(D,L\text{-lysine}\cdot HCl)_{16}$-glycine was in the right handed α-helical conformation in 98% methanol. In water the polymer appears to be a dimer, with the valine block being involved in the formation of an intermolecular β-structure. Nevertheless, examination of the known α-helical regions of proteins of known conformation has indicated that no more than two such residues occur consecutively in sequence in an α-helix (Smith, 1967). Thus it appears that a sequence of three β-branched residues will interrupt an α-helix. Two such sequences occur in the various cytochromes c; position 57 is always Val or Ile, 58 is Ile, Thr, or Val, except for wheat, yeast, and candida, and 59 is always Trp. In Neurospora cytochrome Ile-Ile-Thr occurs in position 94, 95, and 96 respectively.

Smith (1967) considering the distribution of proline residues, constant glycine residues and the sequences of three β-branched residues concluded that there should be little room for any significant amount of helix in cytochrome c. This conclusion was fully supported by the X-ray work of Dickerson et al. (1967). They found no obvious α-helix anywhere in the cytochrome c molecule. Though the helix-disrupting properties of a sequence of three β-branched residues occurring consecutively has not been proven definitively, two such sequences within 33 residues in tropomyosin is of interest since this molecule is considered to be almost 100% α-helical.

The segmented rope model consists of short lengths of straight α-helix inclined to one another so that perfect knob-into-hole packing occurs at the cross-over point, that is, it approximates to a coiled-coil over a short range. The interchain packing of hydrophobic residues deteriorates with increasing length of the inclined α-helices and it appears necessary to postulate a bend in the axis of the α-helix. Parry (1970) postulated 20-30 Å as the length of the segmented rope "unit". If this sequence of 3 β-branched residues fulfills this requirement the length of the segmented rope "unit" would be approximately 40 Å. This result comes from the sequence of peptide X-A2-Cla which contains 26 amino acid residues between proposed regions of bending in the α-helix.

Theoretical correlations between amino acid sequence and α-helical structure is still unclear in the study of globular proteins. A recent review of this area is given by Venkatachalam and Ramachandran (1969). The amino acid sequence of tropomyosin will no doubt be helpful in furthering the study of the relationship of primary sequence to the formation of the α-helix. However our first objective has been to search for possible repeating hydrophobic residues required for stabilization of the two individual α-helical chains in the "coiled-coil" as postulated by Crick (1953) and others (Cohen and Holmes, 1963 and Parry, 1970).

The sequence of 33 amino acid residues represented in Figure 51 does not show a simple regular periodicity of hydrophobic residues as required by these earlier hypotheses. However, it is possible that residues (i.e. lysine or glutamic acid) other than those usually considered as hydrophobes may fulfill this function if their side chains are of sufficient length to permit hydrophobic interactions of their β and/or methylene carbons with other side chains, and at the same time allow their charged moieties to be turned towards the exterior of the coiled-coil or segmented rope structure. Model building studies in our laboratory indicate that this may be possible. It is also possible that the portion of the structure of tropomyosin represented by the sequence of 33 residues shown in Figure 51 is a non-ordered or non-helical region although this seems unlikely because of the high helical content of the protein indicated by the extensive physical-chemical studies previously discussed. Answers to these

questions will hopefully be forthcoming when the complete amino acid sequence of the 17,000 molecular weight CNBr fragment has been elucidated, a project presently under active investigation in our laboratory.

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