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STUDIES ON CREATINE KINASE FROM THE M-BAND OF BOVINE CARDIAC MUSCLE

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled STUDIES ON CREATINE KINASE FROM THE M-BAND OF BOVINE CARDIAC MUSCLE submitted by OKSANA SONIA HERASYMOWYCH in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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J. B. Smillie

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George Kotovych

David E. Hoke
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Date October 25, 1979

This thesis is gratefully dedicated to:

my mother Mary, who instilled the dream,

my father Zeno, who encouraged its pursuit,

and my husband Sergi, who helped to fulfill it.

Their love and understanding made this project possible.

ABSTRACT

This study reports the isolation and characterization of homogeneous creatine kinase which was localized as having its origin within the M-band of bovine cardiac muscle.

The preparative procedure involved the use of low ionic strength extraction of a muscle mince followed by two DEAE-cellulose chromatography steps, a procedure designed to release the enzyme bound to the myofibril. The creatine kinase thus isolated appears to be the MM isozyme, on the basis of evidence from polyacrylamide gels which indicated it co-migrates with the MM forms of CPK from other sources. This conclusion was corroborated by comparison of its amino acid composition with the amino acid compositions from other MM sources.

Comparison of the physical parameters of the cytoplasmic and myofibrillar CPK indicated they are probably the same enzyme. They both have an $E^{1\%}_{1cm}$ at 280 nm of 9.0, and possess equivalent UV spectra. Circular dichroism spectra in both the near and far UV also point to the two enzymes as being identical. Their α-helical content was determined to be 30%, with a β-conformation value of 20%. Sedimentation equilibrium studies for cytoplasmic and myofibrillar enzymes gave an average molecular weight of 80,000 daltons for the native enzyme, and 40,000 daltons for their subunit molecular weight. SDS-polyacrylamide gels showed a characteristic doublet pattern of average molecular weight 40,000 daltons. The $S^{o}_{20,w}$ was 5.1 ± 0.1 S. Both enzymes appear to have an average specific activity of 60 units/mg protein. K_m values are in the range of 0.05 mM for ADP and 2.0 mM for creatine phosphate.

ATPase activity studies indicated bovine cardiac CPK has an inhi-

bitory effect on the ATPase of myosin, heavy meromyosin and subfragment-1, with inhibition values of 26, 20 and 19% respectively. Maximum inhibition was found to occur when the two enzymes were in a 1:1 mole ratio. Sedimentation velocity studies demonstrated that bovine CPK promotes the aggregation of synthetic myosin filaments. The addition of 165,000 dalton protein from rabbit skeletal muscle to bovine CPK was found to inhibit the enzymatic activity of creatine kinase by 13% when the 165,000 dalton protein was in a 2:1 mole ratio to CPK.

Electron microscopy and immunobiological studies revealed no binding bf chicken antibodies to creatine kinase to the M-band of myofibrils from bovine heart. For this reason, reconstitution of the M-band was used to localize the origin of the myofibrillar CPK. Reconstitution of the M-bands of rabbit skeletal muscle and bovine cardiac muscle following their removal by low ionic strength extraction was accomplished. Examination of myofibrils in the electron microscope showed that creatine kinase and the 165,000 dalton component will re-form the M-band independently of each other. In the skeletal system, rabbit skeletal CPK and the 165,000 dalton protein were used, while in the bovine cardiac case, bovine cardiac CPK and rabbit skeletal 165,000 dalton protein were employed. The latter conditions gave results which suggest the presence of a bovine analogue of 165,000 daltons in the M-band of bovine cardiac myofibrils. The fact that bovine creatine kinase will re-form the M-band firmly establishes its presence, for the first time, as an integral part of the M-band of mammalian cardiac muscle.

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ABBREVIATIONS AND SYMBOLS

Units for mass, length, volume and time are abbreviated according to standard procedure.

ATP	adenosine triphosphate
CD	circular dichroism
CNBr	cyanogen bromide
CPK	creatine phosphokinase or creatine kinase
CPM	counts per minute
DDSA	dodecenyl succinic anhydride
DEAE	diethylaminoethyl
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	dithiothreitol
$E^{1\%}_{1cm}$	absorbance of a 1% solution in a 1 cm light path
EDTA	ethylenediaminetetraacetate
HMM	heavy meromyosin
HS	Hasselbach-Schneider's Solution
K_m	Michaelis-Menten constant equal to the substrate concentration at which the velocity is half maximal, in moles/ℓ
LMM	light meromyosin
ln y	natural logarithm of protein concentration in fringe displacement units
mA	milliamperes
mCi	millicuries
NMA	nadic methylanhydride
PBS	phosphate buffered saline solution
r	radial distance from the axis of rotation
rpm	revolutions per minute

S	Svedberg unit of sedimentation velocity (10^{-13} sec)
$S_{20,w}$	sedimentation coefficient corrected to water at 20°C
$S^{o}_{20,w}$	intrinsic sedimentation coefficient
TCA	trichloroacetic acid
Tris	tris-(hydroxymethyl)aminoethane
UV	ultraviolet
$\bar{v}$	partial specific volume
η	solvent viscosity
ρ	density of solvent
$[\theta]_{\lambda}$	mean residue ellipticity at wavelength λ
$[\theta]_{obs}$	observed ellipticity value

CHAPTER I

INTRODUCTION

A. VERTEBRATE STRIATED MUSCLE

The mechanism of muscular contraction centres around the intriguing biochemical problem of how the chemical energy of ATP is transformed into mechanical energy. Since several excellent reviews detailing the mechanism of vertebrate skeletal muscle (1 - 4) have been published in the literature, the following discussion will be confined to presenting a brief general outline of the salient features. Cardiac muscle, like its skeletal counterpart, is a cross-striated muscle. It shares many similarities as well as a few differences which have also been extensively reviewed (5 - 7).

As illustrated in Figure 1, vertebrate muscle when examined in the electron microscope has a striated appearance. A schematic diagram of the structure of the myofibril appears in Figure 2, and is representative of both the cardiac and skeletal muscle systems. The characteristic cross striations one observes are due to the alternation of optically dense and less dense transverse regions along the myofibril. The dark bands are termed anisotropic or A bands; the light bands are called isotropic or I bands. The term anisotropic refers to the property these A bands possess: under polarized light, these bands are strongly birefringent, indicating that their molecular components are asymmetric and oriented in a specific manner. The light I bands, on the other hand, are relatively nonbirefringent and hence are termed isotropic.

In resting muscle the A bands are about 1.6 μ in length and the I bands about 1.0 μ long. The Z line bisects the I band and is seen as

Figure 1. Electron microscopy of sectioned bovine cardiac myofibrils (magnification = 27,300 X)

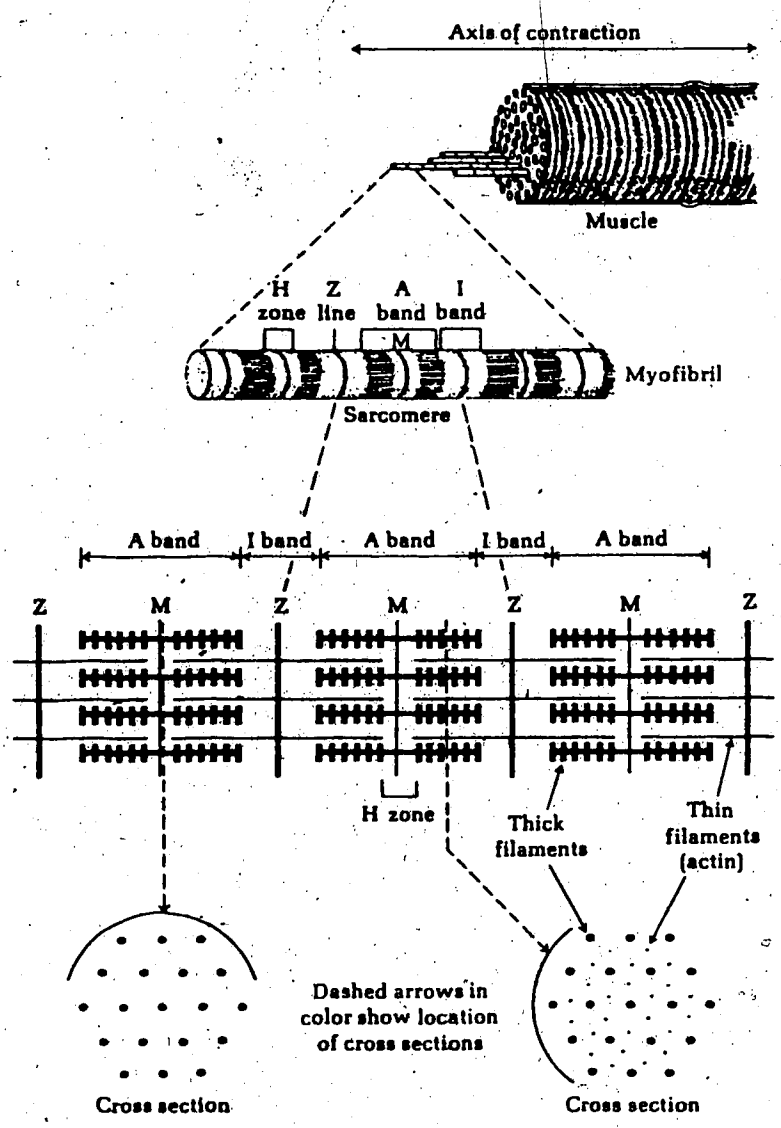


Figure 2. Schematic representation of myofibrillar structure [from Lehninger (97)].

a very dense line about 800 Å wide. The space between two such adjacent lines defines the functional unit of contraction, the sarcomere. The length of the sarcomere ranges from 1.5 to 2.5 μ.

Each myofibril is composed of many parallel myofilaments, of which there are two types. These are designated the thick and thin filaments. The thick filaments are about 150 Å - 170 Å in diameter and 1.6 μ in length, and occupy the central portion of the sarcomere. They consist of myosin molecules which are aggregated in a tail-to-tail manner with the resulting formation producing a bare smooth region at the middle of the filament with myosin heads projecting in opposite directions. These adjacent myosin filaments join in a narrow region of relatively low optical density in the middle of the A band called the H zone. A dark line called the M-line or M-band bisects the H zone. The function of the M-band is believed to be in maintaining the thick filaments in register. A more detailed discussion of the ultrastructure of the M-band follows later.

The thin filaments are about 60 Å in diameter. These filaments interdigitate with the myosin filaments, running from the Z band at the edge of the sarcomere through the I band and into the A band to the edge of the H zone. The A band is the only region within which there is an overlapping of the thick and thin filaments. Electron microscopy studies on cross sections have shown that the thick filaments are approximately 450 Å apart, arranged in a hexagonal pattern, with each thick filament surrounded by six thin filaments, also in a hexagonal array. The formation of interfilamentous cross-bridges between the thick and thin filaments has been observed by the use of electron microscopy techniques.

Figure 3 illustrates the sliding model of muscle contraction (1). Upon contraction the sarcomere shortens, but the length of the A band and the distance to the Z line from the H zone remain constant. The contraction is signalled by a nerve impulse which causes the release of Ca^{2+} from the sarcoplasmic reticulum into the sarcoplasm to give a final Ca^{2+} concentration of 10^{-5} M. The constant formation and disruption of cross-bridges between the thick and thin filaments produces the sliding motion of the filaments past each other. The immediate source of energy for this process is the energy released from the hydrolysis of ATP which accompanies contraction.

The regulation and control of muscle contraction rests with the major proteins of the myofilaments, myosin and actin.

The major protein of the thick filament is myosin, a large molecule comprised of two heavy chains and a total of four light chains with a molecular weight of approximately 470,000 daltons. The myosin function of hydrolyzing ATP is localized at the globular heads. Associated with the heads of the myosin are low molecular weight proteins termed light chains (8, 9). These are non-covalently bound to the myosin and are heterogeneous in charge, composition and size. In skeletal muscle there are three types, with SDS-polyacrylamide gels giving molecular weights of 25,000, 18,000 and 16,000 daltons for A1, DTNB and A2 light chains, present in approximately 1:2:1 mole ratio respectively. The nomenclature stems from the observation that myosin will react with 5,5'-dithiobis-(2-nitrobenzoic acid) (i.e., DTNB) to liberate a light chain fraction which is half the total light chain material. This DTNB chain does not appear to be essential for the enzymatic activity of myosin and its functional role is not understood. The A1 and A2 light chains can only

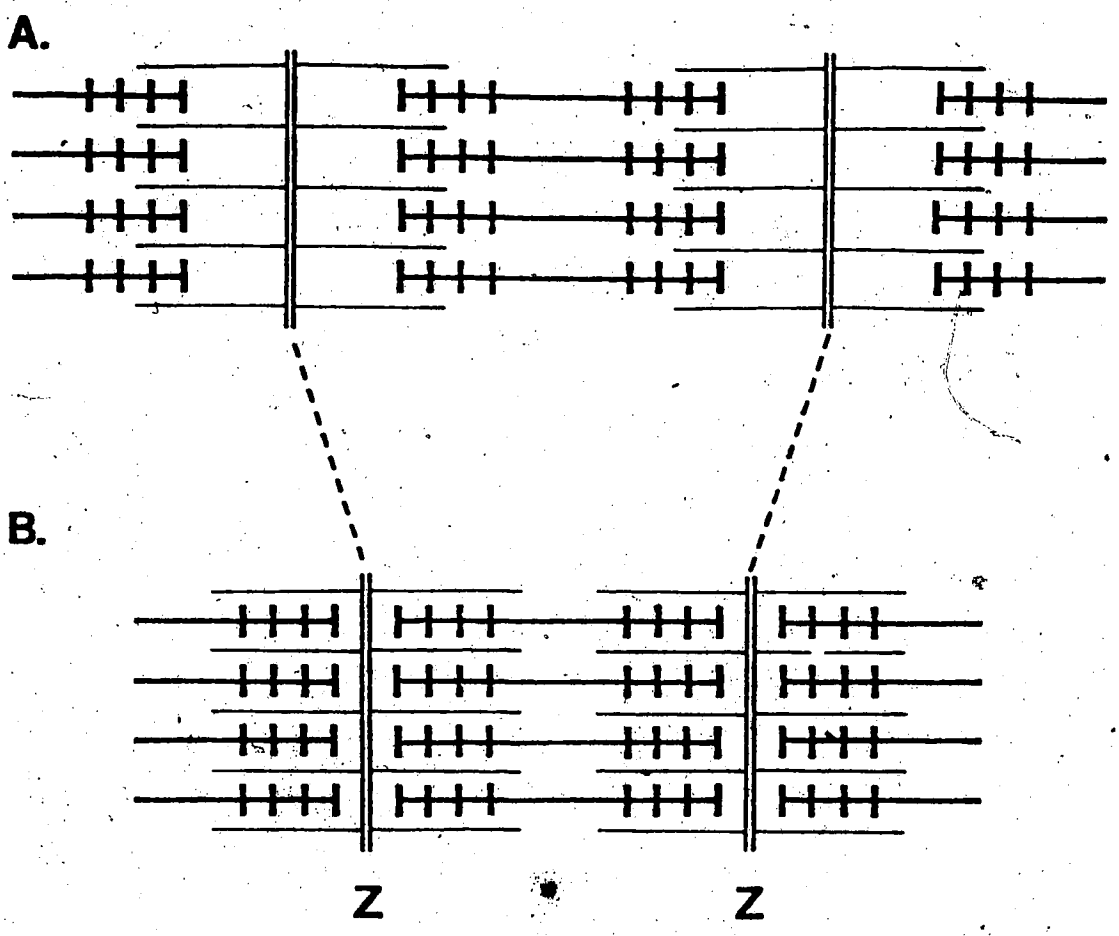


Figure 3. Sliding filament model for contraction of the sarcomere. The diagram displays one complete sarcomere, along with parts of neighbouring ones. A: striated pattern observed in resting muscle. B: striated pattern observed in contracted muscles.

be removed by denaturing conditions such as alkali pH 11.4, and hence are termed alkali light chains. The liberation of these alkali light chains is accompanied by a simultaneous loss of ATPase activity, and denaturation of the myosin. It has therefore been postulated that the role of the light chains in myosin is to act as regulatory proteins in the hydrolysis of ATP. In the case of cardiac myosin, there are only two different light chains present which may be isolated from myosin at alkaline pH. These have been found to possess molecular weights of 27,000 and 18,000 daltons respectively and are present in a 2:1 mole ratio (9). It is thought that the differences in the light chains between skeletal and cardiac myosins, in addition to the structural heterogeneity in the heavy chain components, may account for the variation in ATPase activity between cardiac and skeletal myosins.

The thin filament is comprised of actin, troponin and tropomyosin, existing in a 7:1:1 molar ratio. The major component of the thin filament, actin, is a globular protein of 42,000 daltons which in the presence of salt and ATP polymerizes to form F-actin filaments. Two such F-actin strands wound about each other make up the thin filament. Along the groove of the F-actin filament lies a polymer of tropomyosin molecules; each traverses seven actin monomers and is associated with a single troponin molecule. Together, the tropomyosin and troponin confer calcium sensitivity to the interaction between myosin and actin (4) and so represent the on-off switch for muscle contraction (4, 10).

B. THE M-BAND STRUCTURE

Since this study centres around the investigation of creatine kinase which has been implicated as having a functional or possibly

structural role within the M-band, the ultrastructure of the M-band will be discussed in some detail. As mentioned previously, the region of increased electron density in the centre of the sarcomere called the M-band has been assumed to have the role of maintaining the thick filaments in register, though this has not been established.

Actual information on the structure of the M-band is not extensive. Electron micrographs show the M-band to appear as a line of high electron density having a width of 750 - 850 Å (11 - 13) in the middle of the A band. Transverse sections through the M-band show this high opacity to be due to 3 to 5 (depending on the source) arrays of cross-bridges connecting each thick filament with its six nearest neighbours in a hexagonal array (11, 14 - 18). These cross-bridges are termed M-bridges. Halfway along the M-bridge can be seen a thickened region which has been attributed to the presence of small (40 - 50 Å diameter) longitudinally oriented filaments. Evidence for such filaments has been obtained from longitudinal sections through the M-band (19, 20). These are called M-filaments.

Although Pepe (18, 21) has proposed a model for the M-band, this discussion will focus on two studies: the work of Knappeis and Carlsen (19) who in 1968 proposed a model of the M-band structure, and the recent study of Luther and Squire (22) whose ultrastructure experiments have given firm support to the role assigned to the M-band. The results reported by Luther and Squire are in accord with the three dimensional M-band model proposed by Knappeis and Carlsen, but conflict with the model of Pepe.

Knappeis and Carlsen (19) observed that in transverse sections, the framework of the M-band consisted of 3 (4 or 5, depending on the

muscle fibre type) arrays of transverse M-bridges, 200 Å apart, connecting each A filament with its six neighbours. They also observed that M-filaments, parallel to the A filaments, passed through the M-band and linked each set of M-bridges together. A schematic cross-section of their model appears in Figure 4a. Here the large solid circles represent the A filaments, the small solid circles M-filaments and the open circles the projections of the I filaments to the level of the M region. The dotted areas represent the M-bridges. The authors have suggested that the role of the M-bridge and M-filaments could be to keep each sliding I filament in a prismatic tube, since the I filaments are no longer guided by the projections from the A filaments when they enter the M region during shortening. These findings support the assumption that the main function of the M-band is to keep the A filaments in position in the longitudinal as well as in the transverse direction.

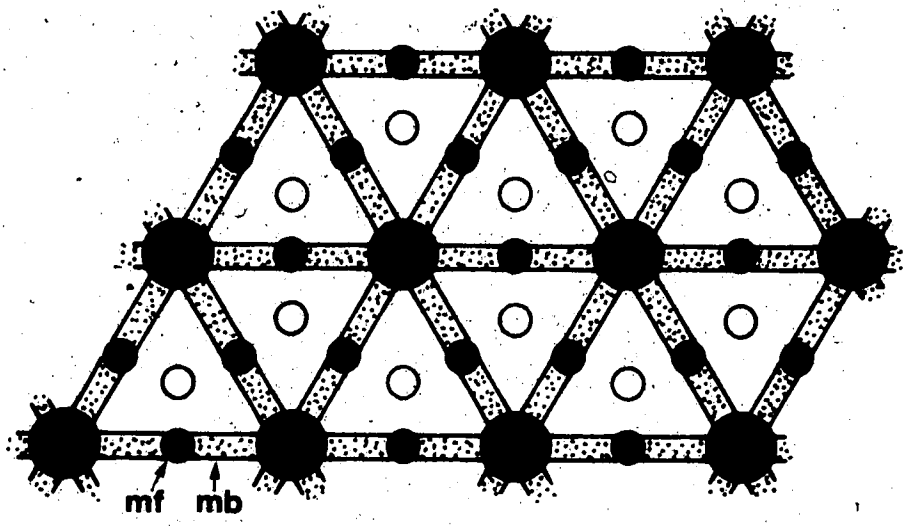
This model conflicts with the model proposed by Pepe (18, 21). Knappeis and Carlsen suggested that at each level of M-bridges, there are six M-bridges which link any myosin filament to its six neighbours in a hexagonal filament array. On each level, the six M-bridges point in one of three directions which are 60° apart (0° and 180° being considered equivalent). Pepe, on the other hand, concluded that only a single pair of diametrically opposed M-bridges occurs on each level, and these link a myosin filament to just two of its six neighbours. He suggested a "thick" transverse section would appear to show a hexagonal array; a "thin" section (less than 220 Å thick) would show M-bridges pointing in one direction only.

Luther and Squire (22) applied accurate sectioning methods to well ordered muscles of frog using <u>in situ</u> fixation, and presented new

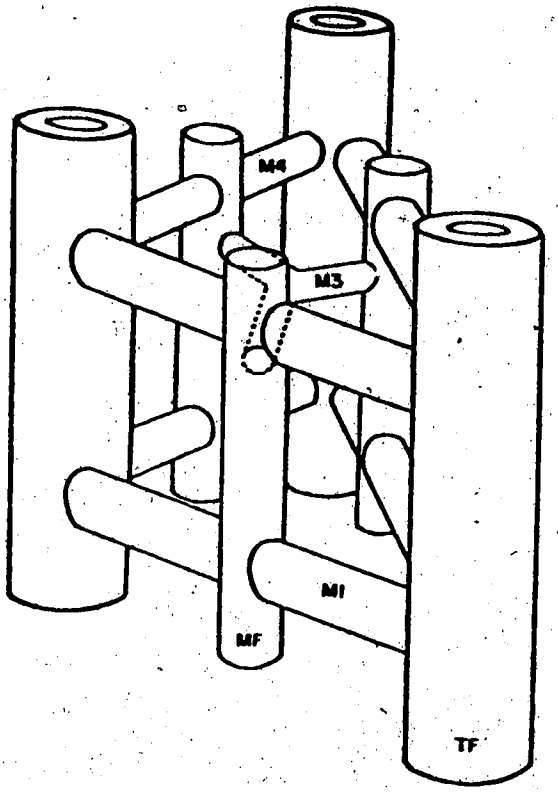
Figure 4a. The Knappeis-Carlsen model of the M-band of striated muscle. The diagram displays a schematic cross-section through the M-band. Large solid circles = A-filaments; small solid circles = M-filaments; open circles = projection of I-filaments to level of M region; dotted bars = M-bridges [from ref. (19)].

Figure 4b. The Luther-Squire model of the M-band of striated muscle. The diagram is a representation of the probable 3-dimensional arrangements of the bridges M1, M3 and M4 on one side only of the M-band. M3 consists of Y-shaped secondary M-bridges linking to the M-filaments (MF). The M-filaments are linked via the main M-bridges to the thick filaments (TF). M1 is at the centre of the M-band, so a similar structure (but inverted) would occur below M1 in this diagram. The secondary M-bridges have been placed at M3 to explain extra (bridging) density at this position in longitudinal sections [from ref. (22)].

a.



b.



evidence incompatible with Pepe's model: even in their thinnest sections (200 Å), M-bridges were found pointing in all three directions 60° apart. Their model is displayed in Figure 4b, and shows a representation of the probable three-dimensional arrangement of the bridges on lines M1, M3 and M4 on one side only of the M-band. The M-filaments (MF) are linked via the main M-bridges to the thick filaments (TF) corresponding to the A filament of the Knappeis and Carlsen model. This model is essentially that of Knappeis and Carlsen with an additional feature: secondary M-bridges (M3). This protein was added to explain an extra bridging density at this position in longitudinal sections as reported by Sjostrom and Squire (23). Their model clearly supports the idea of longitudinal M-filaments in the M-band linking the centre of the main M-bridges. Since the main M-bridges and secondary M-bridges are at different levels, the M-filaments would also be expected to link these bridges together; otherwise the secondary M-bridges could not be connected to the myosin filaments.

One could therefore expect at least three M-proteins and possibly more, since this model incorporates at least three different M-band structures apart from myosin. Whether these proteins are to have a strictly structural role remains to be seen. Luther and Squire feel it is the main M-bridges themselves that have the primary structural role in the M-band and which organize myosin filaments so that the A band has a well-defined structure.

A discussion pertaining to the assignation of roles for proteins which have been extracted from the M-band follows a review of their general properties and localization studies.

C. THE COMPONENTS OF THE M-BAND

Extraction procedures which selectively remove the M-band of skeletal muscle usually reveal the presence of three major components. Two of these have been shown to be the enzymes, creatine kinase and glycogen phosphorylase b (24, 25), while the third is the 165,000 dalton component (21). The fact that two of the three are enzymes seems to imply that it may be functionally useful to have these proteins located at the M-band where they have ready access to the contractile machinery. Nonetheless, these enzymes may also be regularly arranged within the M-band performing a direct structural role. The following discussion will centre around the three proteins implicated in studies to date as possible candidates for having their origin within the M-band. Their general characteristics, as well as a brief survey of the literature, will be presented.

1. Creatine Kinase

a. General Characteristics

In vertebrates, working muscle cells must regenerate the ATP used in the contraction process, and this is presumably accomplished via a phosphotransferase reaction so that the following equation applies:

$$CP + ADP \rightleftharpoons ATP + C \qquad [1]$$

where CP is the substrate creatine phosphate and C is the product creatine.

In such a manner, a high level of ATP is maintained in the sarcomere. The enzyme responsible for catalysis of this important reaction is creatine kinase (ATP:creatine phosphotransferase E.C. 2.7.3.2), sometimes abbreviated as CK or CPK.

The history of creatine kinase began in 1927 when Eggleton and Eggleton (26, 27) and Fiske and Subbarow (28) independently reported finding the first compound to carry a high phosphoryl transfer energy and to be involved in energy transfer. Since then, the enzyme has become implicated in various physiological roles. Clinical interest in the enzyme is very high, and the reviews of Tyler (29) and Sherwin et al. (30) are of benefit in this regard. Creatine kinase has been of special importance in research in the muscular dystrophies, since elevated enzymatic activities in the serum are of great assistance in the early diagnosis of such disorders (31 - 33) or in the detection of the female carrier in the sex-linked Duchenne type of progressive muscular dystrophy (34). Clinical assays of creatine kinase activity have also been proposed as a measure of myocardial infarct size (35 - 37) since levels of creatine kinase activity in serum also show an increase following acute myocardial infarction and perioperative ischemic injury (38).

The following is a brief general discussion of studies performed on creatine kinase which have led to understanding the properties, mechanism and behaviour of the cytoplasmic enzyme, and will be confined to a cursory summary of the main features as gleaned from the review articles of Watts (39), Bickerstaff and Price (40), Eppenberger (41) and Thomson et al. (42).

In vertebrates, cytoplasmic creatine kinase occurs as three forms which are readily distinguishable by their electrophoretic mobility. These are called the muscle or MM type, the hybrid MB, and the brain type BB, in order of increasing mobility towards the anode at pH's greater than 7, and indicating their major tissue of origin.

During development, the brain-type isozyme is the first to

appear in all tissues examined, and this form remains constant in the brain as the animal attains maturity. In chicken heart and mammalian smooth muscle, the BB isozyme remains dominant with a trace of MB. In mammalian heart and red and white striated muscles, the MM form gradually predominates until only a trace of the hybrid remains at the stage of full adulthood. Atrophy of white muscles which can be caused by denervation, vitamin E deficiency or hereditary dystrophy, has been shown to be accompanied by a loss of the MM form and a reappearance to a "fetal state" of BB.

The amino acid composition has been determined for the MM form of creatine kinase from several species. In general, the amino acid compositions for each type are all very similar, irrespective of the source. In the case of BB enzymes, these have been found to contain significantly less of the basic amino acids than the MM forms, explaining their greater electrophoretic mobility. For a given species, the amino acid composition of the MB hybrid is intermediate between that of the parent forms.

Generally, there are eight thiol groups per molecule; two of these are relatively exposed to the solvent, while 6 are buried. No disulfide bonds are present.

Primary structure determinations indicate that less than 15% of the total sequence is known. In general, these studies centre on the peptide sequence containing a reactive thiol group, and it appears that there is considerable homology between this peptide and the corresponding peptide from other isozyme forms.

Two moles of alkylating agent react per mole of enzyme, indicating CPK has 2 reactive sulfhydryl groups, one

in each subunit of rabbit skeletal creatine kinase. The C-terminus has been determined to be valine while the N-terminus could not be detected by either fluoronitrobenzene or phenylisothiocyanate in either the native protein or following denaturation in urea.

Secondary and tertiary structure studies show a compact globular structure containing 25 - 30% α-helix, and approximately 15% β-pleated sheet, as determined by optical rotary dispersion. Preliminary X-ray diffraction studies (43, 44) have indicated the rabbit skeletal enzyme crystallizes in two forms, both of monoclinic space-group C2, and possesses a 2-fold axis of symmetry relating two identical or nearly identical subunits.

The shape and organization of the subunit have also been under investigation. Sedimentation studies under various conditions indicate that creatine kinase consists of two freely dissociable subunits, each consisting of a single polypeptide chain containing no disulfide bridges. The calculated frictional ratio f/f_o was found to be 1.21, with an axial ratio of 4.4 for an assumed anhydrous prolate ellipsoid. The shape is therefore similar to that of a compact cigar-shaped molecule. The use of sodium lauryl sulfate to dissociate the subunits without an apparent loss in structure has permitted similar estimations for the subunits and these values were found to be identical. The molecule can thus be envisaged as consisting of subunits joined side by side rather than end on end. Studies on the calf brain enzyme indicate it also appears to have an identical shape.

The molecular weight now generally accepted for rabbit skeletal creatine kinase is 82,600 daltons. Experimentally determined values for creatine kinase from other sources fall in the range of 78,500 - 85,100

daltons.

Kinetic and enzymatic studies have shown that all the mammalian enzymes have similar specific activities. Detailed investigations indicate no evidence for any phosphorylated enzyme intermediate. The mechanism is believed to be rapid-equilibrium, random-order type with synergism in substrate binding, i.e., the binding of metal nucleotide to the enzyme facilitates the subsequent binding of creatine and vice versa. It is believed that each subunit contains one catalytic site, and each site contains separate binding sites. Both substrates are thought to bind simultaneously, the presence of the first substrate making binding of the second substrate easier, and vice versa.

The reactive thiol group previously termed "essential" has recently been shown to be not directly involved in the catalytic mechanism. Its exact role remains an enigma, since other studies have indicated that preservation of the integrity of this reactive thiol group seems a requirement in forming a ternary complex on addition of creatine.

It appears that the dimeric structure of the enzyme is not essential for the expression of catalytic activity. Bickerstaff and Price (45, 46) linked creatine kinase to CNBr-activated Sepharose with one subunit by using low levels of CNBr activation. This derivative was then treated with denaturing agent and the non-matrix bound subunit washed away. Such matrix-bound subunits were found to be catalytically active with 50% of the specific activity of the soluble enzyme. This suggests that the quaternary structure is not a controlling factor over the expression of catalytic activity.

Some indication of the non-identical behaviour of the subunits has come from studies on the kinetics of modification of the reactive

thiol group on each subunit with a variety of reagents: iodoacetate, 7-chloro-4-nitrobenzofurazan and DTNB (47). The thiol groups were found to react at different rates in the presence of MgADP, creatine and nitrate (a transition state analogue complex). Studies on ADP binding using equilibrium dialysis with the same substrates showed features characteristic of non-cooperativity or non-identical sites. It is thought that this non-identical behaviour may have some possible regulatory significance.

b. Intracellular Compartmentation of Creatine Kinase

Compartmentation refers to the property of a protein to be characteristically located in one or another organelle or intracellular structure. Compartmentation permits control and integration of some intracellular activities in addition to segregating chemically incompatible reactions. Creatine kinase is an enzyme which has been localized as being present in the mitochondria, on myofibrils and microsomes in addition to being present in the cytoplasm.

Creatine kinase is believed to be localized on the outer surface of the inner membrane of the mitochondrion (48). This functional compartmentation of creatine kinase is thought to be the foundation of a method for transferring the intracellular high energy phosphate from its site of production in the mitochondrion to appropriate positions of utilization (49 - 53).

Although initially mitochondrial creatine kinase had been thought to be cytoplasmic creatine kinase absorbed to the mitochondria, several groups of workers (53 - 56) eventually suggested that this form may be distinct from any of the three cytoplasmic forms, MM, MB and BB. Ogunro et al. (57) in their compartmentation studies of creatine kinase used

isopycnic density gradient centrifugation or differential pelleting to subfractionate guinea pig heart. Isozyme analysis of creatine kinase in the subcellular fractions was accomplished by electrophoresis. In addition the enzyme activity recovered in any individual fraction was expressed as a percentage of the total activity recovered in all four fractions. The distribution of creatine kinase showed 4% in the myofibrils, 6% in the mitochondria, 1% in the microsomes and 89% in the cytosol. MM isozyme was found to occur in the cytoplasm, myofibrils and microsomes, while MB was found only in the cytoplasm along with traces of BB. The mitochondrial enzyme turned out to be a totally distinct and additional mitochondrial-specific form of creatine kinase with only a small amount of the total creatine kinase activity (<10%) associated with the mitochondria.

The kinetic studies of Jacobus and Lehninger (52) on intact mitochondria from bovine heart have indicated that the mitochondrial creatine kinase is especially adapted to the formation of creatine phosphate in the mitochondrion, since this enzyme had been found to have a 10-fold greater apparent affinity for ATP than the sarcoplasmic enzyme (i.e., favouring the reverse of reaction [1]). In accord with this idea, several workers (51 - 53, 58) have suggested that the mitochondrial enzyme mediates the transfer of high energy phosphate from the mitochondrial compartment to the cytoplasmic compartment by catalyzing the formation of freely diffusable creatine phosphate. Once formed, the creatine phosphate passes readily into the cytoplasm where it is transphosphorylated back into ATP by cytoplasmic creatine kinase to meet energy requirements.

The most recent investigation of mitochondrial creatine kinase from bovine heart by Hall et al. (59) has shown it to be very different

from the cytoplasmic enzymes. The smallest active form of the dimer is 64,000 daltons with two free thiol groups per subunit. Two active forms were found in solution, one with a cathodal (opposite to cytoplasmic forms) mobility on electrophoresis and a molecular weight of 64,000 daltons; the other possessed a greater mobility with a molecular weight three times larger. Although the forms were separable, they were found to interconvert in solution, depending on the protein concentration and the presence of reducing agent. Attempts to form hybrids with the cytoplasmic MM, MB and BB forms using disruption and reannealing techniques proved unsuccessful. This result implies the mitochondrial creatine kinase may have a very different tertiary structure from the cytoplasmic forms. These authors similarly agree with the supposition that the mitochondrial enzyme acts as an energy "shuttle" to transport energy to sites of utilization for muscle contraction.

Little work has been done on the association of creatine kinase with microsomes other than the work of Kleine (60) who showed like Ogunro et al. (57) that the microsomal fraction is only approximately 1% of the total cellular creatine kinase content. It has been suggested by Baskin and Deamer (61) that in this compartment creatine kinase may function as a local source of ATP for calcium transport when the overall ATP concentration is relatively low as a result of myofibrillar contraction.

Further evidence for compartmentation of creatine kinase isozymes is provided by the localization of MM creatine kinase in the myofibrils (62 - 64) and purified sarcoplasmic reticulum (65, 66). In these positions it is thought that the enzyme regenerates the ATP required for contractile and transport events of the cardiac cycle. In view of the present study, the localization in myofibrils is of special interest.

Ottaway (62) had reported that a portion of creatine kinase appeared bound to the myofibrils of beef heart. Sholte (63), Saks et al. (64) and Alievskaya and Chetverikova (65) similarly found creatine kinase to be located in the myofibrils of skeletal and cardiac muscles of rat. The discussion immediately following on the role of creatine kinases within the M-band, in like manner, is further evidence for the functional potential of their compartmentation.

c. Localization Studies of Creatine Kinase in the M-Band

Localization studies involving the techniques of histochemistry (30, 66) and cell fractionation (60, 62) had indicated that the bulk of creatine kinase is found in the cytoplasm, with lesser amounts associated with mitochondria or with the sarcoplasmic reticulum. However, over the years evidence has arisen that some of the creatine kinase in skeletal and heart muscle cells may be bound to the elements of the contractile apparatus (61, 62, 67 - 71).

Studies by Pepe (72) on the structural organization of the myofibril as revealed by antibody-staining methods had shown that the M-band protein was different from actin, myosin and tropomyosin. Samosudova (73) had earlier demonstrated with electron microscopy that the M-band of striated muscle could be extracted completely by 5 mM Tris buffer pH 8. Using this same procedure, Kundrat and Pepe (74) extracted the M-band and showed that the extracted protein could specifically absorb the M-band labeling antibodies from antibody which labeled both the I band and the M-band. Using the same low ionic strength extraction technique on chicken skeletal myofibrils, Morimoto and Harrington (75) were able to isolate and purify an 88,000 dalton dimeric protein. They identified it as an M-band protein on the basis of the ability of anti-

body prepared against "crude" M-band extract to specifically alter the thickness and stability of the M-band structure as manifested in electron micrographs. Physical studies on the molecule indicated dissociation into two subunits of 43,000±1,000 daltons occurred in 6M guanidine hydrochloride.

Turner et al. (76) later identified this M-band protein to be the MM creatine kinase of chicken skeletal muscle from previously published data (77, 78). A comparison of size, subunit composition, amino acid composition, electrophoretic mobility, solubility properties and specific enzymatic activity provided strong support for the identity of these two proteins. Conclusive evidence for this identity was demonstrated by antibody tests. In addition, the present of MM-creatine kinase isozyme within the M-band was demonstrated by the method of indirect immunofluorescence; myofibrils incubated with antiserum against MM-CPK showed a regular pattern of fluorescent lines running through the middle of each A band.

Subsequent studies by Walliman et al. (24, 79) have indicated that in chicken skeletal muscle, at least 3 - 5% of the total creatine kinase activity present remained with the myofibrillar fraction after extensive washing, and this bound protein became released by a single extraction at low ionic strength. In the case of chicken cardiac muscle (79) a difference was noted; heart muscle contained almost exclusively the BB isozyme, and only 2% of this BB-CPK was found to bind to the myofibril. Electron microscopy coupled with immunofluorescence showed this BB isozyme to have its origin within the Z-band. This latter result may not be so surprising in view of the fact that chicken cardiac muscle, unlike mammalian cardiac, does not possess a distinct

M-band structure as such.

The overall evidence, consequently, seems to indicate that in skeletal muscle at least, MM-CPK is not adventitiously bound, but rather is an integral element in the M-band structure.

d. Interaction Studies of Creatine Kinase with Myosin

Since creatine kinase in the M-band could conceivably be undergoing interaction with its surrounding neighbours, the following discussion will present a brief survey of some biochemical studies on creatine kinase interactions with another contractile component, myosin.

Yagi and Noda (69) had found that when glycerinated myofibrils were incubated with creatine kinase and creatine phosphate, the myofibrils shortened, with a concurrent liberation of creatine, until the substrate creatine phosphate was exhausted. Subsequent work by Yagi and Mase (70) showed that CPK from rabbit skeletal muscle was able to inhibit noncompetitively the hydrolysis of ATP by rabbit skeletal myosin in the presence of divalent cations Ca^{2+} or Mg^{2+}, when the molar ratio of CPK to myosin exceeded 1. Botts and Stone (71) verified this result and found it attributable solely to the direct inhibition of myosin by creatine kinase.

Morimoto and Harrington (75) demonstrated that their 88,000 dalton M-band protein, subsequently identified by Turner et al. (76) to be MM-CPK, was able to promote the aggregation of myosin filaments under particular conditions. This finding seemed to substantiate the concept that M-protein CPK forms cross-bridges between adjacent thick filaments of the A band. Any protein which binds to myofibrillar myosin only at the M-band region would be expected to bind to the rod portions of the myosin molecule, since the central regions of the thick filaments, to

which the M-proteins attach, are thought to be devoid of myosin "heads". Hence the report of Houk and Putnam (80) was of special interest in this regard. Using steady state fluorescence polarization experiments, the authors were able to label CPK with a sulfhydryl specific dye and show it binds to the rod portion of the myosin molecule. This interaction was specific for CPK and myosin. No such interaction was found for subfragment-1.

Botts et al. (81) have since demonstrated with both electron paramagnetic resonance and nanosecond fluorescence depolarization techniques that creatine kinase interacts with myosin, heavy meromyosin and subfragment-1. No effect of creatine kinase on light meromyosin or "rods" was found, contrary to the finding of Houk and Putnam. There was also some indication that thiols may be involved either directly or indirectly in the interaction.

Studies giving further support to the concept that the site of interaction on the myosin is in the "head" region come from the biological activity studies of Mani and Kay (82) and Herasymowych et al. (83), who demonstrated that the ATPase of myosin, heavy meromyosin and subfragment-1 is inhibited by creatine kinase for the case of both rabbit skeletal and bovine cardiac muscle. The circular dichroism studies of Mani and Kay showed (82) this interaction decreases as one proceeds from myosin through to heavy meromyosin to subfragment-1. In addition, low speed equilibrium studies revealed the presence of a complex formed from the interaction of creatine kinase with subfragment-1.

2. The 165,000 Dalton Protein

a. Localization Studies of the 165,000 Dalton Protein in the M-Band

Masaki et al. (84) have reported that extraction of chicken

skeletal muscle with a high ionic strength medium of Hasselbach-Schneider's solution* resulted in the complete removal of the A-band. From this extract, they were able to isolate a component which could specifically absorb antibody which labeled the M-band. Masaki and Takaiti (85) subsequently resolved this M-band protein into two components, one with a polypeptide chain weight of 165,000 daltons, the other of 94,000 daltons, and in a following publication (86) concluded that the 165,000 dalton component was the M-band protein on the basis of fluorescent antibody techniques. This 165,000 dalton component was also shown to possess considerable binding affinity towards myosin, in addition to being somewhat resistant to trypsin digestion.

Landon and Oriol (87) have succeeded in purifying the M-protein from rabbit skeletal muscle by a procedure of washing with intermediate ionic strength followed by extraction with 5 mM Tris pH 8, and purification on DEAE cellulose in the presence of ATP. Two contaminants noted in their preparation were actin and a 100,000 dalton component. Optical rotary dispersion and circular dichroism studies gave a value of 30% β sheet and low α-helical content for the 165,000 dalton protein.

Definitive evidence for the 165,000 dalton protein as being present in the M-band of chicken skeletal muscle has been presented in papers by Trinick (88) and Trinick and Lowey (89, 90), who used a modification of the Masaki procedure of high ionic strength extraction to extract M-band proteins. They found two 170,000 dalton proteins co-migrating on SDS-polyacrylamide gels. Of these, the 7S protein was found to be the glycogen debranching enzyme, while the 5.1S was found to be from the M-band.

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*0.6 M KCl, 0.1 M potassium phosphate pH 6.4, 10 mM sodium pyrophosphate, 1 mM $MgCl_2$.

By direct application of antibodies to the 5.1S component to the myofibril, they were able to present electron micrographs which showed a definite darkening and concurrent thickening of the M-band structure.

Taking these localization studies into consideration, it seems clear that the 165,000 dalton protein is another integral element in the M-band structure of skeletal muscle.

b. Interaction Studies of the 165,000 Dalton Protein with Other Contractile Components

Mani and Kay (91, 92) have isolated, purified and characterized the 165,000 dalton protein from rabbit skeletal muscle. In addition, they have done interaction studies with creatine kinase from rabbit skeletal muscle (the other M-band component). Addition of the 165,000 dalton component was found to inhibit the enzymatic activity of creatine kinase in a competitive manner. Low speed sedimentation equilibrium studies also showed data indicative of a complex formation between the two M-band proteins.

Mani and Kay (92) have also examined interactions of the 165,000 dalton protein with myosin and subfragment-2. Circular dichroism experiments indicated the M-protein interacted with myosin giving a net increase of 600 deg cm^2 $dmole^{-1}$ at 221 nm, and subfragment-2, giving a net increase of 800 deg cm^2 $dmole^{-1}$. Low speed equilibrium runs showed complex formation of 165,000 dalton protein with subfragment-2. Gel filtration allowed separation of this complex and gave an estimated molecular weight of 230,000 daltons. No interaction was indicated when the 165,000 dalton protein was mixed with either light meromyosin or subfragment-1.

More recently, Masaki et al. (93) have reported the binding of I^{125}-labelled 165,000 dalton protein to myosin, and found that the M-

protein bound to myosin in a 2:1 mole ratio at most. This ratio decreased as myosin was allowed to aggregate at low ionic strength prior to incubation of myosin with M-protein. Fluorescent antibody techniques in combination with electron microscopy seemed to indicate that the binding of M-protein was to specific sites on the light meromyosin portion of myosin.

3. Glycogen Phosphorylase b

An examination of the literature available on the M-band proteins discloses numerous reports of a protein of about 100,000 daltons as being present along with either creatine kinase, the 165,000 dalton protein, or both. It was noted by Masaki et al. (85, 86), as well as Eaton and Pepe (94) along with what was later identified as creatine kinase (21). Other workers who have also reported its presence in their M-band preparations include Eaton and Mochan (95), Landon and Oriol (87), Mani and Kay (82) and Herasymowych et al. (83).

Trinick and Lowey (89) isolated and characterized this protein as being glycogen phosphorylase (molecular weight 90,000 daltons). They produced antibodies to the purified protein but were unable to demonstrate binding to the myofibril. This finding seemed to imply that this component may not have its origin in the M-band. Heizmann and Eppenberger (25) recently verified that this protein is indeed phosphorylase b from chicken muscle, and showed that it is immunologically identical with the putative M-band protein termed protein A of Eaton and Pepe (94) or the component II of Masaki and Takaiti (86). More importantly, localization studies by means of indirect immunofluorescence revealed that though the phosphorylase was located mainly in the I-band, it was present also in the H-zone where the M-band is. They concluded that phosphorylase is not

exclusively a M-band protein since it is bound to additional proteins in the thin filaments.

These results suggest that glycogen phosphorylase "may" be a component in the M-band, since it appears firmly attached to the myofibrillar structure. Possibly, it may perform a role in mixed enzymatic and structural functions.

D. THE M-BAND STRUCTURE: THE ASSIGNMENT OF THE COMPONENTS TO THE KNAPPEIS-CARLSEN MODEL

Only one publication to date has endeavoured to integrate the biochemistry of the M-band proteins and their localization studies into the model proposed by Knappeis and Carlsen. In 1975, Walliman, Turner and Eppenberger (96) assembled all the available data at that time and assigned the role of the M-bridge to creatine kinase, and the role of the M-filament to the protein A of Eaton and Pepe (94) (the 100,000 dalton protein). In view of most of the literature presently available on the M-band proteins, this assignation is only half correct; data on creatine kinase still supports its assignation as the M-bridge, while the M-filament is now most likely to be the 165,000 dalton component.

These assignations are in accord with the criterion required by the Knappeis-Carlsen model (see Figure 5a): the M-bridges and M-filaments must be able to bind each other, while M-bridges must be able to bind to myosin.

Creatine kinase has been shown to bind to myosin filaments by Morimoto and Harrington (75) in addition to displaying interaction with myosin in ATPase studies (69 - 71, 82, 83, 96) and in circular dichroism studies (82). These results all tend to implicate the head region of

myosin as at or near the site of interaction, in conflict with the study of Houk and Putnam (80) who found binding to the rod portion. This interaction, at or near the heads, is difficult to reconcile, since the M-band region is supposedly devoid of "heads". Nonetheless, these studies are evidence that creatine kinase does interact with the thick filament and hence fulfills the requirements of the M-bridge.

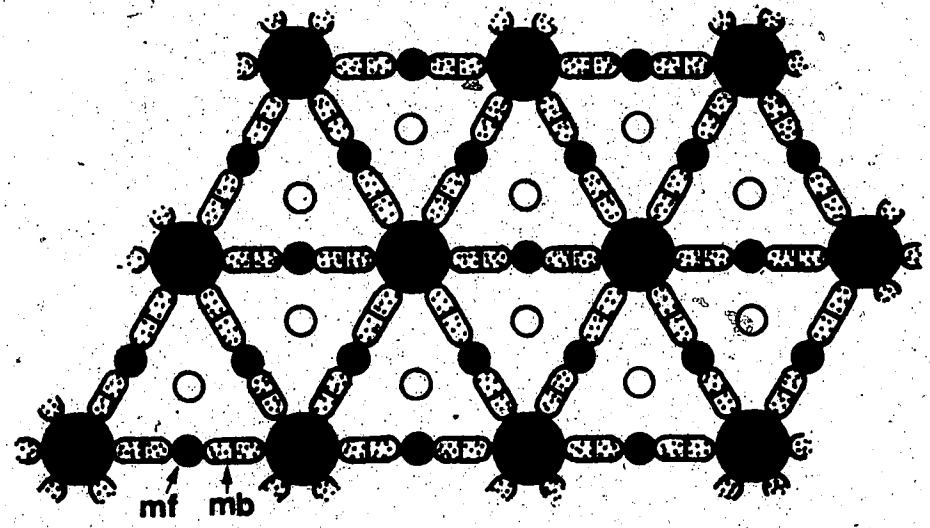
With respect to the role of the M-filament, Walliman had assigned it to the 100,000 dalton component with what was then evidence of it forming large aggregates as well as binding to CPK (94). This 100,000 dalton component has since been identified as glycogen phosphorylase b (25, 90) and shown to bind to the M-band, though not exclusively (25). Further resolution of proteins from M-band extracts have uncovered the 165,000 dalton component which has been shown to undergo aggregation under certain conditions by Mani and Kay (91) and Masaki and Takaiti (86), unlike creatine kinase which remains monomeric (75). This tendency to aggregate would be deemed useful in forming the M-filament structure. Mani and Kay (91) have also demonstrated that the 165,000 dalton component inhibits the enzymatic activity of creatine kinase, and forms a complex with it as shown in sedimentation equilibrium experiments. These results thus tend to support the view that the 165,000 dalton is the most probable candidate for the role of the M-filament.

The model as visualized by Walliman et al. (96) is illustrated in Figure 5, showing a schematic cross-section of the M-band. The dimensions indicated are for the diameters of the thick and thin filaments as determined from electron microscopy (19, 75), in addition to the centre to centre spacing of the thick filaments (75). A comparison of the hydrodynamic data for creatine kinase as determined by Morimoto and

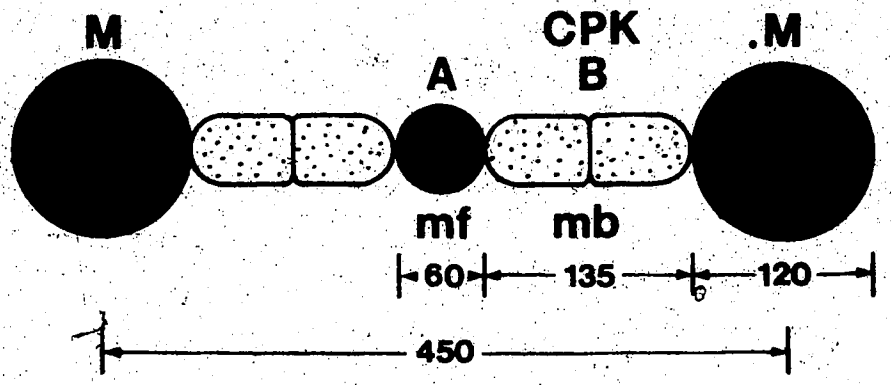
Figure 5a. The Knappeis-Carlsen model of the M-band of striated muscle. The diagram displays a schematic cross-section through the M-band. Large solid circles = A-filaments; small solid circles = M-filaments; open circles = projection of I-filaments to level of the M-region; dotted bars = M-bridges. (Modified by Walliman et al. (96).)

Figure 5b. The integration of the biochemistry of the M-proteins into the Knappeis-Carlsen model according to Walliman, Turner and Eppenberger (96). mb = M-bridge; mf = M-filaments. The Knappeis-Carlsen model is modified to show the mb to be the CPK and the M-filament as being the B-component of Eaton and Pepe (94) (now presumed to be the 165,000 dalton component).

a.



b.



Harrington fits in well with this model. Their calculations indicated CPK to be an equivalent ellipsoid with length 150 Å, and a minor axis of 43 Å. Knappeis and Carlsen (19) have indicated the M-bridge to be 110 - 135 Å for resting muscle.

The role of the glycogen phosphorylase or 100,000 dalton protein could conceivably be visualized as a third M-band component when one considers the Luther-Squire model (see Figure 4b) which has the additional feature of a secondary M-bridge (M3). This M3 protein is proposed to bind to the M-filament, and this is in accord with the results of Masaki and Takaiti (86) who were able to demonstrate association of their M-protein (the 165,000 dalton component) to component II, subsequently identified as phosphorylase.

This model, therefore, with creatine kinase acting as the M-bridge and the 165,000 dalton component acting as the M-filament, takes into account all the relevant information on the M-band structure with one exception: the binding affinity of the 165,000 dalton component to myosin. Masaki and Takaiti (86) found this property to hamper their preparative procedures, since once bound to myosin, the M-protein became difficult to wash out. Mani and Kay (92) have suggested the site of this interaction on myosin as being on the heavy meromyosin subfragment-2 portion. The model as it now stands does not predict M-filaments to interact with myosin. However, it must be kept in mind that the model is static. Perhaps as Mani and Kay (92) have suggested the subfragment-2 "hinge" portion of myosin becomes involved to accommodate the variable interfilament distance during muscle contraction.

E. AIMS OF THIS PROJECT

It would appear from the preceding discussion that in skeletal muscle, creatine kinase and the 165,000 dalton protein have been proven to be integral members of the M-band structure. Models proposed for the M-band are such that both these proteins may be incorporated, and in the case of the Luther-Squire model, can even be used to accommodate the third candidate for the M-band, phosphorylase. The main goal in this project was to determine if an analogous situation occurs within the M-band of mammalian cardiac systems. The choice of bovine cardiac muscle as a source was encouraged by Eplings' micrographs on bovine cardiac myofibrils (97) which show a definitive M-band, as well as Ottaway's finding that bovine cardiac myofibrils contain creatine kinase (62). Skeletal M-band methodology was thus applied to bovine heart muscle. Homogeneous creatine kinase was isolated, characterized and identified. In addition, this creatine kinase from the myofibril compartment was compared with the cytoplasmic enzyme to see if there were any differences. Immunological and electron microscopy techniques were applied to see if the myofibrillar creatine kinase isolated by this method was an integral element of the M-band. Furthermore, since localization of a protein is not enough to identify it as a structural component, reconstitution studies for both the bovine cardiac and rabbit skeletal systems were performed. A positive result from these studies would seem to unequivocably determine the structural function of such proteins. Finally, interaction studies of the myofibrillar creatine kinase with other contractile components were undertaken to assist in elucidating the possible role of the M-band creatine kinase. It was hoped that the integration of all the biochemical data together with electron microscopy would give further

insight into the M-band structure of cardiac striated muscle.

CHAPTER II

EXPERIMENTAL METHODS

A. GEL ELECTROPHORESIS

1. Polyacrylamide Gel Electrophoresis

Disc gel electrophoresis experiments were performed for purposes of determining homogeneity as well as isozyme characteristics of creatine kinase. The method employed was that described by Schaub and Perry (99). The buffer system consisted of a solution of 25 mM Tris, 160 mM glycine at a pH of 8.38. Complete separation of different isozymes was achieved by applying a current of 2.5 mA per gel for one hour.

2. SDS-Polyacrylamide Gel Electrophoresis

SDS-Polyacrylamide gel electrophoresis was performed in accordance with the methodology of Shapiro et al. (100). Samples were prepared in a 2% SDS, 1 mM DTT medium and heated in a boiling water bath for 5 - 10 min. Subsequent electrophoresis on 10% polyacrylamide gels was carried out for 3 hours at 8 mA per gel. Gels were stained with Coomassie Brilliant Blue for 1 hour, and then allowed to destain overnight in 2 ℓ of 7% acetic acid, 7.5% methanol (v/v). Molecular weight determinations were estimated by comparing the migration distance of the protein under study with those of proteins of known molecular weights according to the method of Weber and Osborn (101).

B. AMINO ACID ANALYSIS

Amino acid analyses were performed on either a Beckman Model 120C or a Durrum D500 automated amino acid analyzer.

Freeze-dried samples were hydrolyzed in fired test tubes contain-

ing constant boiling 6 N HCl, with 0.1% phenol added to preserve the integrity of the tyrosine residues. Tubes were evacuated, sealed and incubated for 24, 48 and 72 h at 110°C. Threonine and serine contents were estimated by extrapolation to zero time. Valine and isoleucine contents were determined from 72 h hydrolyses. Tryptophan content was established using the method of Goodwin and Morton (102). The spectrum of the protein was measured in 0.1 N alkali and the mole ratio of tyrosine to tryptophan was determined using the following equations which relate the moles of tyrosine, M_{Tyr}, and tryptophan, M_{Trp}, present at wavelengths of 294.4 nm and 280.0 nm, using respective absorbance values A.

$$M_{Tyr} = 10^{-3} (0.592\ A_{294.4} - 0.263\ A_{280.0}) \qquad [2]$$

$$M_{Trp} = 10^{-3} (0.263\ A_{280.0} - 0.170\ A_{294.4}) \qquad [3]$$

Dividing equation [2] by [3] gives an equation giving the mole ratio of tyrosine to tryptophan. The total cysteine plus cystine content was determined as cysteic acid following performic acid oxidation of the protein sample according to Moore (103). In order to estimate the number of sulfhydryl groups, the DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) coupling method was used (104). "Free" SH groups in the native molecule as well as "buried" groups were measured; in the latter case 6 M guanidine hydrochloride was employed as the solvent. The difference between the total cysteic acid content from performic oxidation and the total thiol content gave an estimate of the number of disulfide bonds present.

C. OPTICAL METHODS

1. Absorption Spectrophotometry

A Gilford 240 Spectrophotometer was used for routine absorbance

measurements in monitoring effluents from chromatography columns. UV absorbance spectra were determined using a Cary 118C recording spectrophotometer.

2. Circular Dichroism Measurements

Circular dichroism spectra were obtained using a Cary model 6001 CD attachment to a Cary 60 recording spectropolarimeter according to the procedure described by Oikawa et al. (105). Calibration of the instrument was done with an aqueous solution of recrystallized d-10-camphor sulfonic acid. Constant nitrogen flushing was employed. Cells of 0.5 mm pathlength were routinely employed in the 200 - 250 nm wavelength region using approximately 0.06% protein solutions. For the near UV region (250 - 320 nm), 1 cm cells were used with approximately 0.2% protein concentration.

The mean residue ellipticity $[\theta]_\lambda$, at a particular wavelength λ, was calculated from the equation

$$[\theta]_\lambda = \frac{\theta_{obs} M}{100 \ell c} \qquad [4]$$

where:

M is the mean residue weight (taken to be 115 in these studies),

θ_{obs} is the observed ellipticity value at the wavelength of interest,

ℓ is the cell pathlength in dm, and

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

The units of $[\theta]_\lambda$ are $degree \cdot cm^2 \cdot dmole^{-1}$.

Apparent α-helix and β-conformation values were estimated using the parameters and equations given by Chen et al. (106). Calculations to determine the fraction of α-helix, f_α, the fraction of β-conformation, f_β (which contains parallel and antiparallel β-sheets as well as β turns),

and fraction of random coil structure, f_R, required the use of ellipticity values at 210, 215, 220 and 225 nm. Solutions of simultaneous equations integrating these ellipticity values were then averaged to give the α-helical and β-conformation contents.

3. Spectrophotometric Titration

The addition of base to a protein solution usually results in a change in the absorption spectrum of the native protein. This change with increasing pH is due to the ionization of phenolic groups of tyrosine residues to form phenolate ions. If spectral changes are determined as difference spectra, ionized tyrosine exhibits a maximum at 295 nm with a molar extinction coefficient of about 2300 (107). A plot of the difference in absorbance ΔA at 295 nm versus pH thus gives the ionization curve of tyrosine residues in the molecule. The pK for ionization of tyrosines accessible to the solvent is usually 10. For tyrosine completely or partially buried within a protein and inaccessible to the solvent, the pK value is higher and tyrosine ionization may exhibit time dependence.

The number of tyrosines ionized was estimated from the increase in absorbance at 295 nm at any pH value using the following equation:

$$n = \frac{\Delta A}{\Delta \epsilon} \times c \qquad [5]$$

where:

c represents the protein concentration,

ΔA is the absorbance difference at 295 nm,

Δε denotes the difference in molar extinction coefficient at 295 nm between ionized and unionized tyrosine and was taken as 2305 (108).

Spectrophotometric titrations were performed using a Gilford 240

spectrophotometer and the solvent system used was 0.5 M KCl, 2 mM potassium phosphate, pH 7. One centimeter quartz cells of 1 ml volume capacity were employed. Samples (3.0 ml) were titrated manually with standardized KOH in a constantly stirred vessel maintained at 25°C. Following the addition of base, an aliquot was removed and the absorbance of the solution measured at 295 nm. The pH of the solution was determined in a Radiometer 64 pH meter after the aliquot was replaced in the titration vessel. Care was exercised to ensure that the protein solution was at equilibrium.

A computer program was used to perform a nonlinear curve fitting iterative procedure which fitted the data for an equation of the form:

$$\Delta A_{295nm} = \sum_{i=1,2} \frac{n_i K_{OH,i} [OH^-]}{1 + K_{OH,i} [OH^-]} \qquad [6]$$

where $pH = 14 - pOH$ [7]

derived from the Henderson and Hasselback equation. In this case, n refers to the relative contribution of each class of tyrosines to the total absorbance:

$$n_1 + n_2 = \text{total } \Delta A_{295nm} \qquad [8]$$

D. CHROMATOGRAPHIC TECHNIQUES

1. Ion Exchange Chromatography

Ion exchange chromatography on DEAE Cellulose DE32 was carried out as recommended in the Whatman literature.

All column chromatography was performed at 4°C. Constant flow rates were maintained by L.K.B. peristaltic pumps. Effluent was collected by a Buchler Fractomat automatic fraction collector, usually in

5 - 6 ml fractions, and the effluent monitored routinely at 280 nm.

Solution conductivities were measured on a Radiometer type CDM 2d conductivity meter. Effluent pH readings were checked using a Radiometer 62 or 64 pH meter equipped with temperature compensator and combination glass electrode.

2. Gel Filtration Chromatography

Gel filtration studies were performed using Biogel A 0.5 m (Bio-rad) packed in a (1.7 x 100) cm column. The equilibrating buffer system consisted of 0.5 M KCl, 2 mM potassium phosphate, pH 7.

Samples of myosin (10 mg) and creatine kinase (2 mg) in a total volume of not more than 1 ml were applied both separately and together on the column. Elution was monitored by taking the absorbance of 1 ml fractions at 280 nm. Chromatograms featured a profile of absorbance versus volume of effluent.

3. Affinity Chromatography

The preparation of an affinity column for purification of chicken antibodies to creatine kinase was performed as suggested by the supplier, Pharmacia. The CNBr-activated resin, Sepharose 4B, (3.0 g) was swelled in 1 mM HCl, and coupled to approximately 40 mg of creatine kinase in 15 ml of 0.1 M $NaHCO_3$, 0.5 M NaCl solution. The reaction was allowed to proceed 2 h with continuous end-over-end mixing. The reaction was then terminated by washing the resin on a glass sintered funnel with 10 ml of the coupling buffer. The absorbance of the filtrates at 230 and 280 nm were determined and the resin was then further washed with 200 ml of the buffer. Unreacted groups in the resin were deactivated by the addition of 7.5 ml ethanolamine, pH 8, for 1.5 h. Non-covalently bound proteins were removed by washing the resin first with 1 M NaCl,

0.1 M sodium acetate, pH 4, and then following with 1 M NaCl, 0.1 M sodium borate, pH 8, using a total of three alternating cycles. A rough quantitative estimate of the amount of coupling could be determined from the differences of the absorbance at 230 and 280 nm of the protein solutions and filtrates.

The resin was packed into a column 0.8 x 9 cm and the column equilibrated with phosphate buffered saline (PBS), pH 7.2, before plasma from chicken (diluted 1:1 with PBS) was applied. Following application of the plasma, the column was further flushed with PBS, and then 4 M guanidine hydrochloride was applied to elute the antibodies. PBS was again put through the column for a final equilibration prior to storage at 4°C. Minimized exposure to guanidine hydrochloride ensured the use of the same column for 3 - 4 preparations. All experiments were performed at 4°C using a flow rate of 3.5 ml/h maintained by an L.K.B. peristaltic pump.

E. BIOLOGICAL ACTIVITY STUDIES

1. Determination of Creatine Kinase Activity

Creatine kinase enzymatic activity was determined according to Eppenberger et al. (77), where 1 unit of creatine kinase activity is defined as the amount necessary to catalyze formation of 1 µmole of ATP per minute. The reaction mixture consisted of 0.15 mM NADP, 3.3 mM $MgCl_2$, 3.3 mM glucose, 0.5 mM ADP, 8 mM creatine phosphate, and approximately 10 µg each of hexokinase and glucose-6-phosphate dehydrogenase (both from Sigma). The creatine kinase concentration was usually in the range of 2 - 5 µg, depending on the freshness of the preparation. The reaction was initiated by the addition of either of two substrates,

creatine phosphate or ADP, to stock solutions of the above components made up in 0.1 M Tris-Cl, pH 7.5, in a final volume of 1 ml. The reaction was monitored by the increase in absorption at 340 nm due to the production of reduced NADPH using an extinction coefficient of 6.3×10^6 cm^2 $mole^{-1}$.

2. Interaction Studies with Cardiac Myosin and Its Subfragments

In order that the effect of creatine kinase on the ATPase of myosin and its subfragments could be examined, cardiac myosin and subfragments thereof produced by controlled enzymatic digestion were prepared.

Myosin from bovine cardiac muscle was isolated according to the procedure of Wolodko and Kay (109, 110) with one important modification. Following batch DEAE cellulose (DE-32) treatment, the cardiac myosin was precipitated and then dissolved up in 0.2 M KCl only. This prevented the carry-over of a large white aggregated contaminant which dissolves at higher than 0.2 M ionic strength. Myosins thus produced from bovine cardiac muscle showed a high degree of purity when examined in sedimentation velocity experiments as well as displayed relatively high ATPase activities of 0.2 - 0.3 µmole P_i per min per mg, as determined at 25°C. The absorbance ratio of 280 nm to 260 nm was routinely measured and generally found to be in the range of 1.74 - 1.76.

Myosin subfragments were prepared according to Lowey et al. (111). Heavy meromyosin (HMM) from bovine cardiac muscle was prepared by the use of tryptic digestion using 1 mg of trypsin to 100 mg of myosin and allowing the reaction to proceed for 20 min at room temperature. The HMM was purified by ammonium sulphate fractionation of the supernatant from the initial digestion mixture, while the initial precipitate was

further treated with 75% ethanol (v/v) and recovered to yield light meromyosin (LMM). The HMM thus prepared was checked for homogeneity in the ultracentrifuge where it sedimented as a single peak around 6S, as well as by determining its specific activity. Usually the value was approximately twice the ATPase specific activity of the parent myosin.

Heavy meromyosin subfragment-1 was prepared by the use of a papain digestion procedure. 2 mg of papain (in a deactivated form from Sigma) was activated by dialysis versus 5 mM cysteine, 2 mM EDTA, pH 5.9, prior to digestion with 200 mg of cardiac myosin for 15 min at room temperature. The reaction was terminated by the addition of 1 mM iodoacetic acid, 2 mM cysteine and the mixture dialyzed versus 50 mM Tris, pH 8, before being applied on a DEAE cellulose DE-32 column equilibrated against the same buffer. Subfragment-1 was eluted by the use of a linear gradient of 0 - 0.4 M NaCl, 1000 ml. Purification was verified by SDS polyacrylamide gel electrophoresis (which gave a molecular weight of 120,000) and by ultracentrifugation (which sedimented as a single peak in the ultracentrifuge with a sedimentation coefficient around 5S). The relative ATPase specific activity for subfragment-1 was usually found to be roughly four-fold the specific activity of the parent myosin.

Assays of ATPase activities of myosin, heavy meromyosin and heavy meromyosin subfragment-1 were carried out by electrometric titration following proton liberation accompanying ATPase hydrolysis in the pH stat using a Radiometer TTI, equipped with a titrator and a titragraph. In addition, the method of Fiske and Subbarow (113) was employed. The latter method required monitoring the liberation of inorganic phosphate ions released on hydrolysis of ATP.

The reaction mixture consisted of 0.5 M KCl, 10 mM $CaCl_2$, 1 mM

glutathione (reduced form) and 2.5 mM ATP, pH 8.0.

The extinction coefficients $E^{1\%}_{1cm}$ at 280 nm used were: for cardiac myosin, 6.06; for HMM, 6.4; for LMM, 3.7; and for subfragment-1, 8.2 (110,112).

3. Interaction Studies with the 165,000 Dalton Protein of Rabbit Skeletal Muscle

The 165,000 dalton component of rabbit skeletal muscle was prepared according to the methodology employed by Mani and Kay (91). The procedure was essentially that used in this study in extracting creatine kinase from the myofibril. Rabbit skeletal muscle was minced in a Waring blender for 1 min and washed several times with 0.1 M KCl, 20 mM potassium phosphate, pH 7. The mince was then allowed to extract overnight in 5 mM Tris, pH 8, following vigorous stirring for 2 hours. The supernatant was recovered and subjected to pH 5 precipitation. The suspension was centrifuged off, and the resulting supernatant further precipitated with ammonium sulfate to 90% saturation in the presence of 10 mM EDTA at pH 7. The final precipitate was dissolved in 50 mM Tris, pH 8, and following dialysis against this buffer, applied to a DEAE cellulose column. Elution was accomplished by a 1000 ml, 0 to 0.2 M NaCl gradient. The 165,000 dalton protein was found to elute at approximately 0.15 M NaCl. To calculate the concentration of protein, a value of $E^{1\%}_{1cm}$ at 280 nm of 12 was employed. Purity was verified by the presence of a single band in SDS-polyacrylamide gels. The 165,000 dalton component was then added at differing mole ratios to creatine kinase and the activity of the CPK was monitored as usual.

F. ULTRACENTRIFUGE STUDIES

Ultracentrifuge studies were routinely performed at 5°C in a Beckman Spinco Model E ultracentrifuge equipped with a photoelectric scanner, multiplex accessory and high intensity light source. The Rayleigh interference optical system was also employed. Low speed sedimentation equilibrium runs were performed according to Chervenka (114).

1. Determination of Protein Extinction Coefficients

The extinction coefficient of a 1% solution of a protein in a 1 cm pathlength cell at 280 nm, ($E^{1\%}_{1cm,280}$) was determined by the method of Babul and Stellwagon (115), using ultracentrifugal synthetic boundary runs with Rayleigh interference optics. An average refractive increment of 4.1 fringes/mg/ml was used to correlate the number of fringes on a photographic plate as measured by a Nikon 6C microcomparater to the absorbance value of a protein solution at 280 nm. In this way, a direct estimate of protein concentration could be determined.

The total protein concentration of supernatants and precipitates at particular stages of the preparation procedure was determined by fringe counts. The values thus obtained were used to express enzymatic activity in terms of per mg total protein, and in this manner; progressive purification of creatine kinase could be monitored.

2. Sedimentation Velocity Experiments

Sedimentation velocity studies were carried out employing the Schlieren optical system with a rotor velocity of 60,000 rev/min and a temperature of 20°C. Photographs were taken at specific time intervals and a Nikon model 6C microcomparater was used to measure the distance from the maximum ordinate of the sedimenting peak to the reference hole.

The equation relating the sedimentation coefficient S (in sec)

to the distance from the centre of rotation to the maximum ordinate (in cm) at time t (sec) and the angular velocity ω (in rad/sec) is given by:

$$S = \frac{1}{\omega^2}\left(\frac{d \ln r}{dt}\right) \qquad [9]$$

Values of S were determined by plotting the natural logarithm of the measured r values versus t and multiplying the resultant slope by $1/\omega^2$. The sedimentation coefficient interval of water at 20°C, $S_{20,w}$, is thus given by the equation

$$S_{20,w} = (S)\left(\frac{\eta_T}{\eta_{20}}\right)_w \left(\frac{\eta}{\eta_o}\right)_T \left(\frac{1 - \bar{v}\,\rho_{20,w}}{1 - \bar{v}\,\rho_T}\right) \qquad [10]$$

where:

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

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

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

ρ_T is the density of the solvent at temperature T, and

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

The value of $\bar{v}$ was estimated from the amino acid composition and taken to be 0.73 (116). Density and viscosity values were taken from the Handbook of Chemistry and Physics.

Intrinsic sedimentation coefficients $S^o_{20,w}$ were determined by plotting $S_{20,w}$ against the initial protein concentration of the sample and extrapolating the results to zero concentration. An equation of the straight line of this plot was fitted by the method of least squares to be given by:

$$S_{20,w} = S^{o}_{20,w} - kc \qquad [11]$$

where:

c is the protein concentration in g/100 ml,

k is the slope of the line and increases in magnitude with increasing protein asymmetry,

$S^{o}_{20,w}$ is the intrinsic sedimentation coefficient at 20°C, and

$S_{20,w}$ is the sedimentation coefficient at a given protein concentration.

3. Sedimentation Equilibrium Experiments

Conventional sedimentation runs were performed on a Beckman Model E ultracentrifuge according to Chervenka (114). Data were recorded by means of either photographic plates using the Rayleigh interference optical system or on chart paper of a photoelectric scanner accessory using UV absorption optics.

The apparent weight average molecular weight M_w of a homogeneous protein is given by the equation:

$$M_w = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \frac{d \ln c}{dr^2} \qquad [12]$$

where:

R is the Universal Gas Constant,

T is the experimental temperature in °K,

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

ρ is the solvent density,

ω is the angular velocity, and

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

In the case where Rayleigh interference optics were employed,

protein concentrations at the meniscus c_m were calculated according to:

$$c_m = c_o - \frac{r_b^2 (c_b - c_m) - \int_{c_m}^{c_b} r^2 \, dc}{r_b^2 - r_m^2} \qquad [13]$$

where:

c_m is the protein concentration at the meniscus,

c_o is the initial concentration of the protein,

r_m is the distance from the meniscus to the axis of rotation,

r_b is the distance from the cell bottom to the axis of rotation,

c_b is the protein concentration at the cell bottom, and

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

A direct measure of protein concentration was determined by adding the fringe displacement at point r to the concentration at the meniscus in terms of fringes.

In the case where the photoelectric scanner was used, the absorption value A at any point along the cell gave a direct measure of protein concentration.

A plot of the natural logarithm of the protein concentration (expressed as either Y, the number of fringes, or by A, absorbance units) versus r^2 yielded a straight line whose slope d ln c/dr^2 was used to determine the weight average molecular weight at any position along the cell by application of equation [12].

G. ELECTRON MICROSCOPY AND IMMUNOBIOLOGICAL STUDIES

Longitudinal sections 60 - 120 nm thick were cut on a Sorvall Porter-Blum MT2-B ultra-microtone knife.

Electron microscopy was performed on a Philips EM 300 electron

microscope operating at 60 kv and using a 30 μm objective aperture. A cold finger was used to minimize contamination.

The measurement of antibody levels by radioimmunoassay was determined using a Packard γ-counter (model 3002).

Further details on preparation of myofibrils and antibody studies are presented in Chapter V.

CHAPTER III

ISOLATION AND CHARACTERIZATION OF CREATINE KINASE

Creatine kinase from bovine cardiac muscle was prepared using two methods. The first of these was a procedure designed to release the enzyme bound to the myofibril, referred to as myofibrillar creatine kinase. The second procedure involves the extraction of cytoplasmic creatine kinase. Both of these enzymes underwent the same purification procedure in order that an estimate of their relative amounts in bovine cardiac muscle could be determined. In addition, both enzymes were simultaneously characterized with respect to their physical characteristics to determine whether or not they are the same enzyme, and to establish any possible differences.

A. MYOFIBRILLAR CREATINE KINASE

1. Isolation

The method of preparation of creatine kinase from the myofibrils of bovine cardiac muscle was essentially that devised by Morimoto and Harrington (75) who originally designed their experiments for chicken skeletal muscle. Two kilogram quantities of fresh or freshly frozen hearts from which all membranes and fat had been excised were processed for each preparation. In general, frozen beef hearts were preferred, since lysis of mitochondria and cells greatly facilitated the ease of washing away proteins not directly bound to the myofibril. The hearts were cut into small pieces and minced for 1 min in a Waring blender with a 3:1 volume of 0.1 M KCl, 20 mM potassium phosphate, pH 7, buffer at 4°C. A total final volume of 7 litres for the resulting suspension was

divided into two batches. Each batch was then centrifuged in a PR-6 IEC centrifuge at 2400 rpm for 10 min, the supernatant discarded and the mince washed repeatedly with the KCl/phosphate buffer. Washing was continued until the supernatant showed an absorbance of less than 0.05 at 280 nm, and no precipitate was detectable upon the addition of a few drops of 10% TCA (trichloroacetic acid) to a ml of the supernatant. Usually at least 10 washes were required for each batch. The washed mince was pooled and extracted with 5 ℓ of 5 mM Tris pH 8. The suspension was agitated by means of an overhead stirrer for 90 minutes (or occasionally overnight) at 4°C. The suspension was then centrifuged in a Sorval RC-3 at 5000 rpm for 10 minutes, the supernatant recovered and subjected to pH 5 precipitation with 0.1 M acetic acid. At this point most of the contaminant, tropomyosin, came out of solution and was sedimented at 5000 rpm in the RC-3. The pH was reinstated to 7 using dilute ammonium hydroxide and EDTA added to make the final concentration 10 mM. The proteins remaining in solution were precipitated by the addition of ammonium sulfate to 90% saturation, recovered following centrifugation and dialyzed versus 5 mM Tris, 0.5 mM EDTA, pH 8.

2. Purification

The dialyzed protein was then applied to a diethylaminoethyl (DEAE) cellulose DE-32 column and the protein eluted with a 0 to 0.2 M NaCl linear gradient. Figure 6 shows the elution profile from an overnight extraction in 5 mM Tris pH 8. The creatine kinase-containing fraction was identified by SDS-polyacrylamide gel electrophoresis and enzymatic activity. Examination of the SDS gel patterns of the first chromatography showed two contaminants, one of which had a molecular weight of 90,000; the other was a low molecular weight coloured protein.

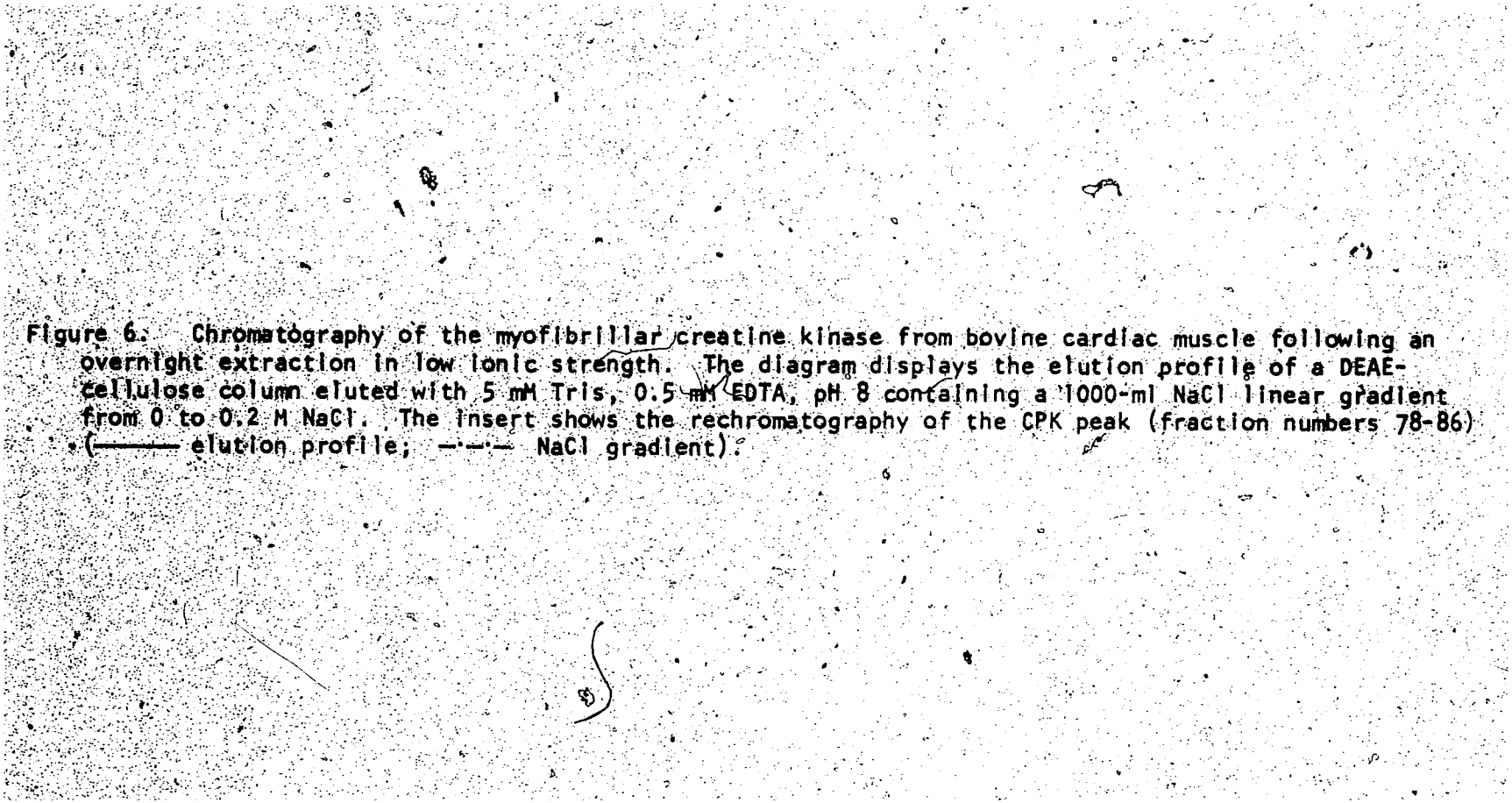
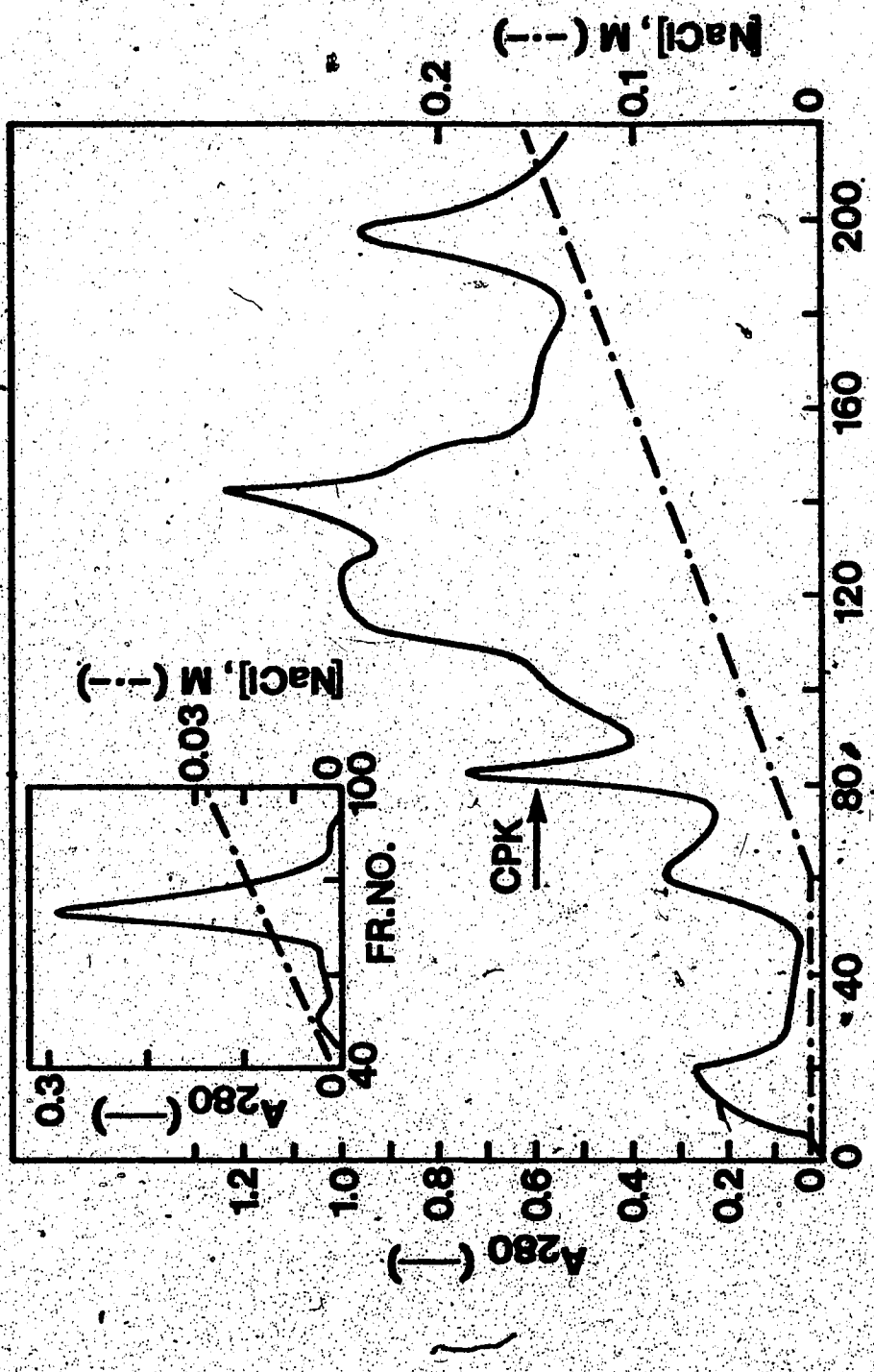


Figure 6. Chromatography of the myofibrillar creatine kinase from bovine cardiac muscle following an overnight extraction in low ionic strength. The diagram displays the elution profile of a DEAE-cellulose column eluted with 5 mM Tris, 0.5 mM EDTA, pH 8 containing a 1000-ml NaCl linear gradient from 0 to 0.2 M NaCl. The insert shows the rechromatography of the CPK peak (fraction numbers 78-86) (——— elution profile; —·—·— NaCl gradient).


[NaCl], M (---)
0
0.1
0.2
A280 (—)
0
0.2
0.4
0.6
0.8
1.0
1.2
0
40
80
120
160
200
FRACTION NUMBER
CPK
[NaCl], M (---)
0
0.03
A280 (—)
0
0.3
0
40
100
FR.NO.

Hence a second chromatography, as seen in the insert in Figure 6, was required before homogeneous creatine kinase was produced.

Figure 7 shows the elution profile of the proteins resulting from a 90 min extraction. The profile appears to have fewer contaminants then that of the overnight extraction. After directly comparing the yields, it became apparent that overnight extractions do not serve to increase the yield, but rather to extract more contaminants over the longer time period. For this reason, 90 minute extractions became the routine procedure for this step of the preparation.

B. CYTOPLASMIC CREATINE KINASE

1. Isolation

The method of preparation of the cytoplasmic creatine kinase from bovine cardiac muscle was essentially that of Eppenberger et al. (77) who had used chicken breast muscle as their source. The procedure for extraction from bovine cardiac muscle was as follows: 500 gm of fresh bovine heart were cut and minced with a Waring blender in 2 volumes of 10 mM KCl, 1 mM EDTA at 4°C for two hours, centrifuged in the Sorval RC-3 at 5000 rpm for 10 min and the supernatant recovered. At this point a modification to the procedure was applied. Isoelectric precipitation to pH 5 was performed, the supernatant recovered and readjusted to pH 7.5. To this solution, 1 mM DTT and ammonium sulfate to 70% saturation were added. The final precipitate was recovered from centrifugation and dissolved in 5 mM Tris, 0.5 mM EDTA pH 8. The protein was dialyzed against this buffer prior to being applied on a DEAE cellulose column.

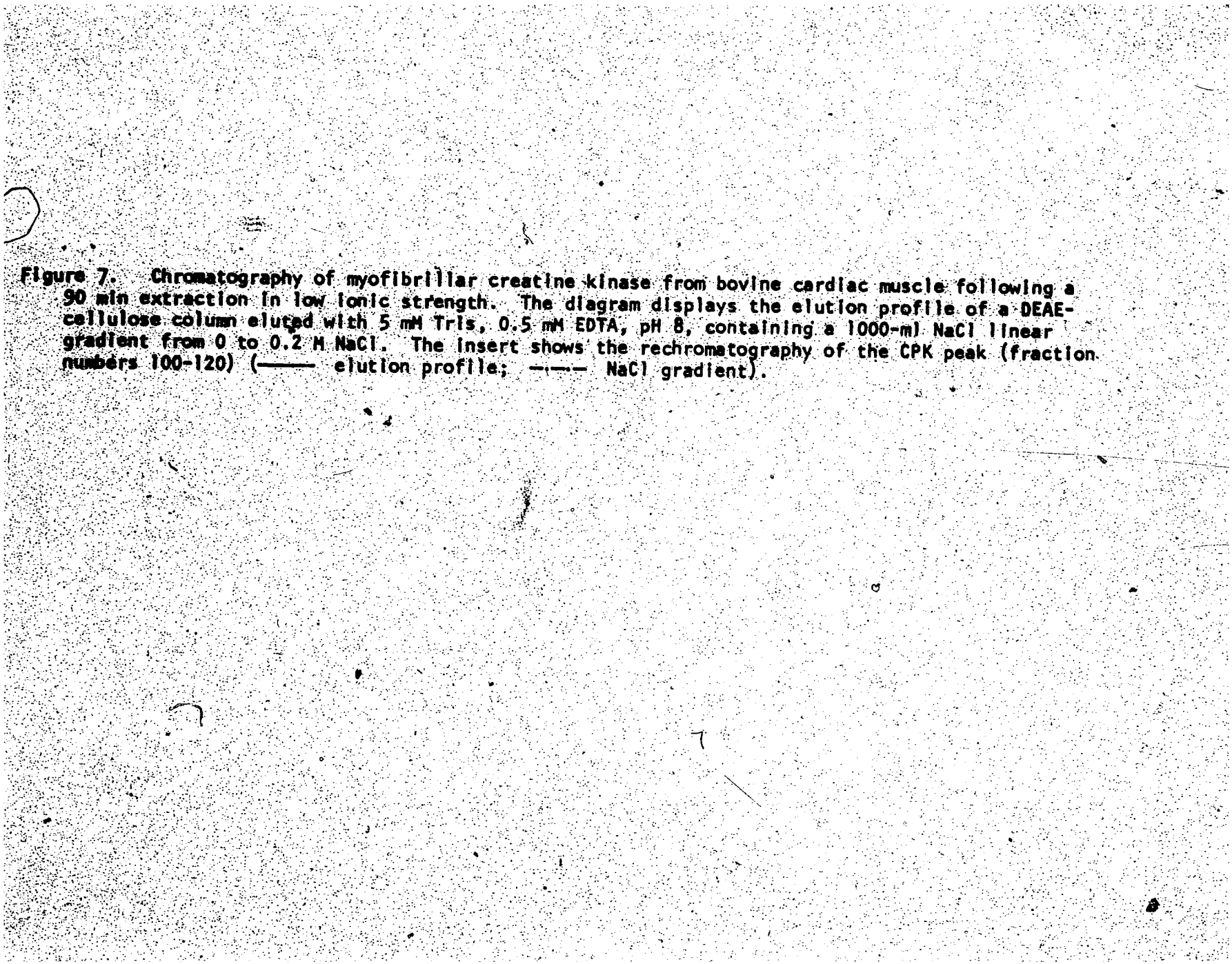
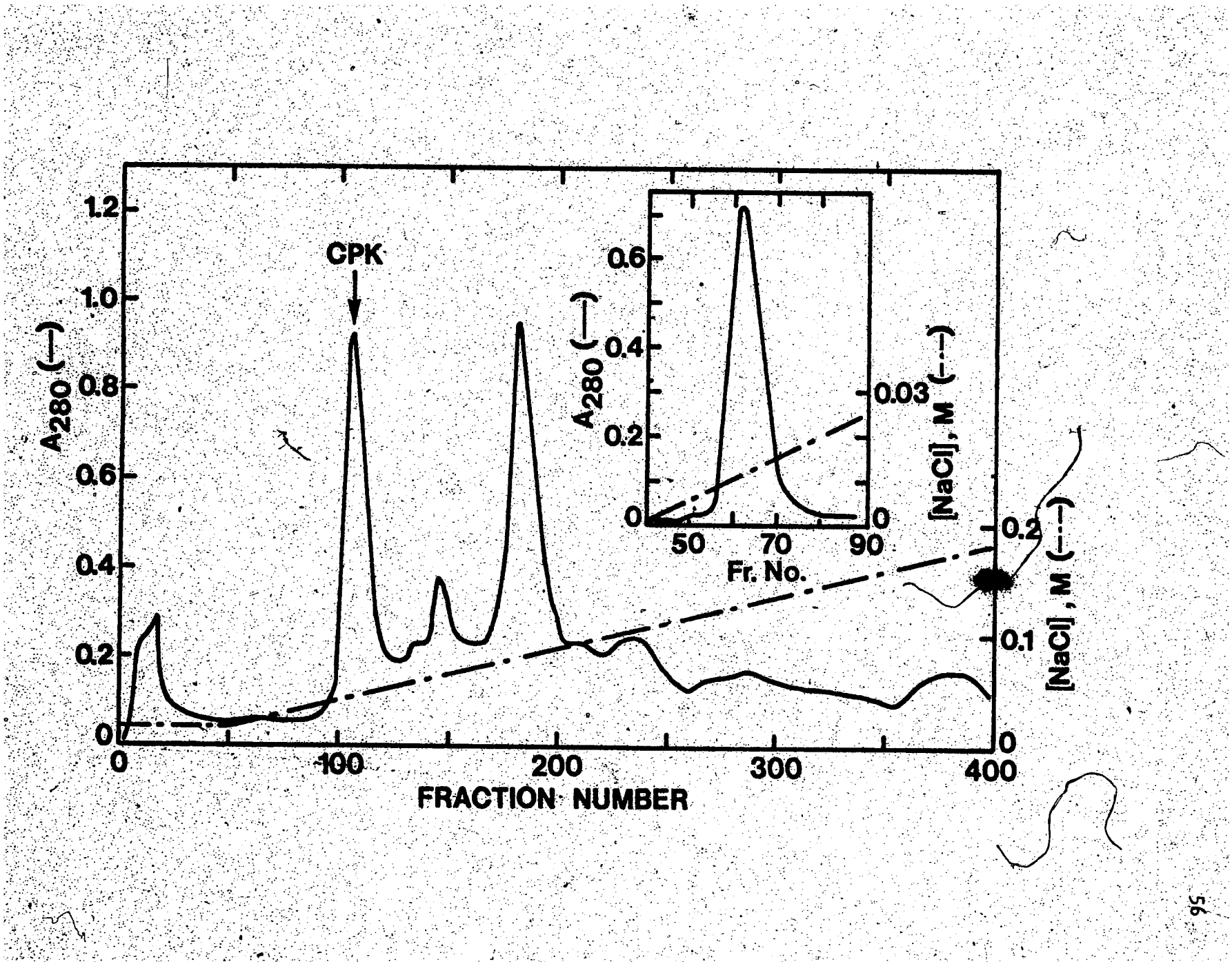


Figure 7. Chromatography of myofibrillar creatine kinase from bovine cardiac muscle following a 90 min extraction in low ionic strength. The diagram displays the elution profile of a DEAE-cellulose column eluted with 5 mM Tris, 0.5 mM EDTA, pH 8, containing a 1000-ml NaCl linear gradient from 0 to 0.2 M NaCl. The insert shows the rechromatography of the CPK peak (fraction numbers 100-120) (——— elution profile; —·—·— NaCl gradient).


CPK
A_{280} (—)
[NaCl], M (– –)
FRACTION NUMBER
Fr. No.
0
0.2
0.4
0.6
0.8
1.0
1.2
0.1
0.03
100
200
300
400
50
70
90

2. Purification

The elution profile of the DEAE cellulose (DE-32) column separating the cytoplasmic components by means of a 0 to 0.2 M NaCl linear gradient is shown in Figure 8. All of the peaks present in the elution profile are highly coloured contaminants with the exception of the cytoplasmic creatine kinase peak. This peak was the only peak possessing any appreciable creatine kinase activity concurrent with the expected pattern for creatine kinase in SDS-polyacrylamide gels. Minor contamination by a few low molecular weight proteins was removed by a second chromatography as seen in Figure 9, producing homogeneous cytoplasmic creatine kinase.

3. Relative Yield Estimation

Cytoplasmic creatine kinase is generally assumed to be the largest source of creatine kinase within the muscle cell (42). Since the purification steps were comparable, an estimate of relative yields from the myofibril and the cytoplasm could be obtained if the assumption is taken that the cytoplasmic portion is almost equal to the total amount of creatine kinase. Since the myofibrillar creatine kinase preparation gave 30 mg of purified material starting from 1 kg of wet muscle, while the cytoplasm yielded 800 mg per kg, then myofibrillar creatine kinase could be estimated to comprise at most 3 - 4% of the total creatine kinase in the heart muscle.

C. CHARACTERIZATION OF MYOFIBRILLAR AND CYTOPLASMIC CPK'S

1. Amino Acid Analysis

Comparative amino acid analysis was employed to indicate any compositional differences between the two creatine kinases. The results of

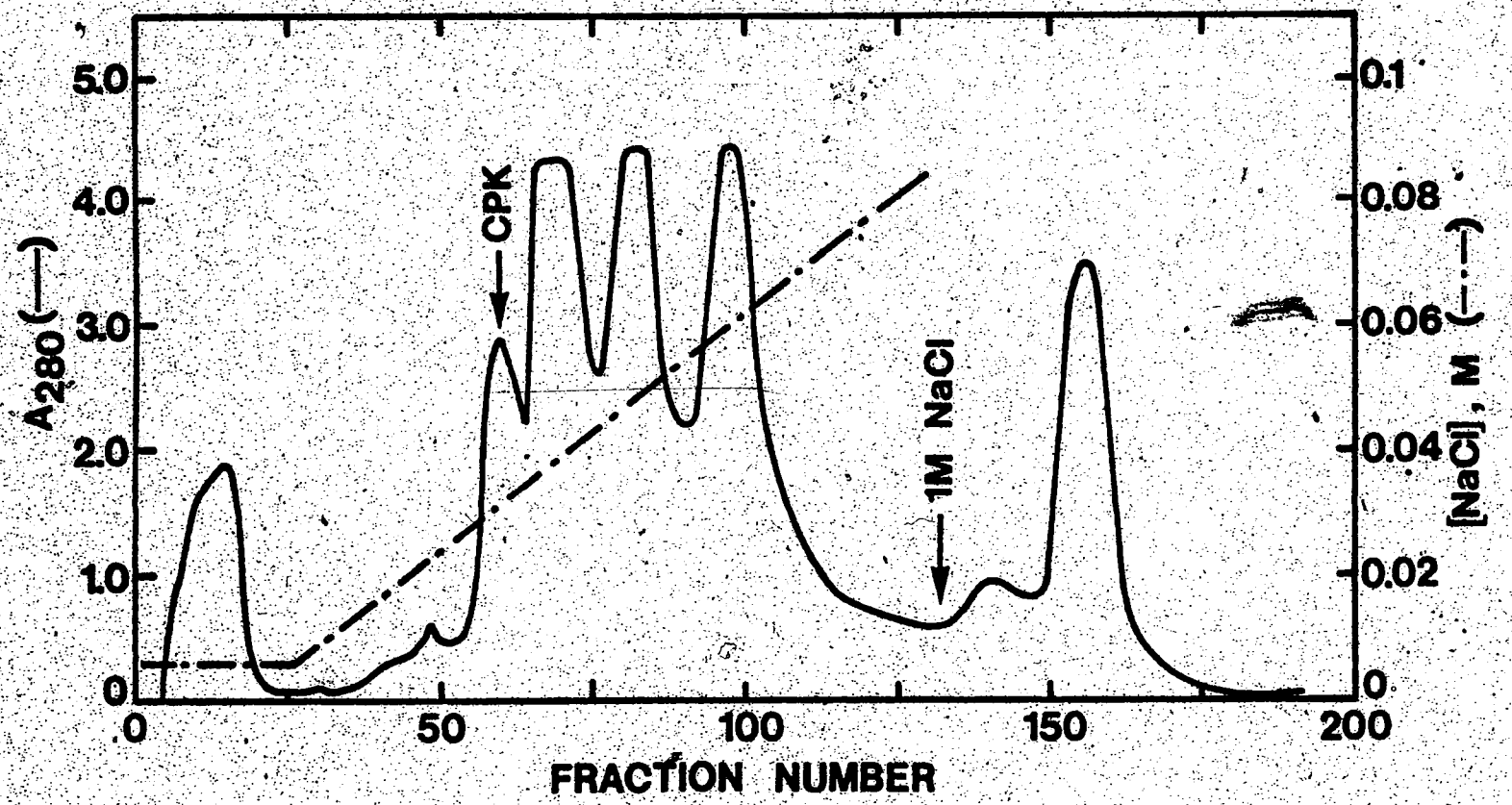


Figure 8. Chromatography of cytoplasmic creatine kinase from bovine cardiac muscle. The diagram displays the elution profile of a DEAE cellulose column eluted with a 1000-ml linear gradient from 0 to 0.2 M NaCl (——— elution profile; -·-·- NaCl gradient).

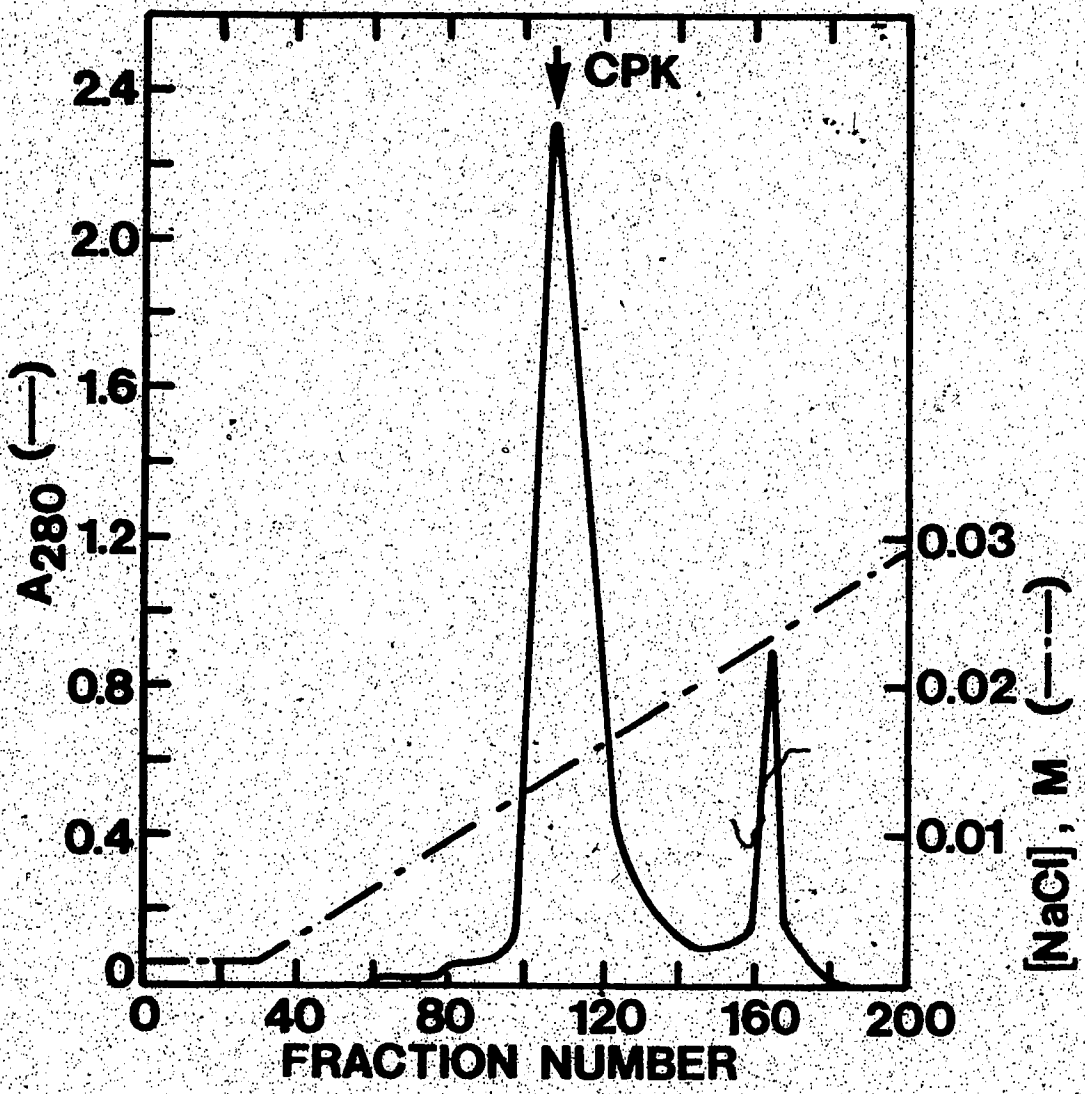


Figure 9. Rechromatography of cytoplasmic creatine kinase from bovine cardiac muscle. The diagram displays the elution profile of a DEAE cellulose column to which fractions 58-62 were applied. The CPK was eluted with a 1000-ml linear gradient from 0 to 0.1 M NaCl (——— elution profile; —·—·— NaCl gradient).

this study, along with published data, are summarized in Table I and are expressed in terms of the number of amino acid residues per 100,000 g of protein. Values shown include those from the enzymes isolated from the myofibril and cytoplasm of bovine cardiac muscle. Comparison of these values with the published data for the MM isozyme from bovine skeletal muscle by Thomson et al. (42) indicates that bovine creatine kinase from either a myofibrillar or cytoplasmic source is probably of the MM form. The similarity in values for the myofibrillar and cytoplasmic creatine kinase further suggests that they could be the same enzyme. In comparing values from other species, it is apparent that the compositional makeup of the enzymes from bovine heart, rabbit skeletal or chicken skeletal muscle are very similar, suggesting an overall homology in the MM form enzymes. On the other hand, data for the bovine brain BB isozyme of creatine kinase (42) shows differences with the MM forms, supporting the proposition that similar isozymes from different species show fewer differences than do the different isozymes of one species.

Tryptophan determinations were carried out according to the method of Goodwin and Morton (102) and a value of 6 residues per mole was determined for the myofibrillar and cytoplasmic enzymes from bovine cardiac muscle. The same number was arrived at for the rabbit skeletal MM isozyme as isolated by Mani and Kay (82). A check on the possible extinction coefficient for the bovine cardiac creatine kinase yielded a probable extinction of $E^{1\%}_{1cm,\ 280nm} = 9.1$, using 2305 for the molar extinction coefficient of tyrosine and 5600 for the molar extinction coefficient of tryptophan.

The thiol content of creatine kinase was determined using DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) and adding a known amount to a

TABLE I

AMINO ACID COMPOSITIONS OF CPK FROM MUSCLE SOURCES

	Residues per 10^5 g of protein					
	Bovine cardiac (myofibril) (1)	Bovine cardiac (cytoplasm) (2)	Bovine skeletal MM (3)	Rabbit skeletal MM (4)	Chicken skeletal MM (5)	Bovine brain BB (3)
Lysine	74.1	77.8	80.2	74.8	73.5	59.3
Histidine	37.5	38.3	35.8	36.6	36.7	30.9
Arginine	40.0	42.2	41.9	44.6	40.1	51.9
Aspartic acid	94.8	98.6	100.0	92.3	93.7	100.0
Threonine	43.5	40.5	37.0	39.4	33.2	46.9
Serine	48.4	48.3	43.0	47.6	44.2	53.1
Glutamic acid	94.0	91.7	97.0	101.2	96.0	116.0
Proline	46.2	45.6	48.0	44.0	44.7	64.1
Glycine	78.5	79.1	80.2	76.6	68.7	85.2
Alanine	45.7	39.7	46.9	38.8	41.8	67.9
Valine	64.9	67.6	66.7	60.7	59.5	61.7
Methionine	21.2	23.4	22.2	20.0	21.1	23.4
Isoleucine	33.2	32.9	37.0	36.9	36.2	37.0
Leucine	83.1	85.3	84.0	84.0	81.3	97.5
Tyrosine	20.0	20.0	19.8	23.1	30.0	24.7
Phenylalanine	36.9	39.3	39.5	38.3	40.4	39.5
Tryptophan	8.0	8.0		8.0	7.0	
1/2 Cysteine	10.2	10.5				

(1) This study, corrected values.

(2) This study, corrected values.

(3) Reference (42), corrected values.

(4) Reference (82), corrected values.

(5) Reference (75), uncorrected values.

known concentration of the protein solution (104). In benign medium 0.1 M Tris, 2 mM EDTA, pH 8, two moles of thiol were bound per mole of creatine kinase from both myofibrillar and cytoplasmic fractions of bovine cardiac muscle. This "free thiol" content was the same in the case of the rabbit skeletal enzyme. When denaturing conditions of 6 M guanidine hydrochloride, 2 mM EDTA, and 50 mM Tris, pH 8, were employed for all three enzymes under study, 8 moles of thiol were found to be in each mole of creatine kinase, and these thiol groups account for all sulfhydryl groups present within the molecule.

The method of Moore (103) was employed to determine the presence of any possible disulfide bonds. Performic acid oxidation and subsequent amino acid analysis produced a 1/2 cystine content of 8 residues per molecule of creatine kinase for both the myofibrillar and cytoplasmic creatine kinase from bovine cardiac muscle, indicating the absence of any disulfide bonds within the molecule. This agrees with data for creatine kinase from other sources (42).

2. Determination of Extinction Coefficients

Figure 10 shows the correlation between the number of fringes and the absorbance of protein solutions of myofibrillar creatine kinase from bovine heart. This linear relationship gives a value of $E^{1\%}_{1cm}$ of 9.0 at 280 nm by the method of least squares. The points plotted were taken over a series of six different preparations. This value was further confirmed by the Lowry method (117) which also gave a value of 9.0. A similar study on the cytoplasmic enzyme from bovine heart gave identical values using both methods. These results agree very well with the calculated value, as determined from the known tyrosine and tryptophan content.

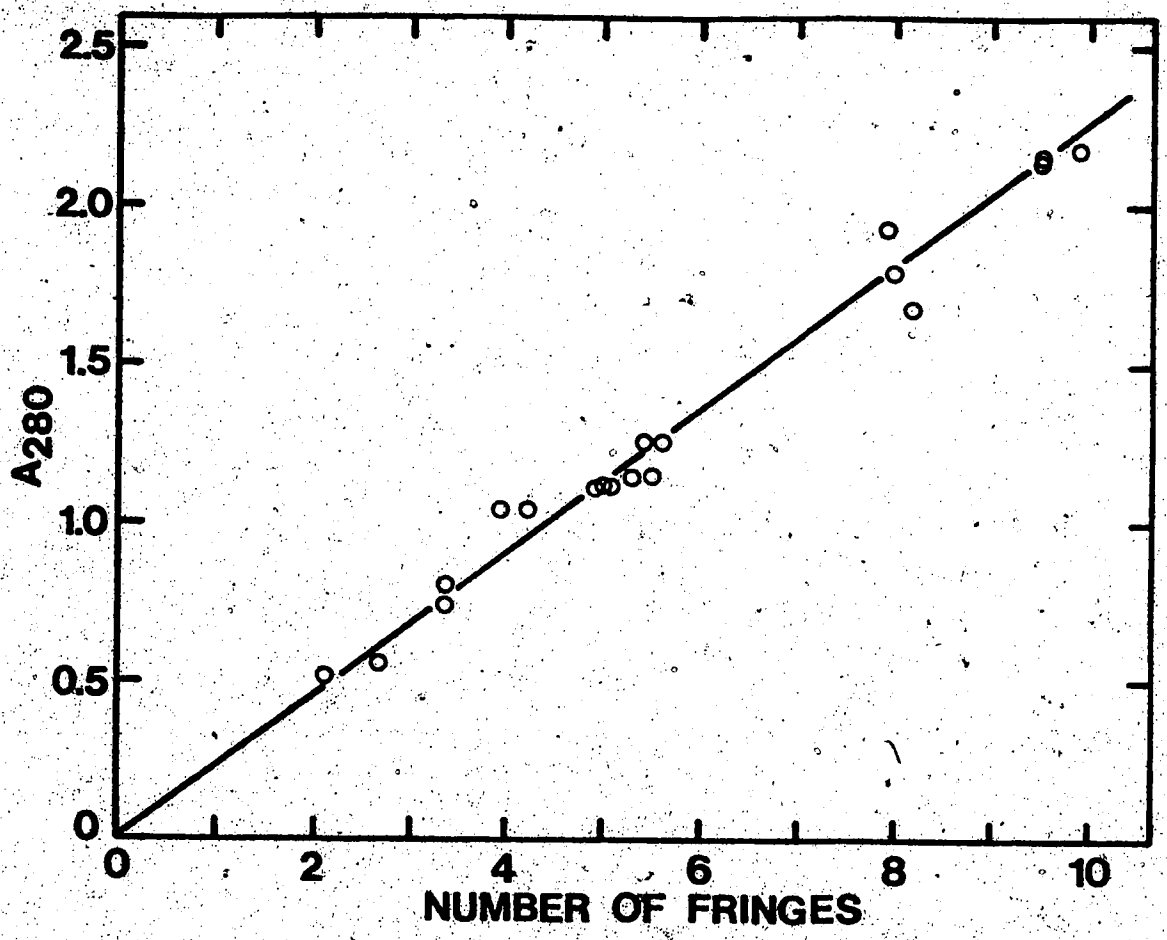


Figure 10. Determination of the extinction coefficient of myofibrillar creatine kinase. The protein was dissolved in 0.5 M KCl, 50 mM Tris, pH 8, and centrifuged at 12,000 rpm. The absorbance value at 280 nm was recorded for each sample, and its concentration determined refractometrically.

3. Determination of Sedimentation Coefficients

Sedimentation velocity studies of creatine kinase from bovine cardiac muscle were performed in 0.3 M KCl, 50 mM Tris, pH 8 for both the myofibrillar and cytoplasmic enzymes. In both cases, the molecule sedimented as a single symmetrical boundary with no evidence of any faster or slower moving components at all concentrations examined.

An intrinsic $S^{o}_{20,w}$ of 5.11 ± 0.1 S was calculated for myofibrillar creatine kinase, while an $S^{o}_{20,w}$ of 5.23 ± 0.1 S was determined for the cytoplasmic enzyme. The linear plots of S versus concentration are shown in Figure 11. These values are in the range reported for chicken skeletal muscle of 5.2 - 5.6 (75). The linear plots of $S_{20,w}$ may be expressed in terms of equations such as $S_{20,w} = 5.23 - (0.0032)c$ for the cytoplasmic enzyme over the range of 1.6 - 10 mg/ml and $S_{20,w} = 5.11 - (0.0015)c$ for the myofibrillar creatine kinase over the range of 2 - 9 mg/ml, where c is the protein concentration in g/100 ml. The low value of the slope terms indicate the molecule to be highly symmetric.

4. Determination of Molecular Weight

a. SDS-Polyacrylamide Gel Electrophoresis

On SDS-polyacrylamide gels, creatine kinase appeared as a characteristic doublet pattern (Figures 12, 13). This was true of both the myofibrillar and cytoplasmic creatine kinases of bovine heart, as well as for creatine kinase from different sources (42)(75). Using the marker proteins indicated in Figure 14, an average molecular weight of 40,000 ± 2,000 was determined for the subunit.

In general, it was found that the relative amounts of the heavier and lighter components which constitute the doublet appeared to vary with each preparation. Particularly at the beginning of this study (Figure

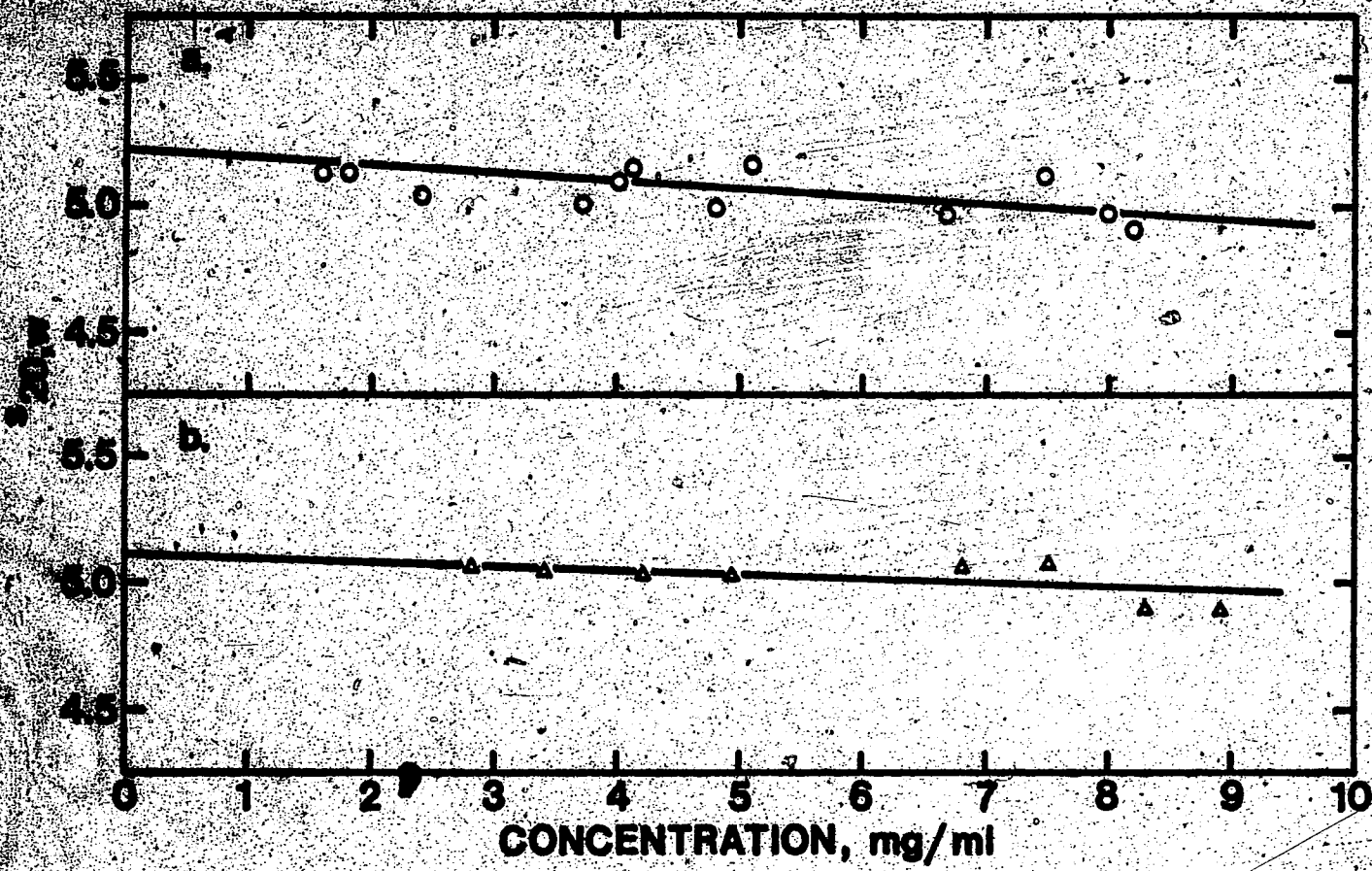


Figure 11. Determination of the intrinsic sedimentation coefficient of cytoplasmic and myofibrillar creatine kinase. (a) cytoplasmic CPK; (b) myofibrillar CPK. Creatine kinase samples of various concentrations were prepared in 0.3 M KCl, 50 mM Tris, pH 8, and centrifuged at 60,000 rpm to determine corresponding $S_{20,w}$ values.

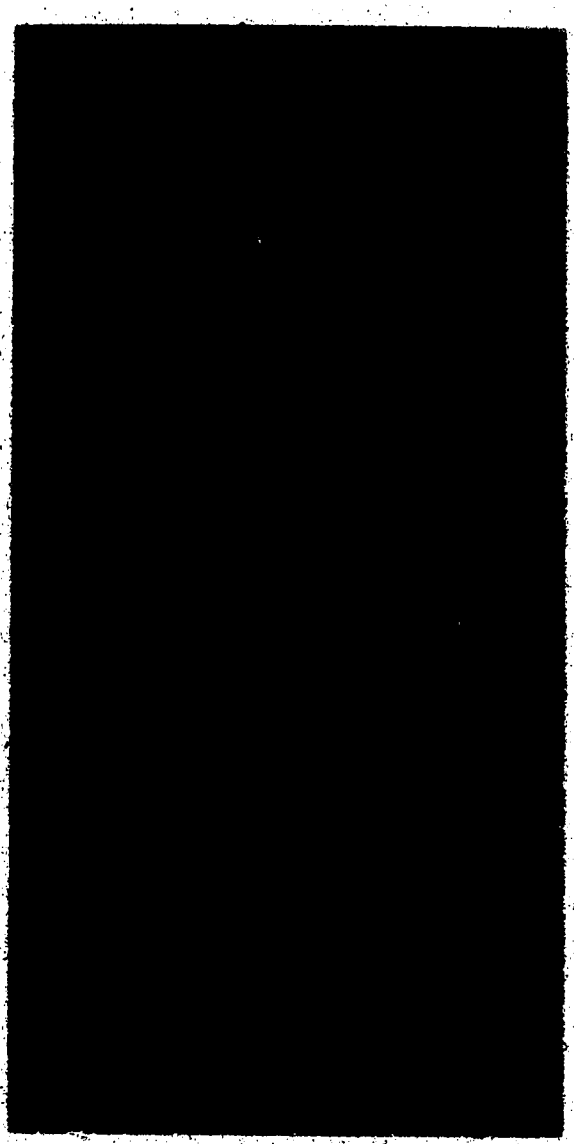


Figure 12. Electrophoresis of commercial and myofibrillar creatine kinase from bovine cardiac muscle. The photographs display the following:
(a) 10%-SDS polyacrylamide gel of commercial MM CPK (Sigma);
(b) 10%-SDS polyacrylamide gel of a preparation of myofibrillar CPK in the early part of this study;
(c) disc gel electrophoresis of myofibrillar CPK, 6% acrylamide concentration.

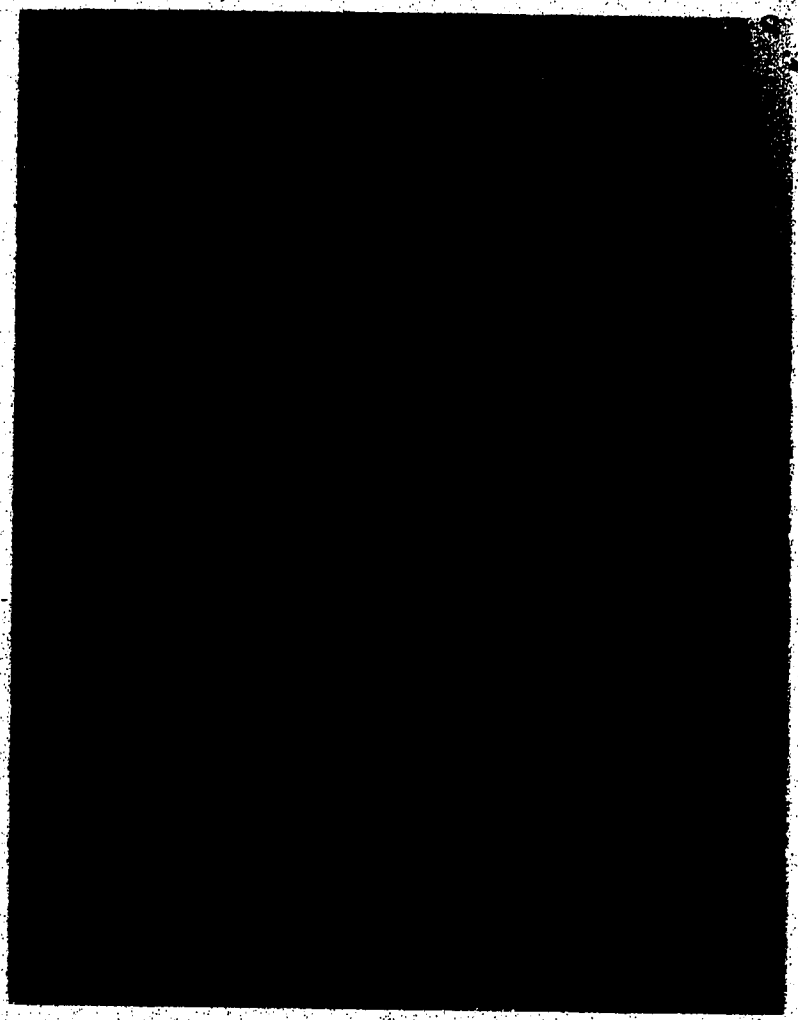


Figure 13. SDS-Polyacrylamide gel electrophoresis of creatine kinases from different sources. The gel concentration was 10%. (a) Commercial BB-CPK from rabbit skeletal muscle (the lower band is a cytochrome contaminant); (b) cytoplasmic CPK from bovine heart; (c) myofibrillar CPK from bovine heart in the later part of this study.

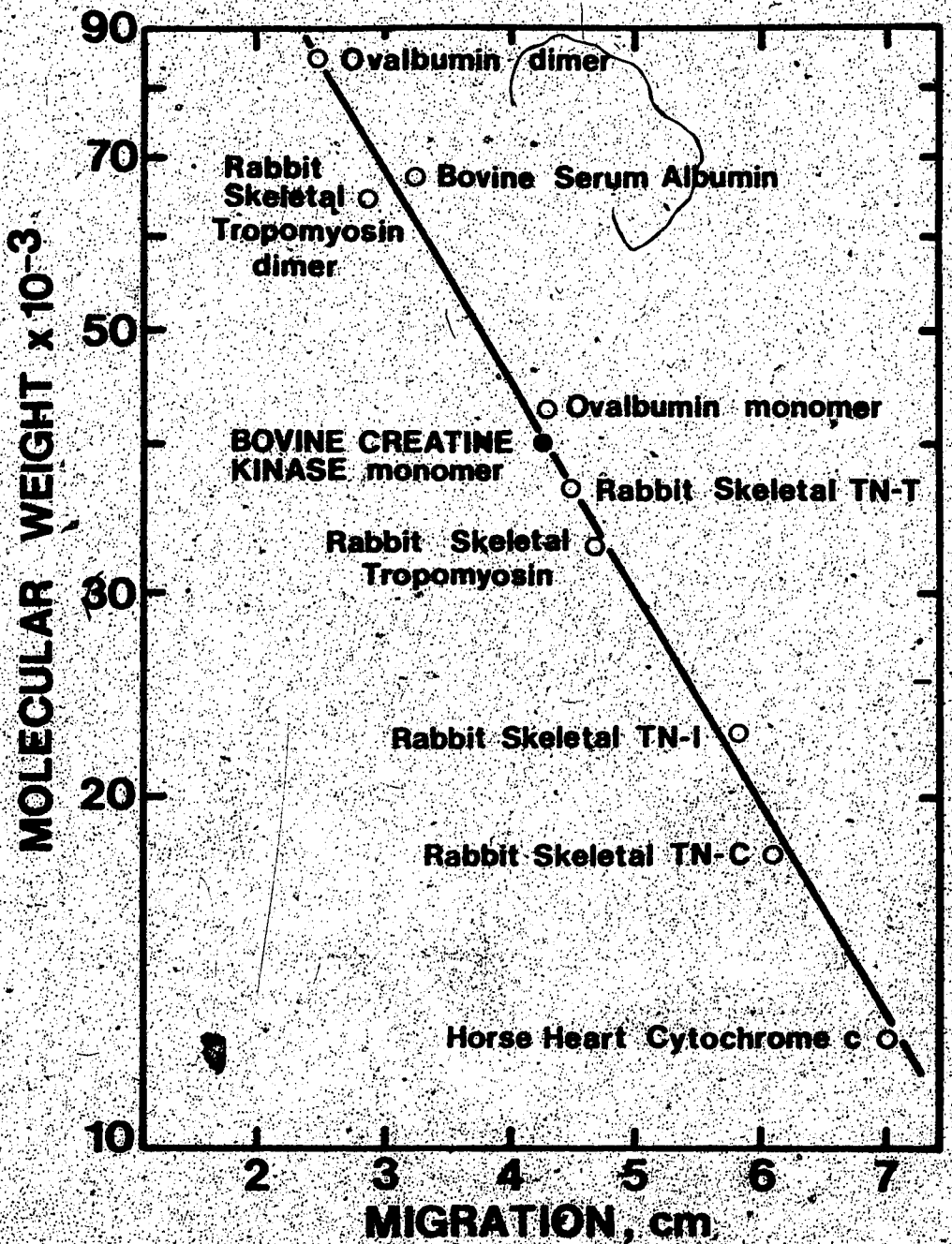


Figure 14. Determination of the molecular weight of the bovine creatine kinase subunit by the use of 10% SDS-polyacrylamide gel electrophoresis. The CPK monomers for both cytoplasmic and myofibrillar enzymes migrated equal distances.

12), the lighter component appeared to be dominant in most preparations. However, with increased efficiency and a shorter purification time (Figure 13), the heavier band predominated. This seemed to imply proteolysis may be a factor in this phenomenon, although no extensive proteolytic studies involving protease inhibition or time studies were undertaken to verify this possibility.

b. Sedimentation Equilibrium Studies

Low speed sedimentation equilibrium studies in the analytical ultracentrifuge were carried out for the native and denatured creatine kinases from the myofibril and cytoplasm of bovine heart.

For native myofibrillar creatine kinase, a molecular weight of 80,000 ± 4,000 was calculated for the protein at an initial loading concentration of 0.55 mg/ml in 0.5 M KCl, 50 mM potassium phosphate, pH 7, at 9.7°C. This value was derived from the slope of a linear plot of ln Y versus r^2 as shown in Figure 15.

Figure 16 shows the results of a similar experiment performed on native cytoplasmic creatine kinase. The slope of this line gave a value of 82,000 ± 4,000 for the molecular weight using an initial loading of 0.58 mg/ml.

Studies on the myofibrillar creatine kinase in a denaturing medium were conducted in 6 M guanidine hydrochloride, 50 mM Tris, pH 7.5, and 1 mM DTT, with an initial loading concentration of 0.72 mg/ml. In this instance a value of 40,000 ± 2,000 was derived from the slope of a linear ln Y versus r^2 plot as shown in Figure 17, using a $\bar{V}$ value of 0.72 for guanidine solutions according to Kay (118). The same experiment, in the absence of DTT, gave a comparable value for the subunit molecular weight of 40,000 ± 2,000, indicating the subunits are not held together

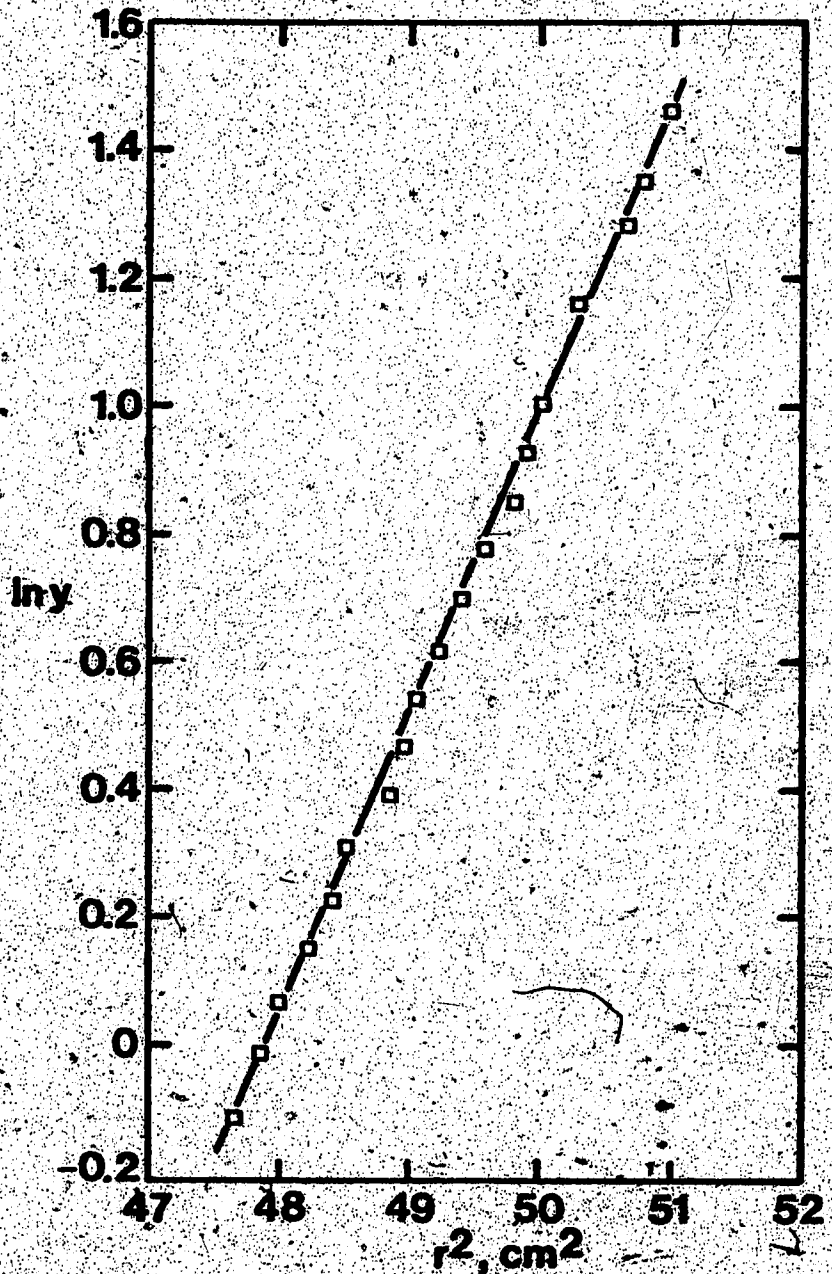


Figure 15. Determination of the molecular weight of native myofibrillar creatine kinase from bovine cardiac muscle. The plot shows the natural log of the concentration as a function of the distance from the axis of rotation for CPK in 0.5 M KCl, 50 mM potassium phosphate, pH 7, at 3.7°C at a rotor speed of 10,000 rpm.

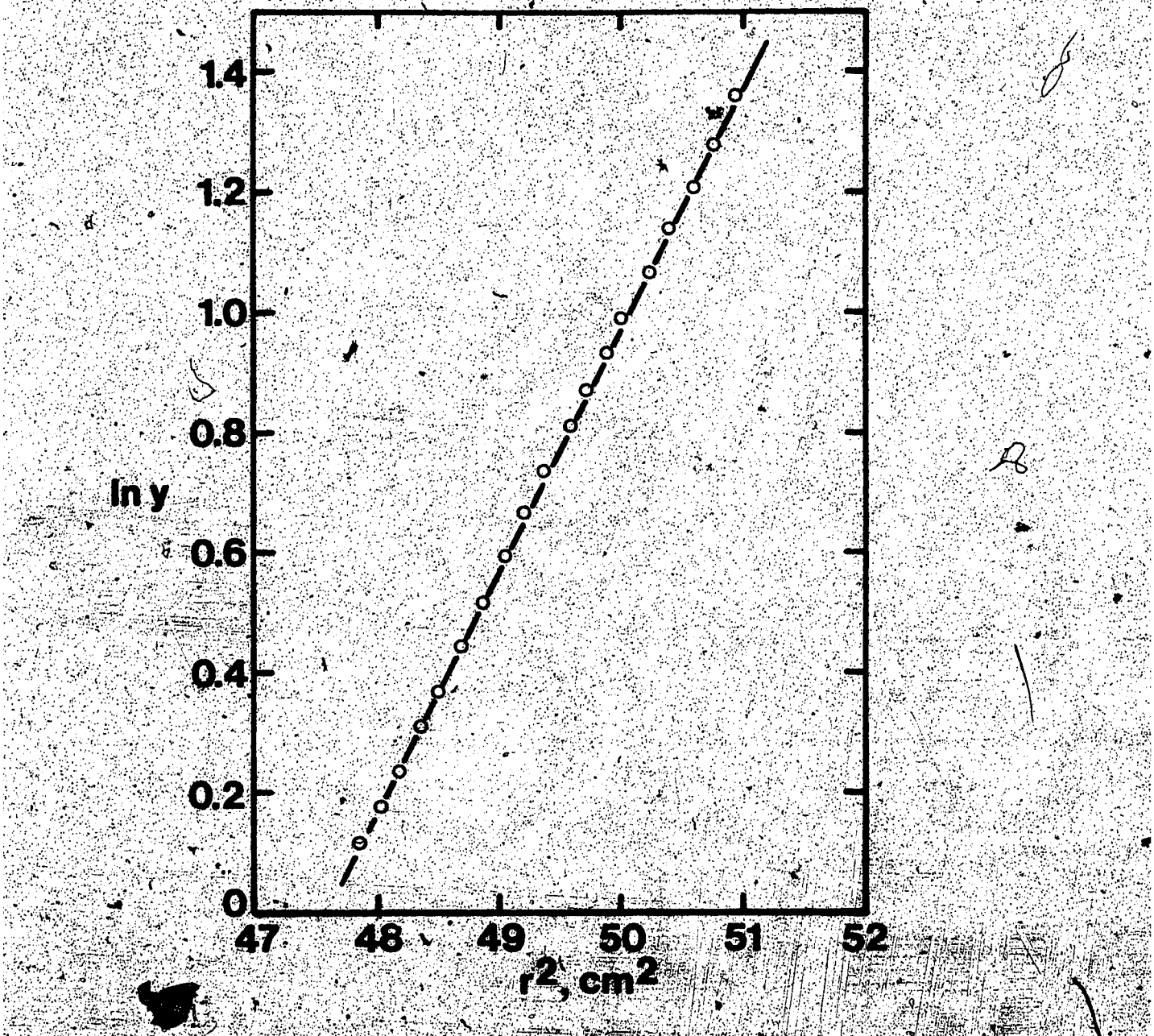


Figure 16. Determination of the molecular weight of native cytoplasmic creatine kinase from bovine cardiac muscle. The plot shows the natural log of the concentration as a function of the distance from the axis of rotation for CPK in 0.5 M KBr, 50 mM potassium phosphate, pH 7 at 5°C at a rotor speed of 3000 rpm.

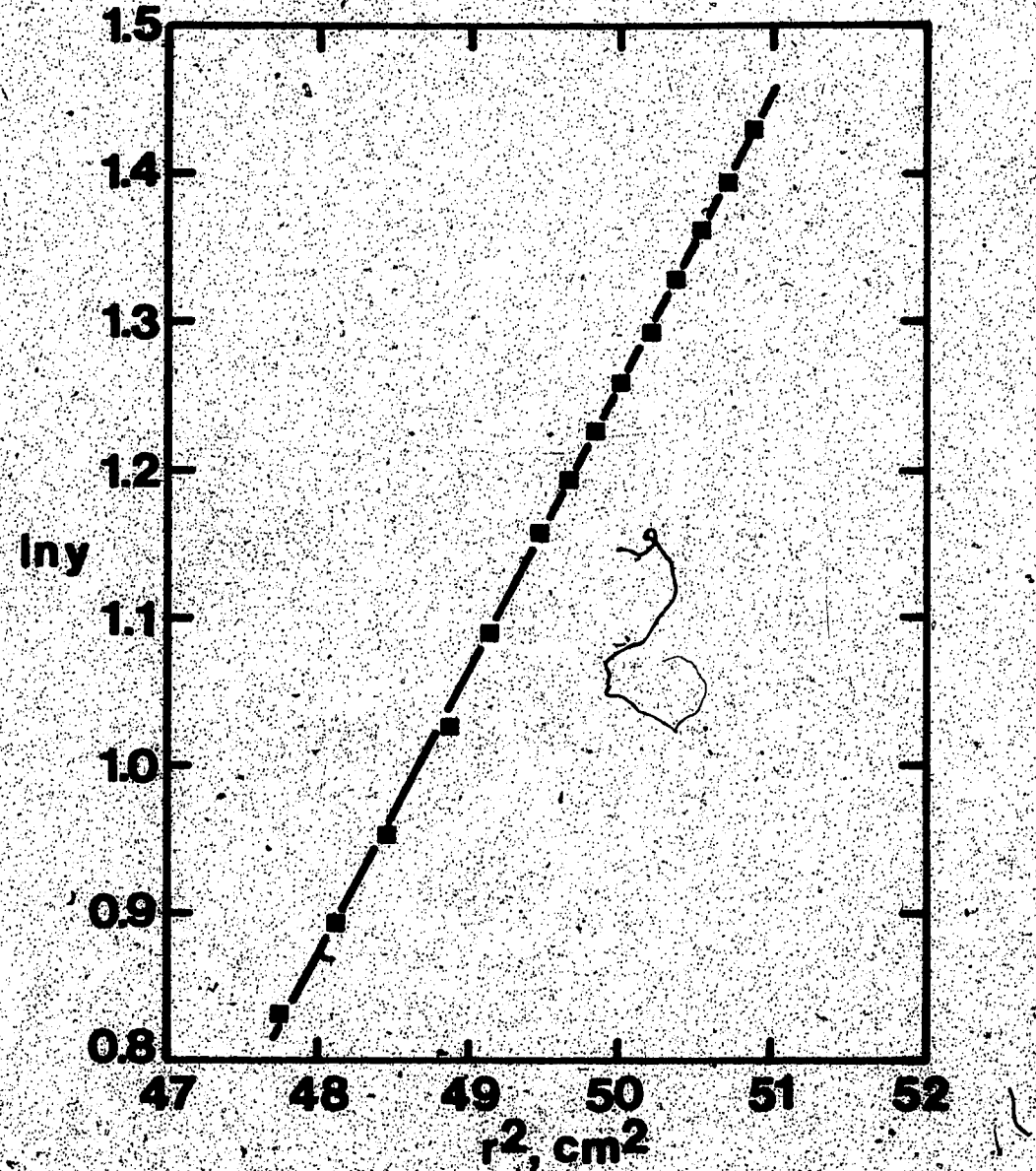


Figure 17. Determination of the subunit molecular weight of myofibrillar creatine kinase from bovine cardiac muscle. The plot shows the natural log of the concentration as a function of the distance from the axis of rotation for CPK in 6 M guanidine hydrochloride, 50 mM Tris, pH 7.5, 1 mM DTT at 20°C and a rotor speed of 11,000 rpm.

by covalent forces.

Figure 18 shows the results of the same experiment repeated for the cytoplasmic enzyme. From the slope a value of 41,000 ± 2,000 for the molecular weight was determined, using an initial loading concentration of 0.83 mg/ml.

5. Ultraviolet Absorption Properties

The UV absorption spectrum of creatine kinase is shown in Figure 19 and is typical of most proteins, displaying a maximum near 280 nm, a minimum near 250, and a shoulder indicative of a tryptophan contribution at about 290 nm. Both the myofibrillar and cytoplasmic creatine kinases from bovine cardiac muscle display identical spectra.

6. Circular Dichroism Properties

a. Far UV Region (190 - 250 nm)

The far ultraviolet circular dichroism spectrum of creatine kinase differs from that of a typical polypeptide chain in the α-helical conformation (119). The α-helix dichroic band normally found at 222 nm is shifted to 218 as shown in Figure 20. The measured ellipticity value at this wavelength is -10,000 ± 400 degrees·cm^2·$dmol^{-1}$, compared with -8,000 ± 400 reported for the rabbit skeletal enzyme at the same wavelength (82). At 208 nm the value for creatine kinase from bovine heart is -11,500 ± 400 degrees·cm^2·$dmol^{-1}$ as compared with -9,000 ± 400 for the enzyme from rabbit skeletal muscle. Calculations according to Chen et al. (106) established an apparent α-helical content for creatine kinase from bovine cardiac muscle of 30%, and a β-conformation value of 20%. Comparable calculations for creatine kinase from rabbit skeletal muscle (82) gave values of 20% and 40% respectively for the α-helical and β-conformation contents. It is clear that both of these enzymes

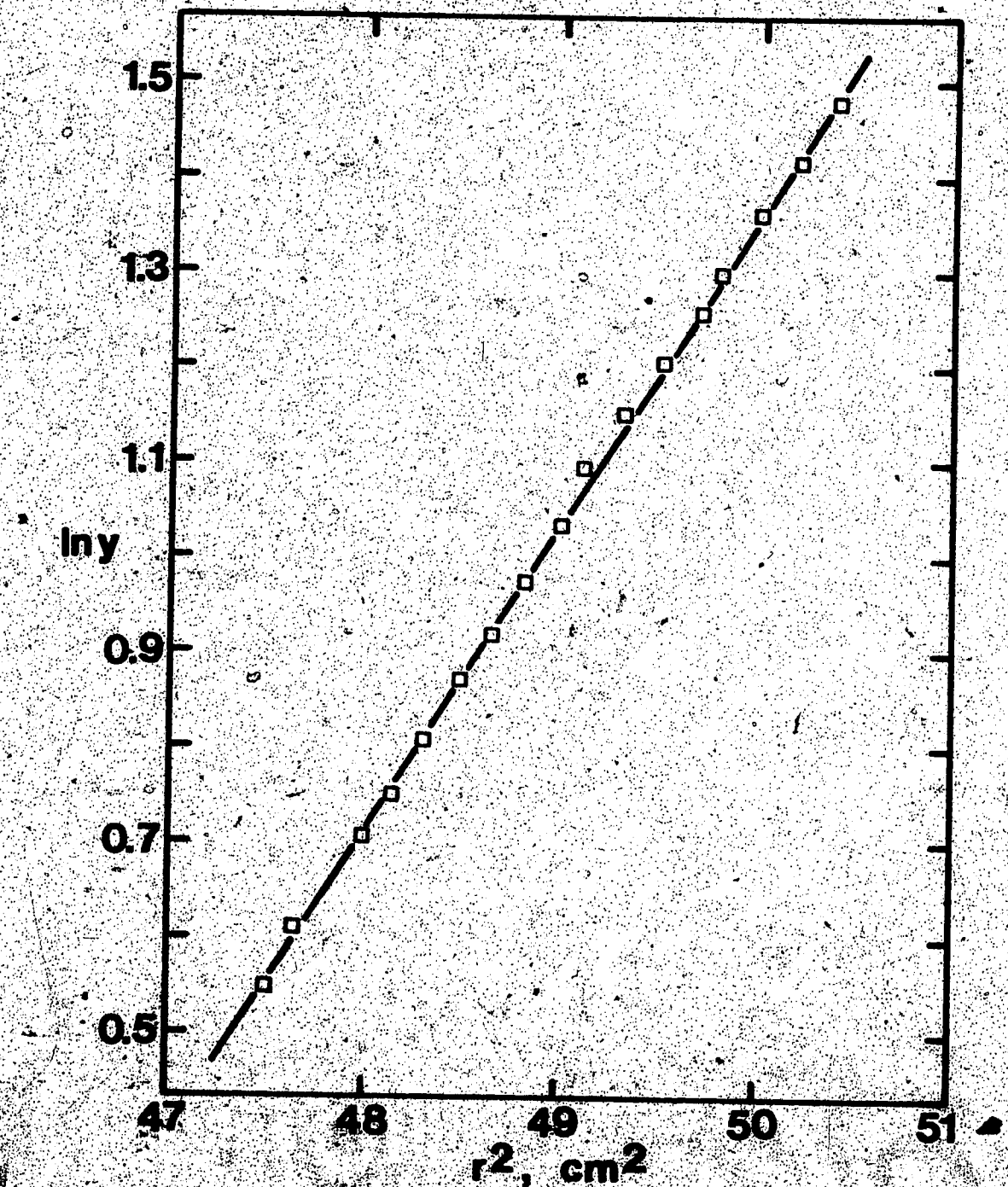


Figure 18. Determination of the subunit molecular weight of cytoplasmic creatine kinase. The plot shows the natural log of the concentration as a function of the distance from the axis of rotation for CPK in 0.4 guanidine hydrochloride, 50 mM Tris, pH 7.5, 1 mM DTT at 25°C and a rotor speed of 16,000 rpm.

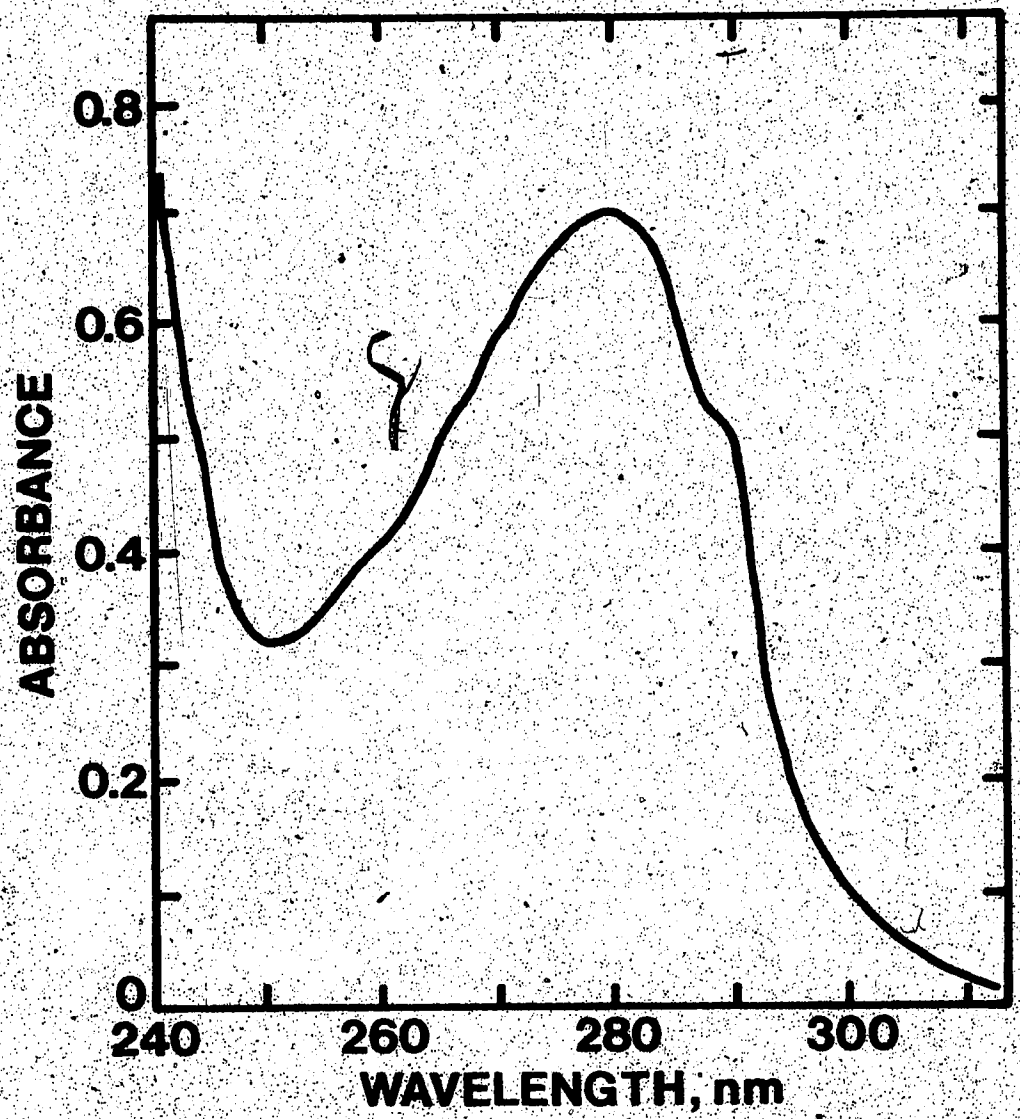


Figure 19. Ultraviolet absorption spectrum of creatine kinase from bovine cardiac muscle. The spectrum shown is for the myofibrillar enzyme. The cytoplasmic enzyme gave an identical spectrum. The concentration of both enzymes was 0.66 mg/ml, in 0.3 M KCl, 50 mM potassium phosphate, pH 7.0, in a cell of path length 1 cm.

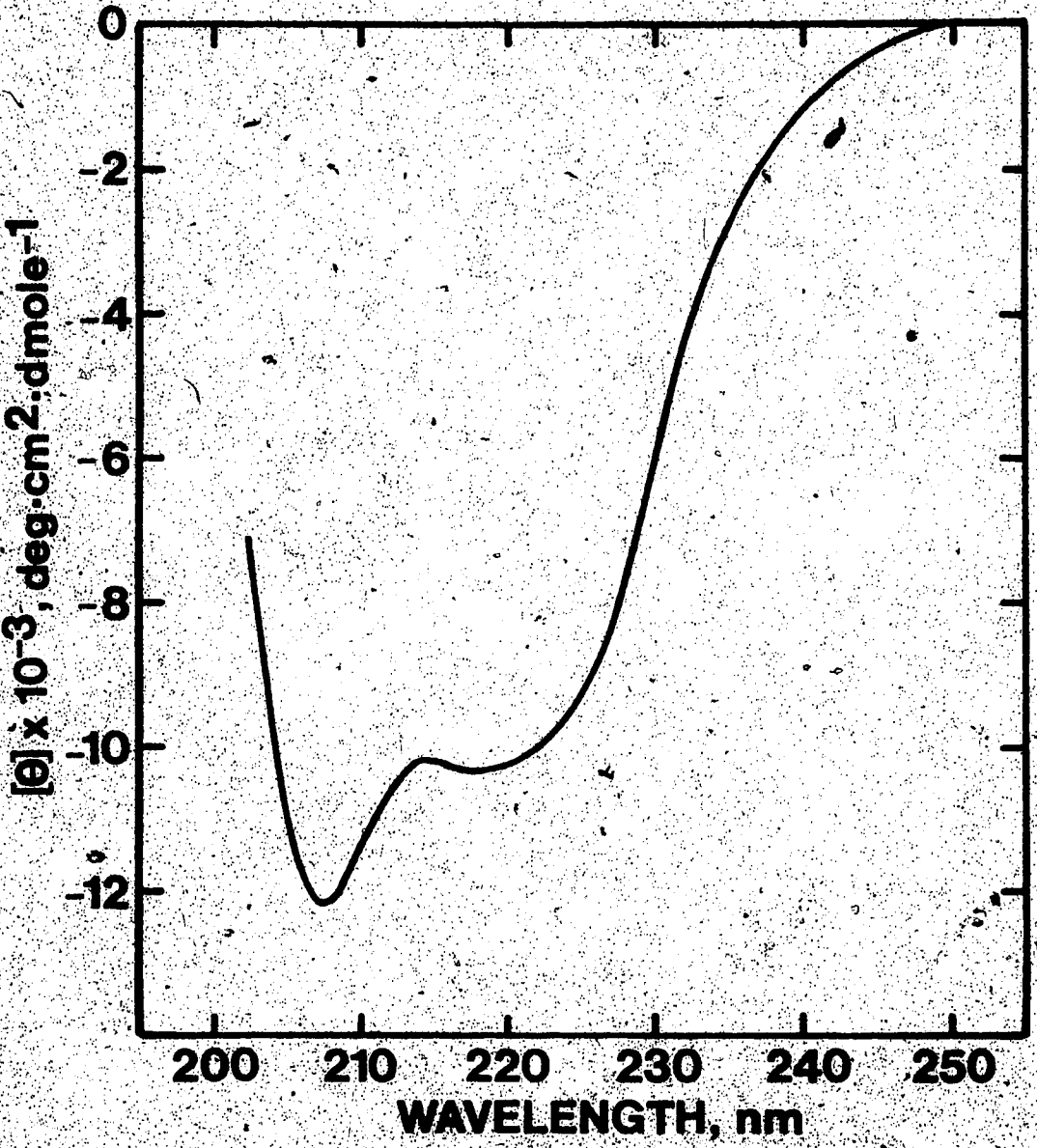


Figure 20. Far ultraviolet circular dichroism spectrom of myofibrillar creatine kinase. The protein was dissolved in 0.5 M KCl, 50 mM potassium phosphate, pH 7.

possess significant amounts of β-structure as reflected in the blue shift of the 222 nm band although they differ in absolute quantities of types of secondary structure.

When the same conditions were employed for the cytoplasmic enzyme, a completely superimposable CD spectrum of the bovine cardiac CPK was obtained. This result suggests the equivalence of these two enzymes in terms of overall structure.

The circular dichroism spectra for the myofibrillar and cytoplasmic creatine kinases from bovine heart in the aromatic region are shown in Figure 21. The spectra appear coincident. Bands due to tyrosines found in an asymmetric environment appear at 275 and 290 nm. In addition, the 290 signal is characteristic of a contribution from tryptophan residues, while the low signals below 275 are due to phenylalanine (120).

7. Spectrophotometric Titrations

The ionization of the tyrosine residues of creatine kinase from both rabbit skeletal muscle and bovine cardiac muscle were measured spectrophotometrically as a function of pH. Circular dichroism measurements in the far UV region were also performed in order to correlate any changes in secondary structure with the tyrosine ionization changes as the pH was increased. The buffer system used for these studies was 0.5 M NaCl, 5 mM borate, at selected pH's.

a. Creatine Kinase from Rabbit Skeletal Muscle

The degree of ionization of tyrosine residues in creatine kinase from rabbit skeletal muscle, measured as a function of pH, is shown in Figure 22. The plateau level of ΔA as one reaches high alkaline pH's corresponds to having all tyrosines present within the molecule accounted for, i.e., n = 19; as obtained from amino acid analysis (82). Two

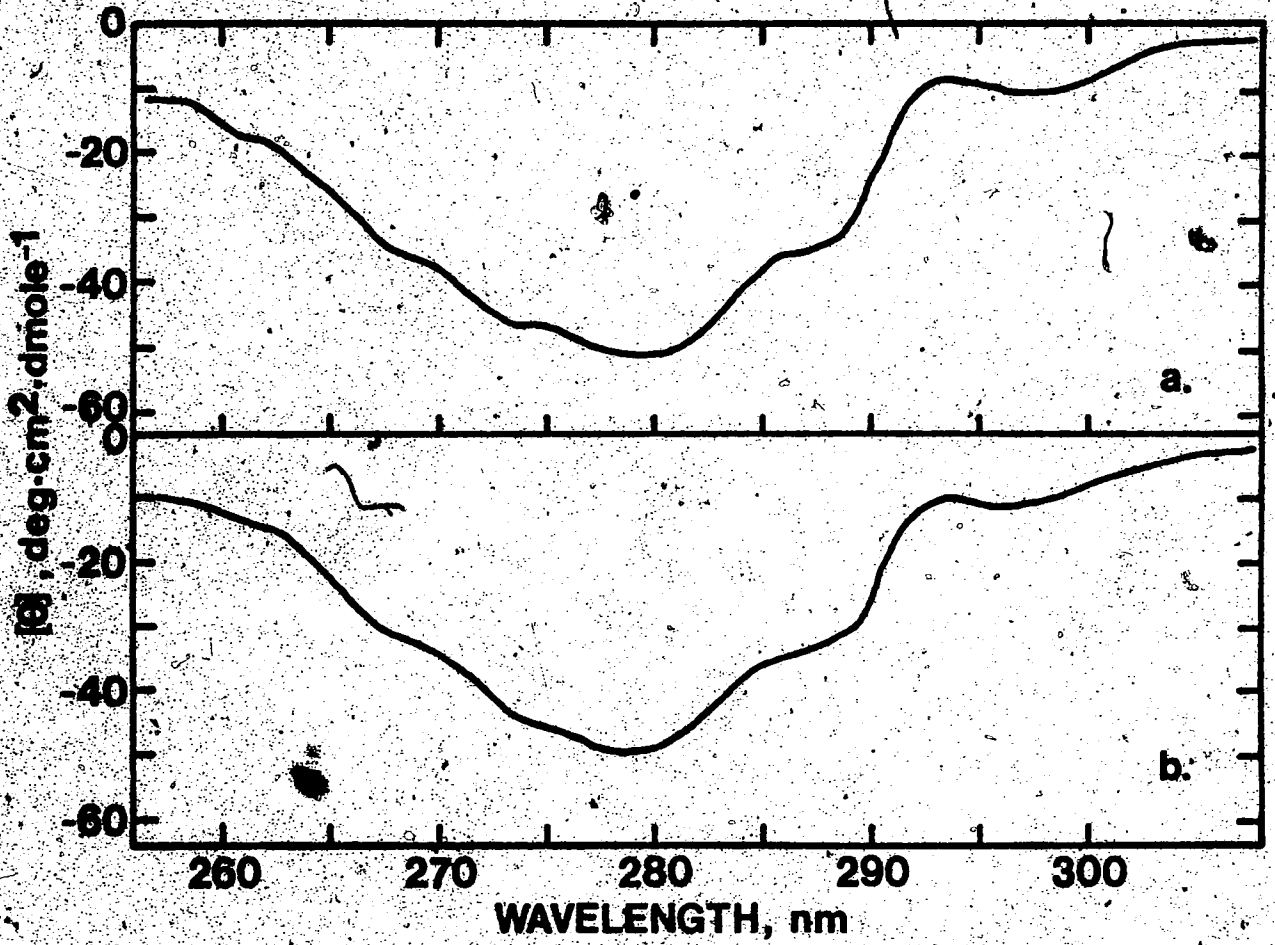


Figure 21. Near ultraviolet circular dichroism spectrum of creatine kinase from bovine cardiac muscle. (a) myofibrillar CPK, (b) cytoplasmic CPK, both in 0.5 M KCl, 50 mM potassium phosphate, pH 7.

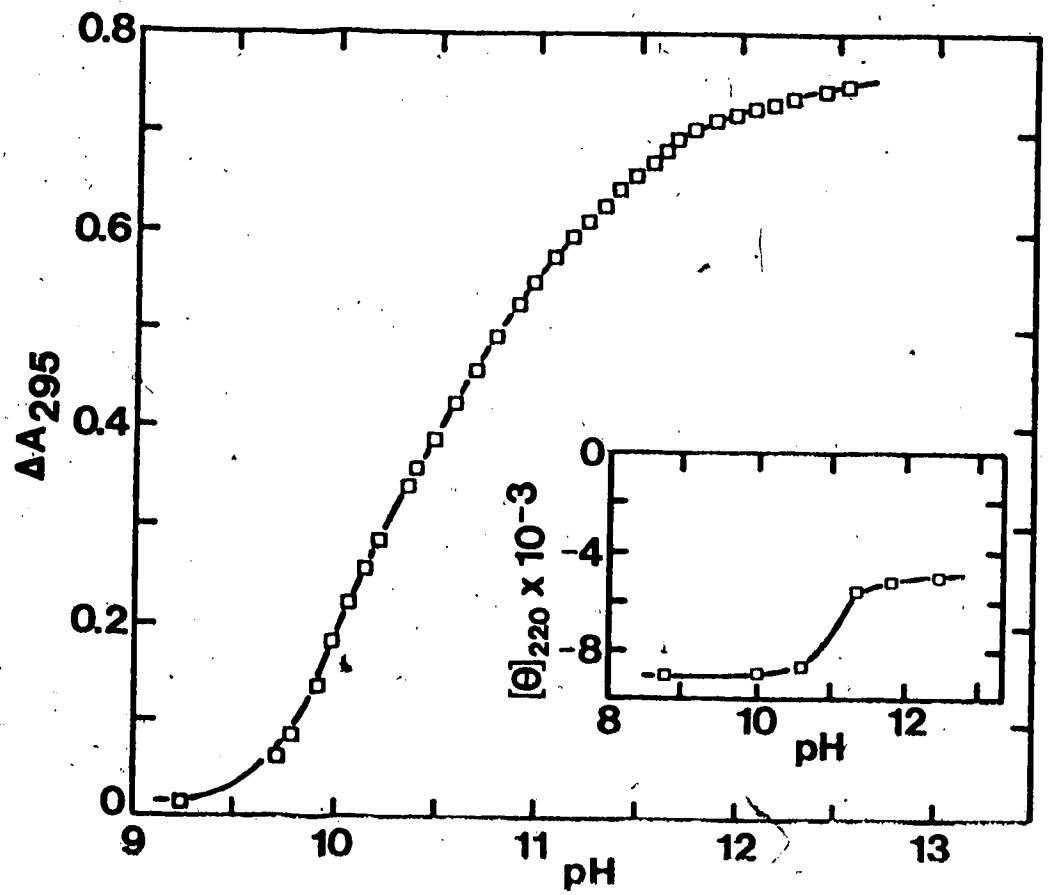


Figure 22. Spectrophotometric titration of creatine kinase from rabbit skeletal muscle at 25°C. The insert shows the plot of $[\theta]_{220}$ values from circular dichroism experiments as a function of pH.

steps of ionization were evident in this curve. A pronounced increase occurred between pH 10.2 and 12. Above a pH of 12.5, the ΔA value became constant; at this point all 19 tyrosine residues had been ionized. Analysis of this titration curve by a non-linear curve fitting program revealed the presence of two classes of tyrosine residues. The first class has a pK value of 10.5 ± 0.1, with 17 tyrosine residues. The second class has a pK of 11.8 ± 0.2 and contains the remaining two tyrosines. Since the pK value of 10.5 is slightly higher than the accepted values of 9.5 - 10 for a fully exposed tyrosine residue within a protein framework, this suggests the residues in this class to be partially buried within the molecular framework.

The insert in Figure 22 shows the concurrent loss of secondary structure with increasing pH as monitored by circular dichroism measurements. In increasing the pH from 8 to 12.5, a decrease in negative ellipticity occurs at 220 nm, indicative of a loss in secondary structure. However, no loss in secondary structure accompanied the ionization of the first class of tyrosine residues since there was no change in θ_{220nm} until pH 10.6. When the remaining two tyrosines were ionized, by pH 11.2, about 40% of the secondary structure disappeared. This suggested that the remaining two groups are well protected from the solvent in the native state.

b. Creatine Kinase from Bovine Cardiac Muscle

Figure 23 shows the pH dependence of the absorbance change ΔA at 295 nm for myofibrillar creatine kinase from bovine cardiac muscle. The cytoplasmic enzyme gave the same results. The ionization curve was seen to have a first order sigmoid shape, with the most pronounced change between pH 10.0 and 11.0. The plateau portion of ΔA at high pH

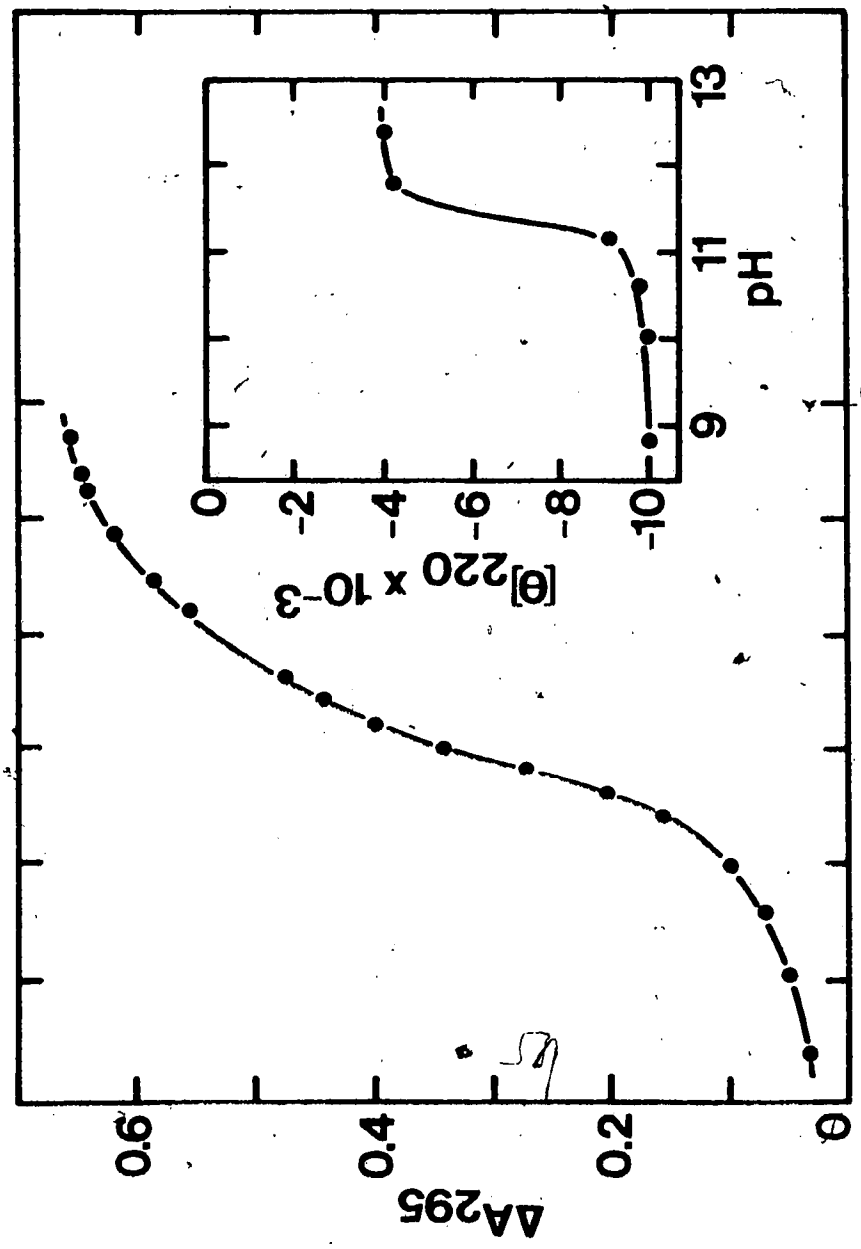


Figure 23. Spectrophotometric titration of creatine kinase from bovine cardiac muscle at 25°C. The insert shows the plot of $[\theta]_{220}$ values from circular dichroism experiments as a function of pH.

corresponded to total ionization of all tyrosine residues, n = 16, as determined by amino acid analysis (83). Unlike the creatine kinase from rabbit skeletal muscle, only a single class of tyrosines with a pK value of 10.6 ± 0.1 was determined to be present within the molecule. This observed pK value is slightly higher than the values usually accepted for tyrosines fully exposed, suggesting these groups are partially buried.

Secondary structure stability data (θ_{220nm}) as a function of pH is shown in the insert of Figure 23. No change was evident in θ_{220nm} up to pH 11.0, suggesting that ionization of tyrosine residues is not accompanied by any loss of secondary structure. By pH 12.5, nearly 40% of the secondary structure has disappeared.

8. Enzymology

a. Progressive Purification Studies

Progressive purification of the myofibrillar creatine kinase throughout the procedure was indicated by determining the specific activity at different stages. The latter quantity was determined by the method of fringe counts. The specific activity of creatine kinase increased from an average value of 2 units/mg protein for the supernatant prior to pH 5 precipitation, to 4 - 6 after ammonium sulfate precipitation and finally to 35 - 40 units for the partially purified creatine kinase after the first DEAE cellulose column. Rechromatography of this protein increased the specific activity to an average of 55 - 60 units/mg for most preparations. At times when the preparation was done very quickly from fresh hearts this activity could go as high as 80 - 85 units/mg. Cytoplasmic creatine kinase preparations which were usually prepared within 4 days gave values as high as 95 - 100 units/mg. Freeze-

drying resulted in a net loss of only 10% activity provided it was done with minimum time loss. The average value of 60 for the myofibrillar enzyme compares with 85 units/mg reported for creatine kinase from rabbit skeletal muscle (82) and a value of 44 for creatine kinase extracted from rat cardiac myofibrils (64).

b. Determination of Kinetic Parameters

Kinetic parameters of myofibrillar creatine kinase from bovine cardiac muscle were determined for the substrates creatine phosphate and ADP.

Figure 24 shows the Lineweaver-Burk plot for the substrate creatine phosphate. The K_m value determined from the intercept on the abscissa was found to be 1.9 mM. A similar study on the cytoplasmic enzyme produced a value of 2.0 mM. The K_m of creatine phosphate for rabbit skeletal creatine kinase was also determined for comparative purposes and found to be 2.1 mM. Published reports using rat cardiac myofibrils as a source have given a value of 1.67 mM for the same substrate (64). All these results are within the same order of magnitude.

Figure 25 shows the Lineweaver-Burk plot of creatine kinase activity for the substrate ADP. Here the value of K_m for ADP was determined as 0.047 mM for the myofibrillar creatine kinase, and 0.050 mM for the cytoplasmic form from bovine heart. This compares with 0.065 mM for the rabbit skeletal protein and 0.077 mM from the myofibrils of rat heart (64). Again all values are in the same order of magnitude.

9. Identification of Isozymes

The amino acid analysis of creatine kinase from bovine cardiac muscle had suggested that the isozyme separated, from both myofibrillar and cytoplasmic sources, was of the MM type. To examine this further,

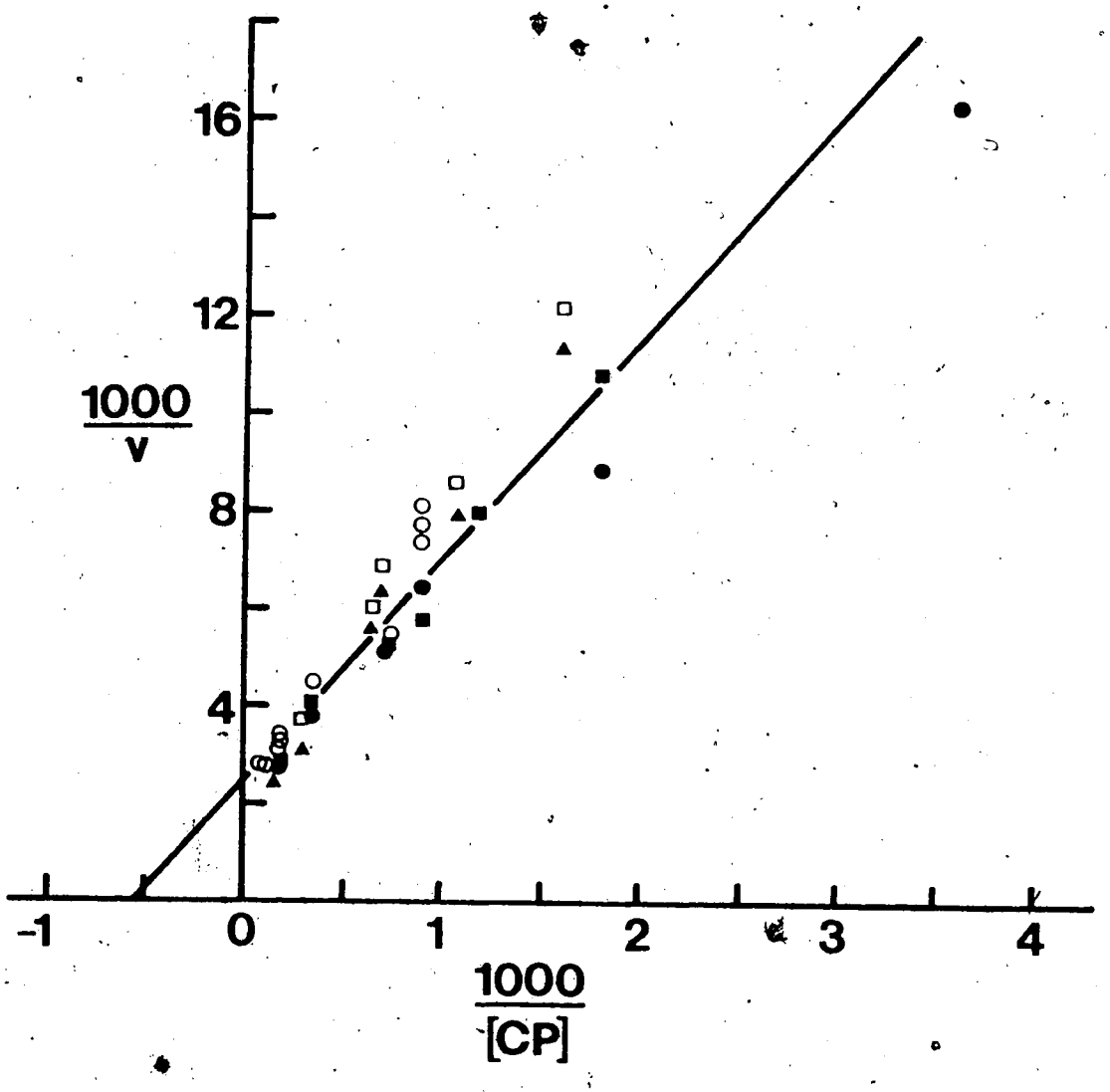


Figure 24. Lineweaver-Burk plot of creatine kinase activity for the substrate creatine phosphate. The reciprocal of the rate is plotted versus the reciprocal of the creatine phosphate molar concentration. The different symbols represent several different preparations of myofibrillar CPK. The intercept on the abscissa gives the value of $-1/K_m$.

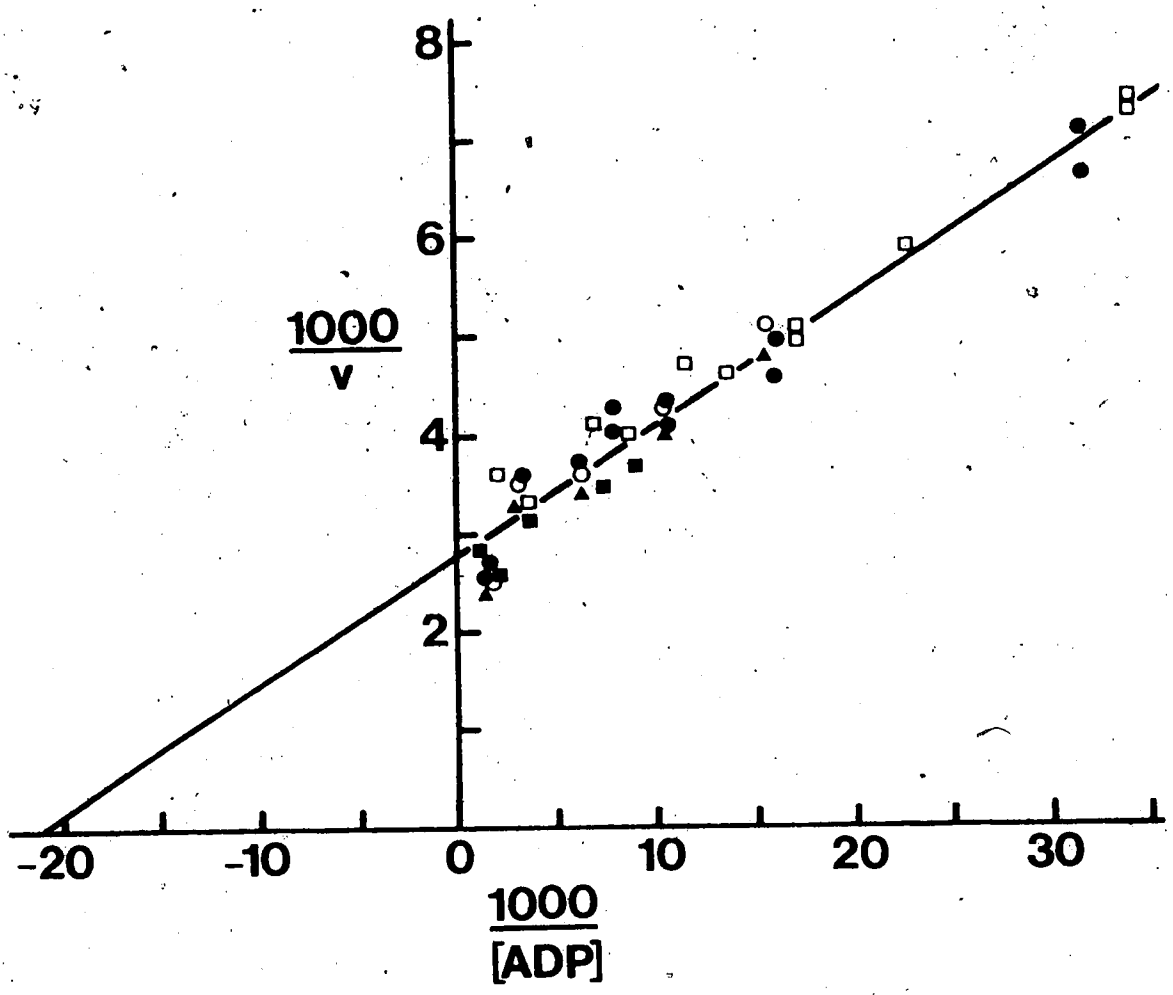


Figure 25. Lineweaver-Burk plot of creatine kinase activity for the substrate ADP. The reciprocal of the rate is plotted versus the reciprocal of the ADP molar concentration. The different symbols represent several different preparations of myofibrillar CPK. The intercept on the abscissa gives the value of $-1/K_m$.

polyacrylamide gel electrophoresis studies in conjunction with the preparation of a viable hybrid MB from two different species were undertaken.

a. Preparation of MB Hybrid

Keutel et al. (121) have reported the pI of calf MM to be 7.3, for MB to be 6.8 and for BB to be 5.6. These calf isozymes were easily separable using a chromatographic system comprised of a DEAE cellulose DE-32 column equilibrated at pH 7 with a succinate-Tris buffer.

Rabbit BB isozyme is highly similar to calf BB (42), hence a substitution of commercial BB from rabbit skeletal muscle for bovine BB was deemed feasible. Rabbit skeletal BB enzyme (Sigma) was thus hybridized with bovine creatine kinase according to the method of Keutel et al. (121) and subsequently purified.

The technique of preparing the hybrid was as follows: approximately 20 mg of bovine creatine kinase (from a myofibrillar source) was added to 10 mg of commercial rabbit BB in a total volume of 0.5 ml. To this was added 4.5 ml of a solution containing 4 M guanidine hydrochloride, 50 mM Tris, 2 mM DTT and 5 mM EDTA at pH 7.6, and the temperature was raised to 30°C for 5 min. Reannealing of the subunits was accomplished by immediate dilution with 31 ml of ice cold solution containing 50 mM Tris, 5 mM EDTA, 1 mM DTT, pH 7.6. The protein was then dialyzed against 20 mM succinate-Tris, 1 mM EDTA, 1 mM DTT, pH 7.0, and put on a DEAE cellulose DE-32 column equilibrated against the same buffer. The separation is shown in the chromatogram of Figure 26.

It should be noted that all of the bovine creatine kinase extracted from the myofibril, as well as that from the cytoplasmic preparations, consistently eluted in the position of the MM peak. In both instances, neither of the preparations showed traces of any MB or

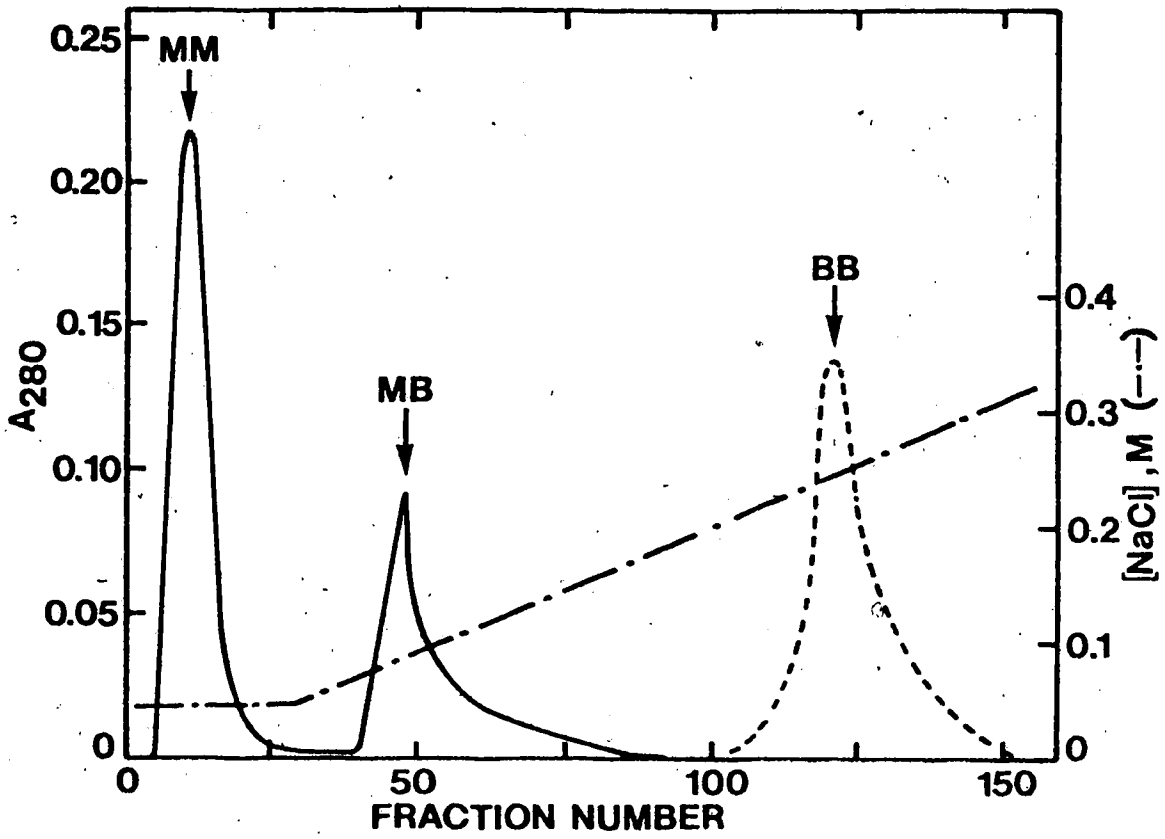


Figure 26. Chromatographic separation of the hybrid MB isozyme of creatine kinase. The diagram shows the elution profile of a DEAE cellulose column eluted with 20 mM succinate-Tris, 1 mM EDTA, 1 mM DTT, pH 7, buffer containing a 1000-ml linear gradient of 0 to 0.4 M NaCl. The dotted line indicates the position rabbit BB-CPK would have if it were present. (——— elution profile; —·—·— NaCl gradient)

BB forms, indicating that the isolation procedure is highly selective for the MM form. Elution profiles of rabbit skeletal CPK from the M-band also indicated this enzyme to be of the MM type.

The same column equilibrated in 6 M urea, 50 mM succinate-Tris, 2 mM DTT, 5 mM EDTA, pH 8.0, gave only one peak, indicative of identical subunits being present, for bovine myofibrillar and cytoplasmic CPK's.

b. Polyacrylamide Gel Electrophoresis

The chromatography results were verified by polyacrylamide gel electrophoresis. Figure 27 shows the results of electrophoresis performed in 10% acrylamide concentration in 0.16 M glycine, 25 mM Tris, pH 8.38 at 2.5 mA/gel for 1 hour. The gel pattern establishes that the mobilities of the bovine myofibrillar, bovine cytoplasmic and rabbit skeletal enzymes are all the same. The MB hybrid formed shows a mobility intermediate between the MM forms and the rabbit BB form.

During the course of this study, a unique phenomenon was noted. The gel patterns seen in Figure 28 are all creatine kinases of the MM form which were seen at the same conditions as mentioned above, but for 2 hours longer. It is apparent that some very minor differences in charge are present within the purified MM creatine kinases. This phenomenon has been reported by Armstrong et al. (122), who found that despite high purification verified by sedimentation velocity and chromatography, as many as seven minor protein bands could be demonstrated by disc electrophoresis, and these multibanded patterns were observed consistently. The presence of thiols did not discourage this pattern's appearance. Since some of these bands were still antigenic, the authors suggested that an extraneous protease may be responsible for the heterogeneity of the purified enzyme, or possibly these contaminating proteins

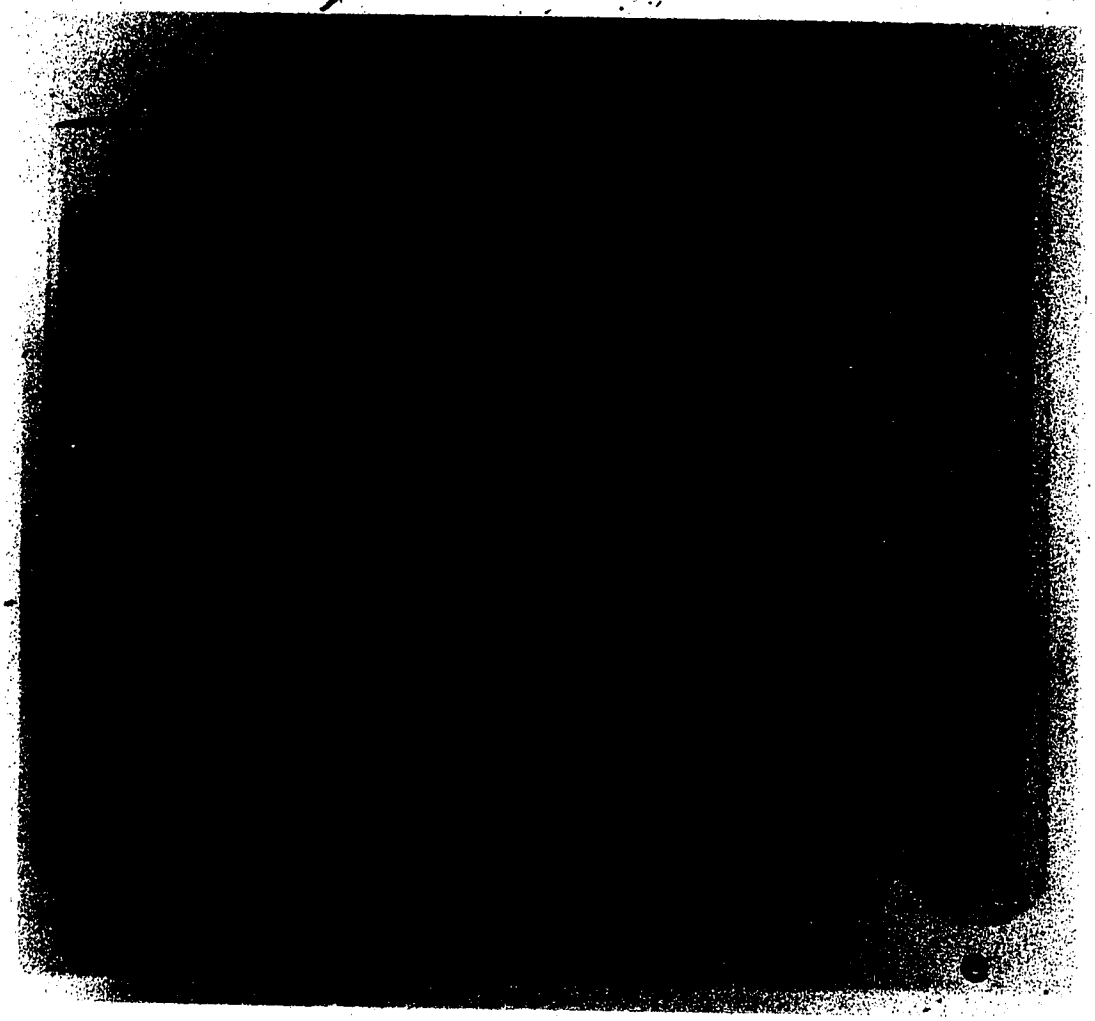


Figure 27. Disc gel electrophoresis of creatine kinase isozymes. The acrylamide concentration was 10%, in a buffer consisting of 0.16 M glycine, 25 mM Tris, pH 8.38. The electrophoresis was performed for 1 h at 2.5 mA/gel.

(a) myofibrillar CPK from bovine cardiac muscle;
(b) commercial rabbit BB (Sigma) from brain tissue;
(c) MM + MB hybrid formed from excess bovine MM and rabbit BB in 4 M guanidine hydrochloride;
(d) MM-CPK from rabbit skeletal muscle;
(e) purified MB hybrid following chromatography on DEAE cellulose.

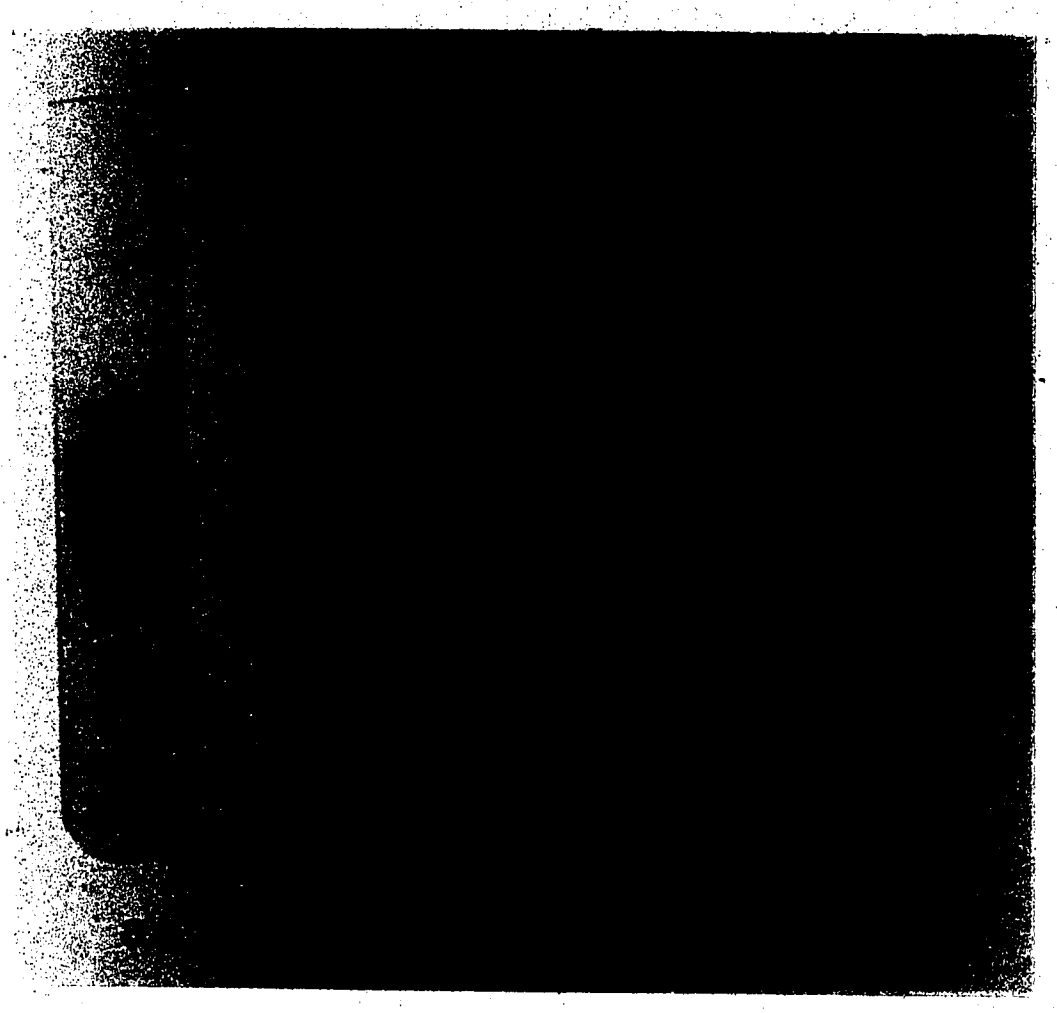


Figure 28. Prolonged disc gel electrophoresis of creatine kinase isozymes. The acrylamide concentration was 10%, in a buffer system consisting of 0.16 M glycine, 25 mM Tris pH 8.38. The electrophoresis was performed for 3 h at 2.5 mA/gel. All of the samples are of the MM type from:
(a) myofibrillar bovine cardiac muscle;
(b) cytoplasmic bovine cardiac muscle;
(c) commercial (Sigma) bovine cardiac muscle;
(d) rabbit skeletal muscle;
(e) commercial (Sigma) rabbit skeletal muscle.

may arise during the purification procedure. The latter suggestion implies the ability of creatine kinase to adopt more than one kinetically stable conformation. The fact that for the MM isozyme this pattern consistently crosses species barriers indicates a common factor of "weak links" within the molecule itself may be responsible for these small differences in charge. It may also be due to these cleavages that the characteristic doublet pattern seen in SDS-polyacrylamide gels arises.

D. SUMMARY

The myofibrillar creatine kinase extracted via a low ionic strength medium from bovine cardiac muscle appears to be the MM isozyme. This conclusion is based on evidence from polyacrylamide gels which indicates the cardiac myofibrillar enzyme to co-migrate with the MM forms of CPK from other sources, and is corroborated by comparison with the amino acid composition of the MM creatine kinases from other sources. Comparison of the physical parameters of the cytoplasmic and myofibrillar creatine kinases indicates that they are probably the same enzyme. They have the same extinction coefficient, $E^{1\%}_{1cm}$ at 280 nm of 9.0, and possess equivalent ultraviolet absorption spectra. An examination of the circular dichroism spectra in both the near and far UV also points to the two enzymes as being identical. The α-helical content for both enzymes was calculated to be 30%, with a β-conformation value of 20%. Sedimentation equilibrium studies for the cytoplasmic and myofibrillar CPK gave a molecular weight in the range of 80,000 daltons for the native enzymes and 40,000 daltons for their subunit molecular weight. The $S^{o}_{20,w}$ is 5.1-5.2. Both appear to have an average specific activity of 60 units per mg protein. K_m values are in the range of 0.05 mM for ADP and 2.0 mM for

creatine phosphate. Most of these values show a great deal of similarity with the values determined for the skeletal CPK counterparts from chicken and rabbit muscle. Differences with creatine kinase from rabbit skeletal muscle are small; rabbit skeletal CPK has a molecular weight of 84,000 ± 4,000 daltons while circular dichrojsm calculations gave an α-helical content of 20% with a β-conformation value of 40% (82). As well, spectrophotometric titrations show that rabbit skeletal muscle has two classes of tyrosines with the second class having a pK of 11.8, while in the bovine cardiac case, CPK has only a single class of tyrosines with a pK of 10.6. This slight difference may prove of use in studies involving the environment of the tyrosine chromophores, particularly in interaction studies with other contractile components. In this context, small subtle changes around the tyrosines could conceivably be picked up by fluorescence measurements.

CHAPTER IV

INTERACTION STUDIES

A. INTERACTION STUDIES OF CREATINE KINASE WITH MYOSIN AND ITS SUBFRAGMENTS

Creatine kinase and myosin are functionally related. It is reasonable to surmise the former enzyme regenerates the ATP used by the latter during hydrolysis, and in this role, creatine kinase may serve as a regulator of ATP concentration in muscle contraction. Reports in the literature have demonstrated interaction between creatine kinase and myosin (70, 71, 80). Studies by Mani and Kay (82) established that an interaction occurs between creatine kinase and myosin, heavy meromyosin and subfragment-1 from rabbit skeletal muscle. As part of an overall comparative study of these systems it was of interest to determine whether a parallel interaction occurs between creatine kinase and myosin, using bovine cardiac muscle as the source.

1. ATPase Activity Studies

Work by Yagi and Mase (70) and Botts et al. (71) has shown that addition of creatine kinase to myosin results in a lowered ATPase of myosin, presumably due to interaction between the two enzymes. Figure 29 shows the inhibitory effect induced by bovine creatine kinase on the ATPase of cardiac myosin, heavy meromyosin and subfragment-1. Maximum inhibition was found to occur when the two enzymes were in a 1:1 mole ratio. ATPase inhibition values of 26% for cardiac myosin, 20% for heavy meromyosin and 19% for subfragment-1 parallel the results reported for the rabbit skeletal system (82), which gave values of 25, 20 and 18%, respectively, for inhibition of skeletal myosin and its subfragments

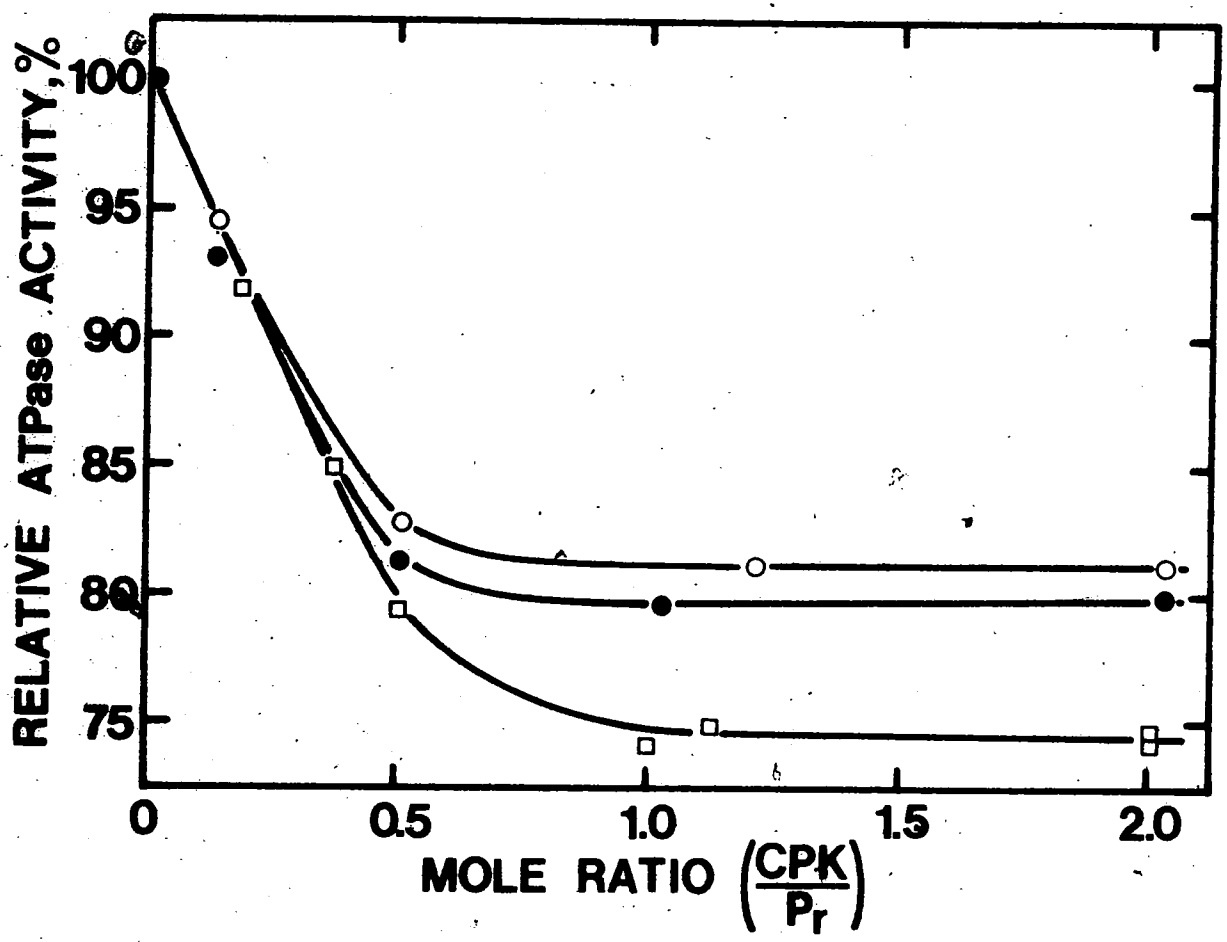


Figure 29. Inhibition of ATPase activity of cardiac myosin and its subfragments by creatine kinase from bovine cardiac muscle. The diagram displays the relative ATPase activities of the proteins P_r, where P_r represents myosin = open squares; heavy meromyosin = closed circles; subfragment-1 = open circles.

by creatine kinase from rabbit skeletal muscle.

2. Association of Creatine Kinase with Myosin Filaments

Morimoto and Harrington (75) had reported that creatine kinase promoted aggregation of synthetic skeletal myosin filaments in solution. By dialyzing myosin in 0.5 M KCl versus 0.13 M KCl, 2 mM barbital, pH 8.3, they were able to prepare synthetic myosin filaments with a very sharp size distribution and a smooth central region similar to natural thick filaments. The addition of creatine kinase to this system resulted in an increase in the sedimentation coefficient of the fast-moving polymer peak from 25 to 39S, and the formation of a larger aggregate species. An attempt to perform the same experiment using a cardiac myosin system did not prove feasible, since under the same conditions cardiac myosin did not remain soluble, but totally precipitated out of solution. Alternatively, a new set of conditions had to be determined.

A range of different pH's and ionic strengths were investigated: from pH 6.8 to 8.0, and ionic strengths of 0.12 to 0.15 M KCl. Using different combinations and different times of dialysis; it was found that cardiac myosin from bovine muscle formed aggregate synthetic myosin filaments after 16 hours in 0.14 M KCl, 10 mM Tris, pH 7.52. This system was found to be highly time dependent; prolonged exposure to dialysis showed such a high degree of aggregation that eventually no myosin was left in solution. Figure 30 shows the sedimentation profiles of cardiac myosin in the upper frame, and the same myosin with a known added quantity of creatine kinase (3.7 mg/ml) taken at different times after reaching 20,000 rpm.

Examination of the sedimentation velocity patterns shows that the addition of creatine kinase to this system results in a decrease in

Figure 30. Effect of added creatine kinase on the sedimentation profiles of cardiac myosin. The sedimentation profiles of myosin (5 mg/ml) are shown in the upper frame, and myosin and CPK (3.7 mg/ml) in the lower frame. The frames were taken at 16, 32, 48 and 64 min after reaching 20,000 rpm. The solvent was 0.14 M KCl, 10 mM Tris, pH 7.52 at 5°C. The first frame is taken as 0 min.

TABLE II

The Effect on S Values of Synthetic Cardiac Filaments by the Addition of Creatine Kinase

mg CPK added	$S_{20,w}$ of myosin (5 mg/ml)	$S_{20,w}$ of myosin + CPK
0.66	65.2, 66.7	88.6, 90.1
1.8	66.4, 102.7	79.5, 128.6
3.7	64.9, 109.2	76.2, 130.4

the area of the faster moving peaks and additionally an increased sedimentation coefficient. A larger aggregate species was found to sediment to the base of the cell prior to reaching operating speed. This larger aggregate is probably a combination of creatine kinase with the cardiac myosin filaments and is interpreted to be the direct cause of this change in the sedimentation patterns. Such changes in sedimentation patterns were found consistently, although exact values were not necessarily reproducible.

An indication of the effect on the S values of the myosin filament doublet peaks by the addition of creatine kinase is given in Table II, accompanying Figure 30.

3. Circular Dichroism Studies

Work by Mani and Kay (82) using circular dichroism experiments had indicated that creatine kinase isolated from the M-band of rabbit skeletal muscle interacts with skeletal myosin and its subfragments, and furthermore, this interaction decreases as one proceeds from myosin through to heavy meromyosin and to subfragment-1. CD measurements demonstrated conformational changes which were revealed as net increases in negative ellipticity at 221 nm as a result of interaction between the two enzymes. The net increases in ellipticity were 2400 degrees·cm^2·dmole^{-1} for myosin, 2000 for heavy meromyosin and 1000 with subfragment-1 upon interaction in each case with creatine kinase from rabbit skeletal muscle at a 1:1 mole ratio.

Comparable experiments were undertaken using cardiac myosin from bovine muscle. Cardiac heavy meromyosin and subfragment-1 were prepared by the method of Lowey et al. (111). Circular dichroism spectra were performed for myosin, heavy meromyosin and subfragment-1, each

at 1:1 mole ratio, with creatine kinase from bovine heart. A change in ellipticity was found only for the case of the myosin:creatine kinase system, giving a net increase in negative ellipticity of 500 degrees·cm^2·$dmole^{-1}$. This value is just outside experimental error. The CD results of this study reflect a difference in behaviour between the cardiac and skeletal systems in that with the cardiac components the fact that there is no change could reflect no interaction or possibly conformational changes are not detected by CD. The evidence of ATPase and myosin aggregation studies seems to favour the latter conclusion.

4. Gel Filtration Studies

Gel filtration studies were undertaken to examine the nature of this interaction between myosin and creatine kinase from bovine cardiac muscle. A Biogel A 0.5 M column was used to attempt isolation of a complex of these two components should it be present. Figure 31 shows the elution profile of a 1:1 mole ratio of the myosin creatine kinase mixture applied. No evidence of a myosin-CPK complex was observed. The applied quantities of myosin and creatine kinase could be accounted for in the two separated peaks. Furthermore, subsequent SDS-polyacrylamide gel studies showed no creatine kinase contamination present in the myosin peak. These results imply that the interaction may be of two types: either an equilibrium type of complex formation, or possibly an electrostatic one. Certainly, there is no evidence to support the formation of a tight complex such as that found for the 165,000 dalton component—subfragment-2 complex by Mani and Kay (92), who used similar gel filtration studies to isolate their complex.

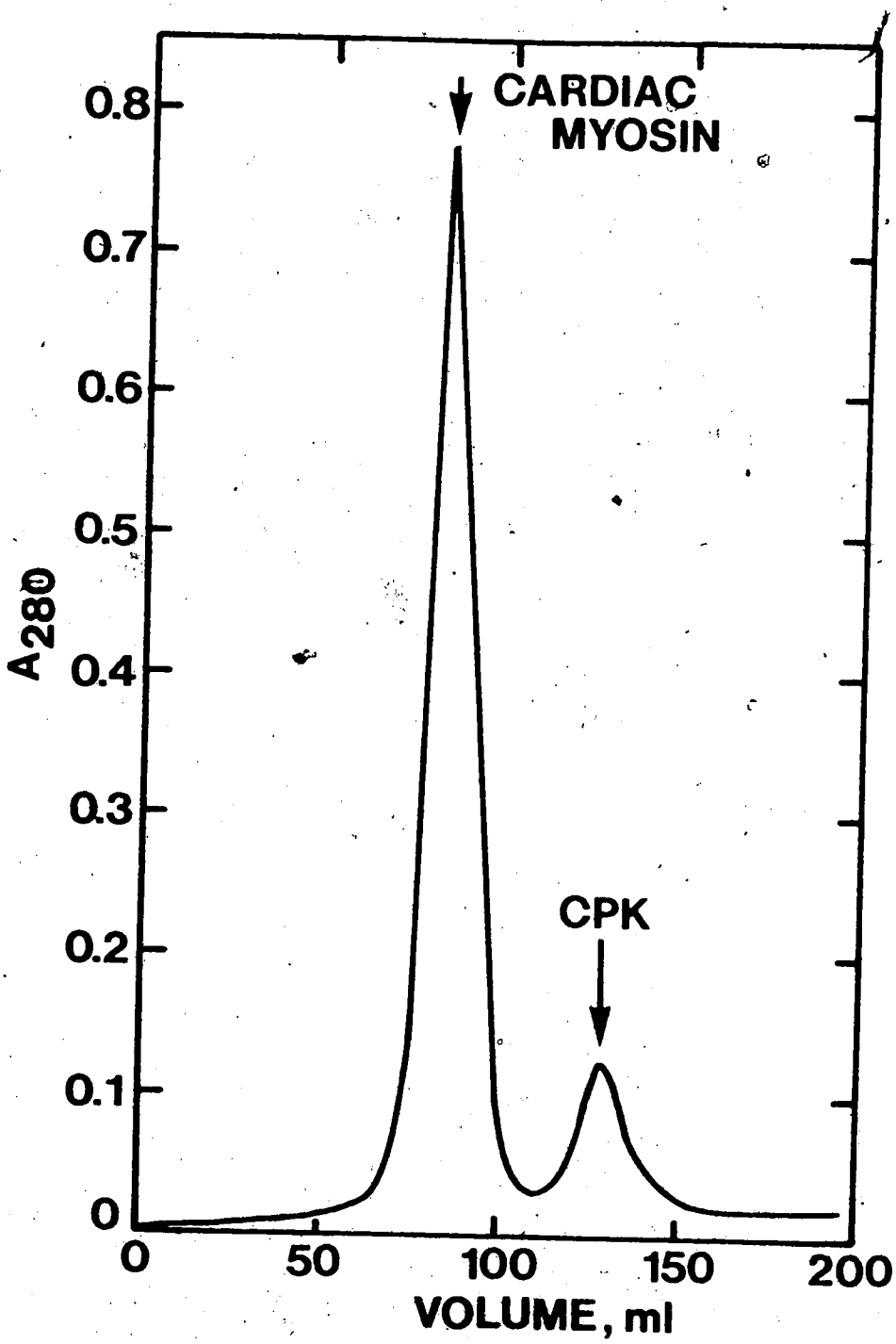


Figure 31. Gel filtration of a 1:1 mole ratio mixture of myosin and creatine kinase from bovine cardiac muscle. The diagram shows the elution profile of such a mixture applied to a Biogel A 0.5 M column in 0.5 M KCl, 2 mM phosphate, pH 7.

B. INTERACTION STUDIES OF CREATINE KINASE WITH THE 165,000 DALTON COMPONENT

1. Investigations Using a Bovine Cardiac Muscle Source

The presence of a second component of the M-band of striated muscle has been established and examined by several workers (86, 89, 91). This protein was extracted along with creatine kinase in a low ionic strength medium from rabbit (91) and chicken skeletal muscle (86, 89). Because the present study also used a low ionic strength extraction of creatine kinase, it was of interest to determine if an analogous 165,000 dalton protein was present within the extracts from bovine cardiac muscle.

Preliminary examinations of SDS-polyacrylamide gel patterns of the low ionic strength extract revealed the presence of a possible candidate for the 165,000 dalton component; however, unlike the case of rabbit skeletal muscle (91), separation proved highly difficult because of the low yield and the presence of 6 - 7 other, more abundant contaminants even after the first DEAE cellulose chromatography. For this reason, this method of preparation was abandoned and as an alternative procedure, the standard methods of Masaki and Takaiti (86), who employed both Guba Straub and Hasselbach-Schneider's solutions (HS) for extraction, were attempted. Their procedures, as designed for chicken skeletal muscle, disrupted the myofibril by virtual extraction of the myosin and in this manner they were able to isolate the 165,000 dalton component. Application of these same techniques to bovine cardiac muscle proved to be unsuccessful. It was found that the 165,000 dalton candidate appeared to possess a high affinity towards myosin, and consequently was lost along with the myosin in separating procedures.

Figure 32 shows a representative set of gel patterns at different stages of an HS preparation. It is evident that after the myosin has been removed all traces of the 165,000 dalton candidate have vanished.

The other standard published procedure of Trinick and Lowey (89) was also attempted. This method, involving extraction with high salt (0.6 M KCl), also proved ineffectual since the same difficulties were encountered. For this reason, studies of a hybrid nature were undertaken.

2. Hybrid Interaction Studies of Bovine Creatine Kinase with Rabbit Skeletal 165,000 Dalton Protein

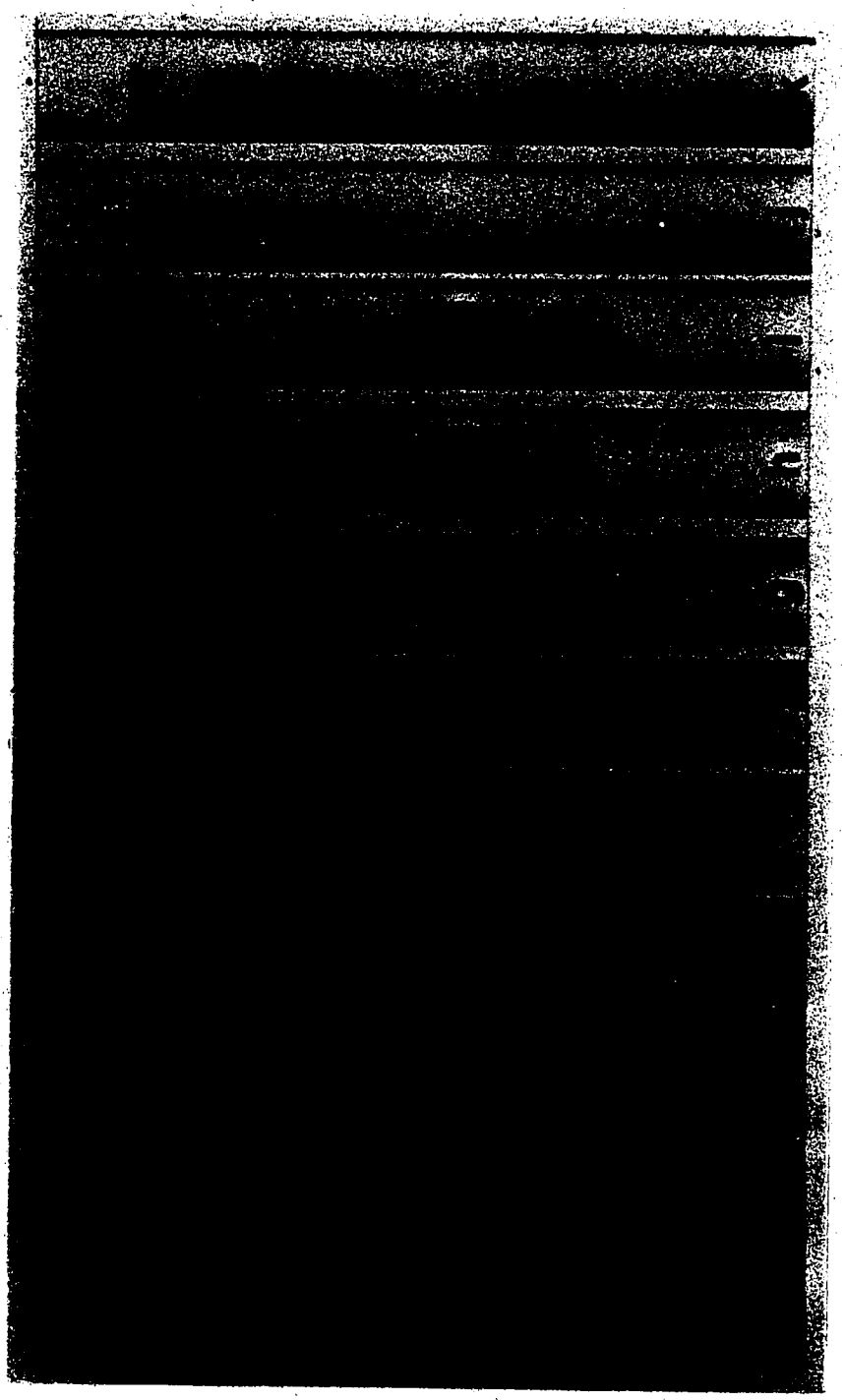
Mani and Kay (91) had found that the 165,000 dalton protein component from rabbit skeletal muscle inhibited the enzymatic activity of creatine kinase from the same source, and furthermore, had established the nature of this inhibition to be competitive. A maximum inhibition of nearly 30% was observed when the 165,000 dalton component and creatine kinase were present in a 2:1 mole ratio respectively.

Accordingly, the same experiment was repeated using the 165,000 dalton component from rabbit skeletal muscle and observing its effect on the activity of creatine kinase from bovine heart. A maximum inhibition of 13% was demonstrated at a 2:1 mole ratio of 165,000 dalton protein to CPK as shown in Figure 33. Considering this is a hybrid system, this value, though low, is significant.

A similar study with a chymotryptic fragment of the 165,000 dalton component (123) which has a molecular weight of 100,000 also showed inhibition, but only 8%.

Figure 32. 7% SDS-Polyacrylamide gel electrophoresis of a preparation of the 165,000 dalton component from bovine cardiac muscle. Gel patterns are of:

(a) initial extract of Guba-Straub procedure;
(b),(c) initial extracts of HS procedure at 25 and 50 µl loading concentrations. Note similarity to Guba-Straub pattern;
(d),(e) 0 to 40% ammonium sulfate precipitate from HS extract at 25 and 50 µl loading. Myosin precipitates;
(f) supernatant of 0 - 40% ammonium sulfate fraction from HS extract;
(g),(h) 40 - 55% ammonium sulfate precipitates from HS extracts at 25 and 50 µl loading;
(i) 55 - 85% ammonium sulfate precipitate from HS extract;
(j) supernatant following 55 - 85% ammonium sulfate precipitation. All traces of the 165,000 dalton candidate have disappeared;
(k) standard 165,000 dalton component from rabbit skeletal muscle.



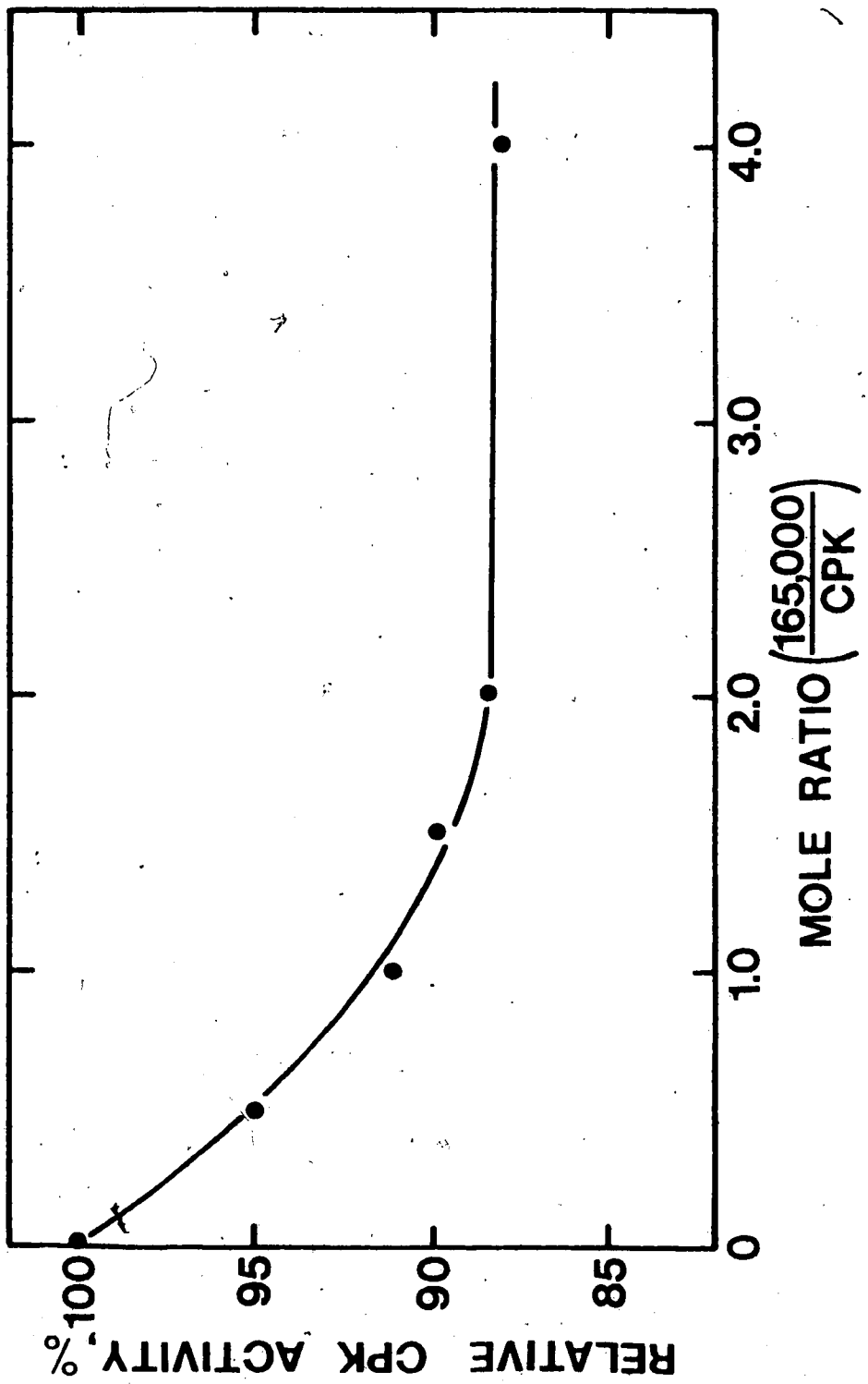


Figure 33. Inhibition of the enzymatic activity of creatine kinase from bovine cardiac muscle by the 165,000 dalton component of rabbit skeletal muscle.

C. SUMMARY

Creatine kinase from the myofibrils of bovine cardiac muscle has been shown to interact with its parent myosin and its subfragments. In the case of CD experiments, this interaction is different than that found in the skeletal case, but in ATPase studies bovine cardiac results parallel findings in the rabbit skeletal system. Similarly, creatine kinase also tends to promote filament formation in the cardiac myosin system as has been demonstrated in the skeletal case. The fact that the hybrid systems show a positive interaction implies two things. Firstly, bovine creatine kinase possibly could substitute in studies of the reconstitution of the M-band in myofibrils from other sources such as chicken or rabbit skeletal muscle, and secondly, it suggests that an analogue of the 165,000 dalton component is present in the low ionic strength extracts of bovine cardiac muscle.

CHAPTER V

IMMUNOBIOLOGY AND ELECTRON MICROSCOPY

A. INTRODUCTION

Bovine cardiac myofibrils, like their skeletal counterparts, possess a definite M-band structure (97). The methodology employed in the extraction of creatine kinase had been shown by Morimoto and Harrington (75) and subsequently by Walliman et al. (24) and Eppenberger et al. (124) to selectively remove the M-band from chicken skeletal muscle. Since the method of preparation of bovine cardiac CPK was the same as that used in earlier studies, it seemed imperative that an investigation be undertaken to localize the origin of the myofibrillar creatine kinase. This chapter therefore centres on antibody and ultrastructure studies designed to determine if the creatine kinase so extracted has the M-band as its source.

B. METHODOLOGY FOR ELECTRON MICROSCOPY OF BOVINE CARDIAC MYOFIBRILS

1. Preparation of Myofibrils

Glycerinated myofibrils were prepared according to Pepe (72). Prior to treatment, the myofibrils were cut into small 1 - 2 mm cubes. The myofibrils were then washed approximately 5 or 6 times with 0.1 M KCl, 20 mM potassium phosphate. A portion of these myofibrils were set aside as a control. The remaining myofibrils were subsequently extracted for 90 min in 5 mM Tris, pH 8.0, and a sample was removed at the end of this time period and placed back into 0.1 M KCl; 20 mM phosphate. The remaining myofibrils were allowed to be extracted overnight. Gentle agitation was allowed throughout these washes and extractions.

2. Fixation and Embedding Procedures

The myofibrils following washing treatment were further sectioned to 1 mm cubes and fixed in 2% glutaraldehyde (Polysciences), 0.2 M sodium phosphate buffer, pH 7.2 for 60 min. They were then washed three times with phosphate buffered sucrose (6.84%) over a 60 min period and fixed at room temperature with 2% osmium tetroxide (Polysciences) in phosphate buffer for 30 - 60 min. A final couple of washes with 0.2 M phosphate buffer for 15 - 30 min preceded the dehydration which was carried out in a step-wise fashion. The myofibrils were washed once with 50% ethanol for 5 min, twice with 70% ethanol for 10 min, twice with 95% ethanol for 10 min and twice with absolute ethanol over a 30 min period. Following dehydration, the myofibrils were placed in propylene oxide (Polysciences) with two changes for 30 min before being infiltrated overnight with a 1:1 mixture of propylene oxide:complete resin.

The preparation of the epoxy embedding resin was as follows: the resin was made up in two parts, A and B, with the final resin 3 parts A to 7 parts B in order to obtain the selected hardness for ease of section cutting.

In making up 50 ml of resin, 7.3 g of EPON 812 Epoxy Resin-epoxy equivalent 150±5 was added to 11.1 g of dodecenyl succinic anhydride (DDSA) to make solution A. 23.4 g of EPON 812 was then added to 19.66 g of nadic methyl anhydride (NMA) to make up solution B. All chemicals were from Polysciences. Solution B was poured into A in order to minimize the dilution of A. To make up enough for the overnight infiltration, 10 ml of the complete resin (i.e., 11.9 g) were removed and 12 drops of DMP-30 accelerator were added. This was then diluted 1:1 with propylene oxide, placed over the myofibrils, and allowed to infiltrate overnight.

The overnight infiltration mix was removed from the myofibril next day, and replaced by the rest of the complete resin to which 40 drops of DMP-accelerator had been added. Infiltration was allowed to proceed for 1 - 3 hours, with at least two changes of fresh resin. The myofibrils were then orientated in the smallest drop of resin in a Beem capsule and prepolymerized for 60 min at 60°C. The capsules were labelled, filled with complete resin and polymerized for two days at 60°C. Sections were then cut 60 - 120 nm thick on a microtome knife.

Sections were stained for 5 min with lead citrate at a 1:4 dilution with 0.1 N NaOH. (The stock solution was made up of 1.33 g lead nitrate and 1.76 g sodium citrate with 8 ml of 1 N NaOH in 50 ml total volume.) The sections were rinsed with 0.1 N NaOH, then water and dried. Further staining was done with saturated aqueous uranyl acetate for 10 min followed by a rinse with water before drying.

C. METHODOLOGY FOR IMMUNOBIOLOGICAL ASSAYS

Trinick and Lowey (89) had unequivocally established the presence of the 165,000 dalton component in the M-band of chicken skeletal muscle by the use of antibodies. Their results showed definite thickening of the M-band concurrent with a highly increased electron density in that region. An attempt to perform a similar experiment with the myofibrils of bovine cardiac muscle and antibodies to creatine kinase from bovine myofibrils was therefore undertaken. Since rabbits proved to be a poor source of antibodies, young roosters were chosen for the immunization procedures.

1. Immunization Procedures

Homogeneous creatine kinase from bovine cardiac muscle, 0.5 mg

in 0.5 ml of 50 mM Tris, pH 8, was emulsified with an equal volume of Freund's Complete Adjuvent (Difco Laboratories). Prior to injection, the two roosters were bled to provide a plasma control sample. The emulsion was injected into the breast muscle of the young roosters. One week later, a booster shot of 0.5 mg creatine kinase with Freund's Incomplete Adjuvent was given to each rooster. Three weeks after the first injection, the roosters were bled, and their plasma recovered after centrifugation.

2. Radiolabelling of Creatine Kinase

Homogeneous creatine kinase was labelled according to the Chloramine T procedure as described by Greenwood et al. (125). Approximately 100 µg/ml of the protein solution in phosphate buffered saline (PBS), pH 7.2, was taken up in a small tube and Na ^{125}I (0.5 - 1 mCi (millicurie); at 50 - 100 µl of 10 mCi/ml) in PBS was added followed by a freshly prepared solution of Chloramine T (25 - 50 µl) from a 5 mg/ml stock solution. The reaction mixture was thoroughly mixed by a Vortex mixer and left 4 - 6 min at room temperature. 50 - 100 µl of freshly prepared sodium metabisulfite (from a stock 12 mg/ml) was added and after 5 min the contents were transferred to a dialysis bag using 1% potassium iodide solution (0.5 - 1 ml) to rinse the glass tube. The protein was then dialyzed against PBS overnight at 4°C. The ^{125}I-labelled protein was stored in a glass container at -20°C and could be used for up to 6 - 8 weeks.

3. Radioimmunoassay Procedures

Radioimmunoassays were performed according to Hunter (126). The 3% polyethylene glycol used in this assay selectively precipitates antigen-antibody complexes (127, 128) of both IgM and IgG class.

The ^{125}I-labelled creatine kinase (25 µl) was taken up into a small tube and 25 µl of plasma were added followed by 0.5 ml of 0.1 M Tris-HCl, pH 7.2, and normal rabbit serum (1:1). The mixture was vortexed and left at room temperature for 30 min and then overnight at 4°C. A control sample received normal plasma instead of immune plasma. 7% polyethylene glycol at 4°C was then added and the mixture vortexed. The tubes were centrifuged at 2000 rpm for 60 min at 4°C. The supernatants and pellet were separated and the pellets washed once with 3% polyethylene glycol and centrifuged as before. The washings were added to the supernatant obtained above. Both supernatants and pellets were counted in a γ-counter. The percent antigen bound was expressed as:

$$\% \text{ antigen bound} = \frac{\text{CPM in pellet}}{\text{CPM in pellet} + \text{CPM in supernatant}} \times 100 \qquad [14]$$

where CPM refers to counts per min.

In both immunized roosters, radioimmunoassays demonstrated a high percentage of bound antigen, 78% in one rooster and 80% in the other, indicating an excellent immune response.

4. Purification of Antibodies

Antibodies to creatine kinase were purified by affinity chromatography (see Experimental Methods) using essentially the method employed by Trinick and Lowey (89). Plasma from both roosters was pooled, diluted 1:1 with PBS and placed on the affinity column. Elution of antibodies was accomplished by flushing with 4 M guanidine hydrochloride, 50 mM Tris, pH 7.5. The antibodies were then immediately dialyzed versus 0.15 M KCl, 0.015 M K_2HPO_4, 0.004 M KH_2PO_4 (a potassium analogue of PBS) to minimize exposure to guanidine. Samples were then frozen and stored at -20°C until required.

5. Labelling of Myofibrils

The labelling of bovine cardiac myofibrils with purified antibodies was performed by applying the technique of Trinick and Lowey (89). Small bundles of glycerinated myofibrils were first cubed, washed with 0.1 M KCl, 20 mM potassium phosphate, pH 7, to remove glycerol, and then incubated overnight in a 2 mg/ml solution of antibody in the same buffer. The myofibrils were then washed extensively and prepared for electron microscopy as described previously. A second sample was incubated in plasma that had antibodies to creatine kinase present.

D. RESULTS OF ULTRASTRUCTURE AND IMMUNOLOGICAL STUDIES

1. Low Ionic Strength Extraction

Figure 34 shows the effect of low ionic strength extraction on the ultrastructure of the myofibrils of bovine cardiac muscle. Figure 34a shows the intact Z and M lines. Figure 34b displays the result of a 90 min extraction with low ionic strength. Definite leaching of the M-band has occurred. Figure 34c shows the overnight extraction, and here again the M-band has been selectively removed, leaving behind a remarkably intact structure sans the M-band. These results parallelled the findings of Morimoto and Harrington (75) and encouraged the suggestion that the creatine kinase in this study may have the M-band as its origin.

2. Antibody Binding Studies

Figure 35 shows the effect of putting crude immune plasma and purified antibody on myofibril preparations. Examination of the three frames in Figure 35 reveals all samples to be virtually identical. No thickening of the M-band is apparent, nor is there an apparent increase

Figure 34. Electron microscopy of sectioned bovine cardiac myofibrils showing the effect of low ionic strength extraction (magnification = 27,300 X). An arrow indicates the position of the M-band.
(a) "control" glycerinated myofibrils washed with 0.1 M KCl, 20 mM potassium phosphate, pH 7;
(b) myofibrils following 90 min extraction with 5 mM Tris, pH 8;
(c) myofibrils following overnight extraction with 5 mM Tris, pH 8.
The apparent differences in sarcomere length are due to fixation at different stages of contraction.

Figure 35. Electron microscopy of sectioned bovine cardiac myofibrils showing the effect of incubation with chicken antibodies to bovine creatine kinase (magnification 27,300 X). An arrow indicates the position of the M-band.

(a) "control" glycerinated myofibrils washed with 0.1 M KCl, 20 mM potassium phosphate, pH 7;

(b) myofibrils following overnight incubation with purified antibody (2 mg/ml) in PBS, pH 7.2;

(c) myofibrils following overnight incubation with crude plasma diluted 1:1 with PBS, pH 7.2.

The apparent differences in sarcomere length are due to fixation at different stages of contraction.

in the electron density with either the addition of purified antibody or crude immune plasma.

Since the M-band could be expected to act like an affinity column and selectively bind the antibodies present, these results suggested three possibilities. The first was that perhaps the creatine kinase isolated in this study may not have its origin in the M-band but may be adventitiously bound to the myofibril. The latter point appeared to be ruled out since no binding at all was demonstrated throughout the myofibril. Secondly, perhaps the aggregation phenomenon of chicken antibodies (a process which increases as purification proceeds) hampers their infiltration into the myofibrils during incubation. Thirdly, and most likely, the antigenic sites on creatine kinase may become inaccessible once it is bound to the myofibril. The question of whether or not creatine kinase has its origin at the M-band, therefore, had not been conclusively proven one way or the other by the use of antibodies.

E. RECONSTITUTION OF THE M-BAND

Because binding of chicken antibodies to the cardiac myofibril could not be established, an attempt was initiated to try and re-form the M-band structure using its known components creatine kinase and the 165,000 dalton protein. During the course of this study, the presence of a possible candidate for the 165,000 dalton protein had been noted in low ionic strength extracts of bovine cardiac muscle (see Chapter IV). Although purification of this candidate proved unsuccessful, hybrid experiments had suggested that the 165,000 dalton component from rabbit skeletal muscle could conceivably substitute for its bovine analogue. Rabbit skeletal 165,000 dalton protein was thus used in recon-

stitution studies in the cardiac system of bovine muscle. In addition, reconstitution studies in an all skeletal system were also performed in order that a comparison of the skeletal and cardiac systems would be complete.

1. Methodology of Reconstitution

Glycerinated myofibrils were prepared as already discussed. Extensive washing was accomplished with 0.1 M KCl, 20 mM phosphate pH 7, and a portion of these myofibrils were set aside to serve as controls. The remaining myofibrils were subsequently extracted for two hours in 5 mM Tris pH 8, and a sample was removed at the end of this time period to serve as an "extracted control". The rest of the extracted myofibrils were used for reconstitution studies.

The myofibrils were placed in solutions of creatine kinase (1 mg/ml) and 165,000 dalton component (2 mg/ml) using PBS, pH 7.2, as the reconstituting buffer system, and subjected to gentle agitation. Samples in which the two components were added sequentially were gently stirred for two hours with only one M-band protein present followed by the second component at about a 1:1 mole ratio. All reconstituted samples were then allowed to incubate overnight. The excess protein was washed away with 0.1 M KCl, 20 mM phosphate pH 7 before preparation of the myofibrils for electron microscopy as described previously.

2. Results of Reconstitution Studies

Figure 36 shows the results for the cardiac system. The washed myofibrils of bovine cardiac muscle appear intact with distinct Z and M bands evident in Figure 36a. Extraction with low ionic strength shows definite leaching of the M-band as seen in Figure 36b. The addition of creatine kinase in Figure 36c, and of the 165,000 dalton component in

Figure 36. Reconstitution of the M-band of bovine cardiac myofibrils. Shown are the electron micrographs of sectioned bovine cardiac myofibrils (magnification 27,300 X). An arrow indicates the position of the M-band.

(a) "control" glycerinated myofibrils washed with 0.1 M KCl, 20 mM potassium phosphate, pH 7.0;

(b) myofibrils following extraction with 5 mM Tris, pH 8.0 for two hours;

(c) Tris-extracted myofibrils following overnight incubation with creatine kinase (1 mg/ml in 0.15 M phosphate buffered saline) from bovine cardiac muscle;

(d) Tris-extracted myofibrils following overnight incubation with the 165,000 dalton (2 mg/ml in 0.15 M phosphate buffered saline) from rabbit skeletal muscle.

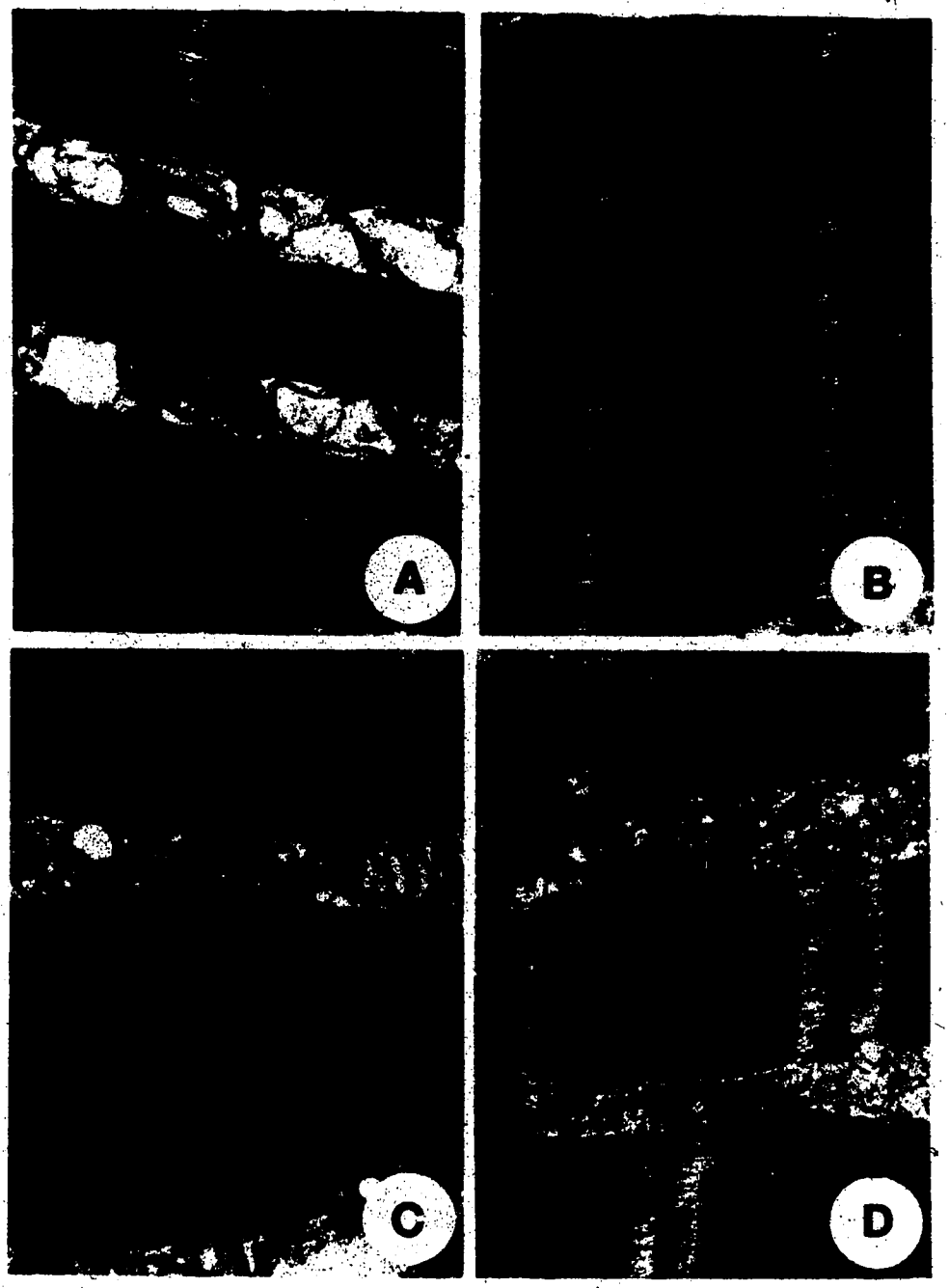

A
B
C
D

Figure 36 continued.

(c) as before, previous page;

(d) as before, previous page;

(e) Tris-extracted myofibrils following 2 h incubation with bovine CPK (1 mg/ml), then overnight with added 165,000 dalton protein (2 mg/ml) from rabbit skeletal muscle in 0.15 M phosphate buffered saline;

(f) Tris-extracted myofibrils following 2 h incubation with 165,000 dalton component from rabbit skeletal muscle (2 mg/ml), then overnight with added bovine CPK (1 mg/ml) in 0.15 M phosphate buffered saline.

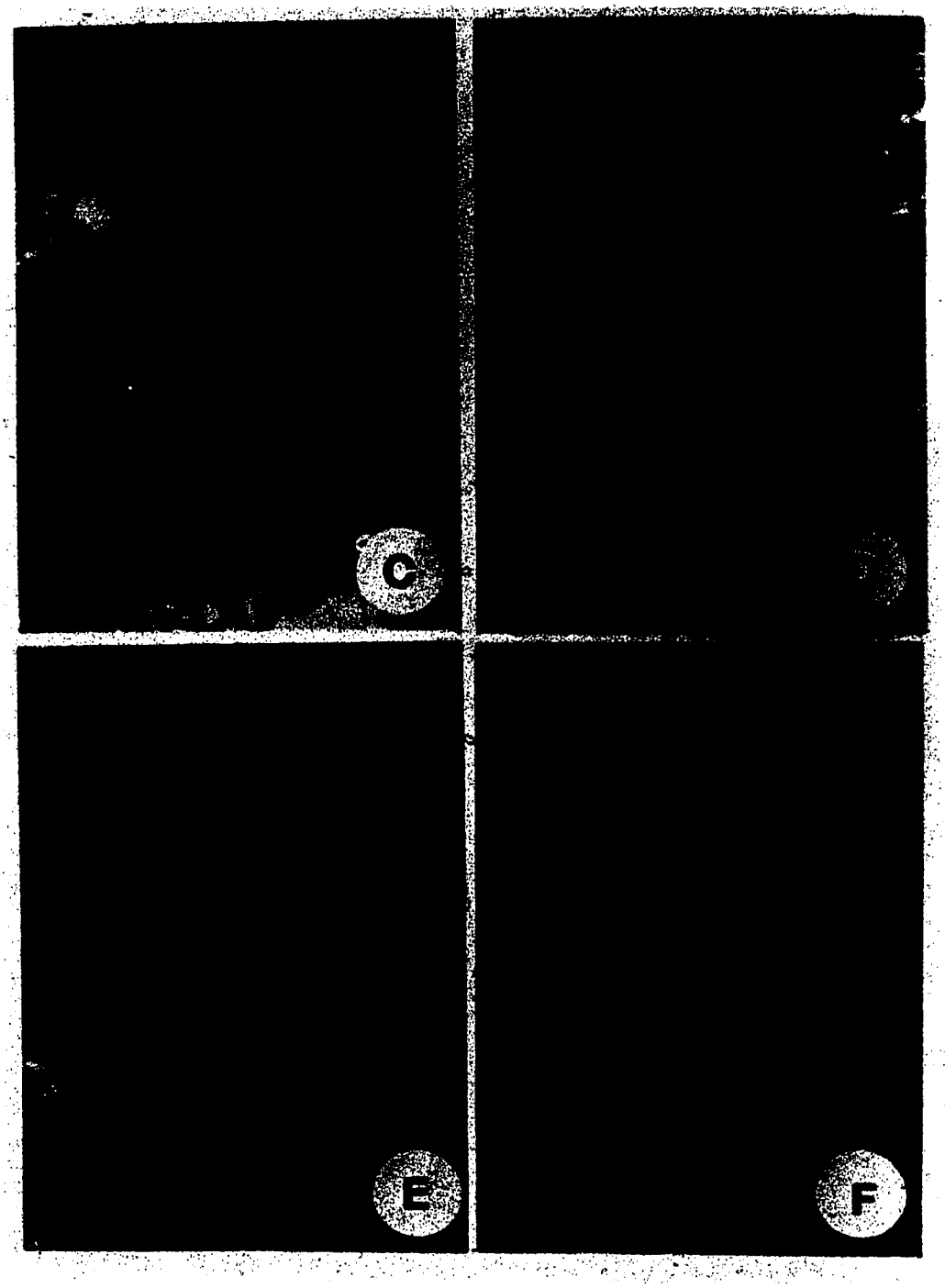

C
E
F

Figure 36d, show a distinct reappearance of the M-band structure. Sequential additions of creatine kinase and the 165,000 dalton component, and that with the order reversed, produced electron micrographs as shown in Figures 36e and 36f. Examination of the relative intensities of the Z and M bands in these micrographs reveals that the M-band reappearance with both components present appears virtually identical to that seen with only one component present.

The myofibrils of rabbit skeletal muscle show a parallel display in Figure 37. Figure 37a shows intact Z and M bands, Figure 37b shows leaching of the M-band, and Figures 37c and 37d show its reappearance. The sequential additions with both components present are shown in Figures 37e and 37f, and here again the relative intensities of the Z and M lines of these micrographs are indistinguishable from those with only one component.

F. SUMMARY

Ultrastructure studies using electron microscopy have shown that the use of a low ionic strength extraction procedure selectively removes the M-band of the myofibrils of bovine cardiac muscle. Despite the lack of success with antibody studies, these experiments did serve to illustrate the possible ambiguity of negative results using antibodies. When binding by antibodies to the myofibril is demonstrated, it proves conclusively that the antigen must be present within the myofibril. However, should no such binding be evident, this fact in itself is not definitive proof that the antigen is not present, since stereochemical factors may be accounting for this non-binding. The fact that creatine kinase seems to re-form the overall structure suggests its origin to be within the

Figure 37. Reconstitution of the M-band of rabbit skeletal myofibrils. Shown are the electron micrographs of sectioned rabbit skeletal myofibrils (magnification = 27,300 X). An arrow indicates the position of the M-band.

(a) "control" glycerinated myofibrils washed with 0.1 M KCl, 20 mM potassium phosphate, pH 7.0;

(b) myofibrils following extraction with 5 mM Tris, pH 8.0, for two hours;

(c) Tris-extracted myofibrils following overnight incubation with creatine kinase (1 mg/ml in 0.15 M phosphate buffered saline) from rabbit skeletal muscle;

(d) Tris-extracted myofibrils following overnight incubation with the 165,000 dalton (2 mg/ml, in 0.15 M phosphate buffered saline) from rabbit skeletal muscle.

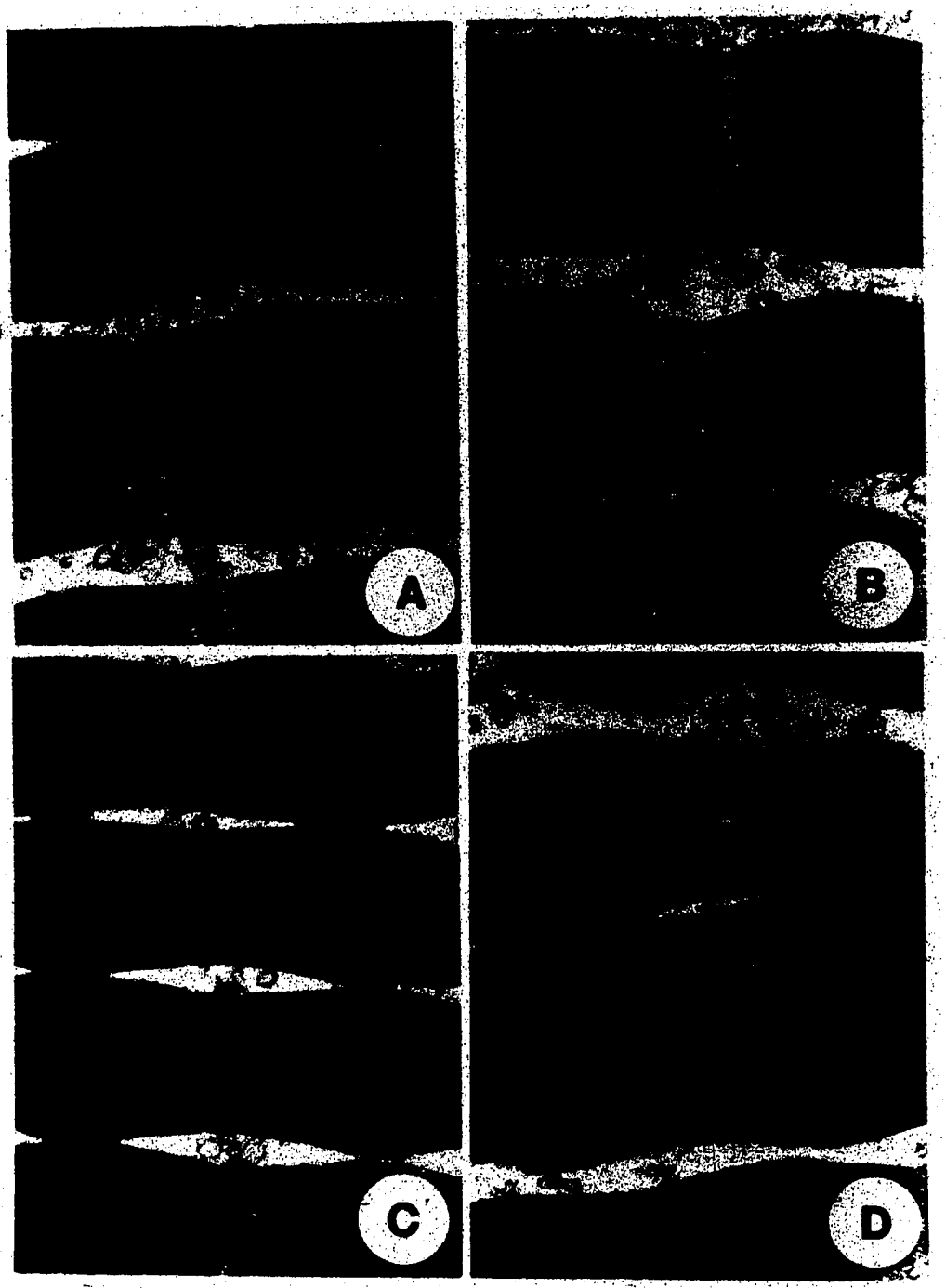

A
B
C
D

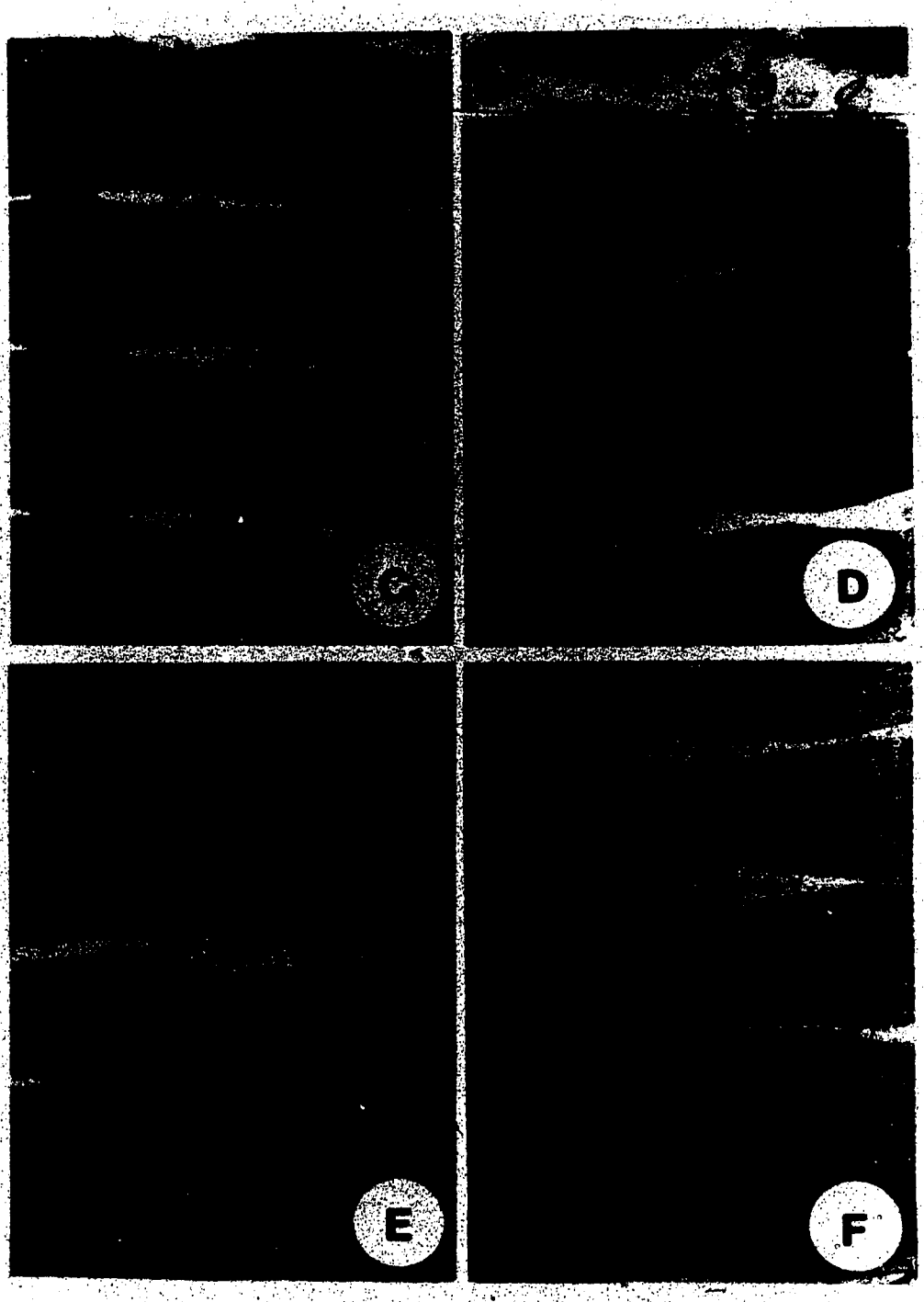
Figure 37 continued.

(c) as before, previous page;

(d) as before, previous page;

(e) Tris-extracted myofibrils following 2 h incubation with rabbit CPK (1 mg/ml), then overnight with added 165,000 dalton protein (2 mg/ml) from rabbit skeletal muscle in 0.15 M phosphate buffered saline;

(f) Tris-extracted myofibrils following 2 h incubation with the 165,000 dalton component (2 mg/ml) from rabbit skeletal muscle, then overnight with added rabbit creatine kinase (1 mg/ml) in 0.15 M phosphate buffered saline.


C
D
E
F

M-band region of bovine cardiac myofibrils. Furthermore, the ability of the 165,000 dalton component from rabbit skeletal muscle to substitute in reconstituting the M-band of bovine cardiac myofibrils argues for the probable existence of a bovine analogue in the M-band of cardiac muscle. The ability of both creatine kinase and the 165,000 dalton protein to re-form the M-bands independently of each other indicates that their binding sites within the M-band are probably different. This is consistent with earlier findings by Mani and Kay (82) and Herasymowych et al. (83) that creatine kinase interacts with the head region of myosin, while the 165,000 dalton protein interacts with the S-2 portion of the myosin molecule (92).

CHAPTER VI

CONCLUDING DISCUSSION

The previous chapters have focussed on characterization and localization studies of the creatine kinase extracted from bovine cardiac muscle at low ionic strength, as well as determining some of the possible interactions which may be operating in the myofibril at both enzymic and structural levels. The purpose of the following discussion is to summarize the main conclusions and, in particular, the implications arising from this study. In this context, an attempt will be made to reconcile the biochemical data of this investigation with the literature available on the M-band proteins from other sources, with the intent of integration, if possible, with the existing model of the M-band, the Knappeis-Carlsen model. In addition, suggestions for further studies arising from this investigation are presented.

A. CHARACTERIZATION STUDIES ON CREATINE KINASE

As indicated in detail in Chapter III, the creatine kinase isolated, purified and characterized from bovine cardiac myofibrils has been shown to possess most of the general characteristics found for cytoplasmic creatine kinases. In particular, comparisons of amino acid composition, ultracentrifugal studies, polyacrylamide gel electrophoresis, α-helical content and kinetic parameters show similar features (39, 41, 43). In addition, it would appear that the myofibrillar enzyme possesses all the characteristics of the MM form found in the cytoplasm of beef heart. This binding of MM-CPK in the myofibrillar compartment could be distinctly advantageous, since the regeneration of ATP would

thus occur close to the contractile apparatus. This same conclusion has been proposed by Walliman et al. (96) in their studies on chicken skeletal muscle, and by Ogunro et al. (57) who examined compartmentation in skeletal guinea pig muscle.

The MM and BB forms have been shown by Eppenberger et al. (124) to be capable of rebinding at both the M and Z bands of chicken skeletal and cardiac muscle which have been previously extracted to remove all traces of creatine kinase. However, there appeared to be a distinct preference for the MM form to bind at the M-band, and the BB form to bind at the Z band. In the present investigation, there was no evidence of MB or BB isozymes present in the myofibrillar preparations, although in bovine cardiac muscle, the MB isozyme which is usually found in much smaller amounts than the MM form could be expected (42). This suggests that in this study, the preparative procedure used was highly selective for the MM form, or that the MM isozyme is the only one associated with the M-band. In view of the results from the skeletal studies on chicken (24, 75, 79, 96, 124) and rabbit (82), the latter suggestion is probably likely.

A suggestion for further studies in reconstitution of the M-band which would be of interest therefore could include the substitution of the other isozymes of creatine kinase, MB and BB. Although the different forms are very similar, perhaps small differences are fundamental in interactions with the components of the contractile apparatus. Evidence for specific or preferential binding of the different isozymes may possibly reveal distinct functions for the MM, MB and BB forms. Mechanisms involving such distinct functions are attractive in that they may account for the existence of creatine kinase isozymes with

stage and tissue-specific distributions (129).

B. INTERACTION STUDIES OF CREATINE KINASE WITH MYOSIN AND ITS SUB-FRAGMENTS

1. ATPase and Circular Dichroism Studies

The ATPase studies involving the inhibitory effect of CPK from bovine cardiac muscle on the ATPase of cardiac myosin, heavy meromyosin and subfragment-1 have been shown to parallel the findings of Mani and Kay (82) in the rabbit skeletal system. The inhibition of ATPase of myosin, heavy meromyosin and subfragment-1 was determined to be 25, 20 and 19% respectively, when the two enzymes were in a 1:1 mole ratio. These values compared favourably to the values of 25, 20 and 18%, respectively, for the rabbit skeletal system.

Mani and Kay (82) had further demonstrated this interaction using circular dichroism to indicate conformational changes which were revealed as net increases in negative ellipticity at 221 nm. They reported net increases in ellipticity of 2400 degrees$\cdot cm^2 \cdot dmole^{-1}$ for myosin, 2000 for heavy meromyosin and 1000 for subfragment-1 upon interaction in each case with rabbit skeletal CPK at a 1:1 mole ratio. In contrast to these results, comparable experiments with the cardiac system revealed a net increase in negative ellipticity of only 500 degrees$\cdot cm^2 \cdot dmole^{-1}$ with myosin only. This result implies either no interaction is occurring, or no conformational changes are detectable by circular dichroism upon interaction. This disparity is not difficult to reconcile when one considers the inherent differences of skeletal and cardiac myosins. The ATPase results suggested the interaction to be at or near the site of the globular heads of myosin. Cardiac myosin shows a distinct dif-

ference from its skeletal counterpart at the level of the light chains. Each molecular of cardiac myosin has 3 polypeptides of 2 different types of alkali chains, while skeletal myosin has 4 polypeptides of 3 types. Since the ATPase function appears to be associated with an interaction of light chains with the heavy chains, the source of the disparity may be at the level of the light chains. With respect to these interaction studies, the CPK in both cardiac and skeletal systems probably inhibits the ATPase in a similar fashion, but in terms of a conformational change upon this interaction, the results as detected by circular dichroism may be somewhat different.

The ATPase results of this investigation, along with that for the rabbit skeletal system (82), are in accord with the reports by Yagi and Mase (70) and Botts et al. (71, 81). This general phenomenon of CPK interacting with the "head" region of myosin is difficult to reconcile with the model of the M-band, since the M-band is supposedly an area devoid of "heads". In addition, it seems in conflict with the result reported by Houk and Putnam (80) who found CPK to interact with the "rod" portion of myosin. This latter result is far more consistent with the model of the M-band. One possible explanation for this discrepancy is perhaps due to the dual nature of the myofibrillar CPK. It will be recalled that the vast bulk of CPK is found in the cytoplasm (57, 124), presumably bathing the myosin found throughout the A band. At this level, perhaps the ATPase results reflect some type of regulatory control for both the cytoplasmic and myofibrillar compartments. The structural role of CPK, on the other hand, could conceivably be localized on the rod portion of myosin, as Houk and Putnam's data suggested (80). Perhaps the techniques of electron paramagnetic resonance

and nanosecond fluorescence polarization used by Botts et al. (81) and circular dichroism used by Mani and Kay (82) are not able to detect these changes.

2. Aggregation of Synthetic Cardiac Myosin Filaments

Since creatine kinase supposedly forms cross-bridges between adjacent thick filaments of the A-band, the results of the aggregation studies proved to be of special interest. Creatine kinase from bovine cardiac muscle was found to promote aggregation of synthetic myosin filaments from bovine cardiac muscle. Although conditions had to be varied, this result parallelled the findings for rabbit skeletal myosin (75). Morimoto and Harrington, in addition to reporting an increased sedimentation coefficient of 25 to 39S, observed these aggregates in the electron microscope, and found a synthetic filament of indefinite length and thickness. In cardiac myosin, the aggregation was not examined in the electron microscope. Hence, a suggestion for further studies in this area would be to examine the filaments in the electron microscope.

C. ISOLATION PROCEDURES OF THE 165,000 DALTON COMPONENT FROM BOVINE CARDIAC MUSCLE

A primary objective for further studies arising from this investigation would be to accomplish isolation and purification of the 165,000 dalton candidate from bovine cardiac muscle. The presence of this protein in bovine cardiac muscle was implied by the inhibitory effect of the 165,000 dalton protein on the enzymatic activity of cardiac creatine kinase (13%) and further established by the reconstitution studies where the rabbit skeletal 165,000 dalton protein was able to

substitute for its bovine analogue.

The difficulties encountered in trying to extract the 165,000 dalton component from bovine cardiac muscle parallel the problems of Masaki and Takaiti (86), who elected to use a technique (Hasselbach-Schneider's solution) which effectively removes the myosin and disrupts the entire myofibrillar structure. One outstanding characteristic which was noted during this study was the apparent affinity of the 165,000 dalton candidate to myosin, a property also reported by Masaki and Takaiti (86). Therefore, one approach to isolate this protein could be to use Hasselbach-Schneider's solution for extraction, but modify the ensuing procedure by maintaining the myosin in a soluble form and subsequently separate it from the 165,000 dalton candidate by gel filtration and DEAE cellulose methods. Should either of these chromatographic techniques be unsuccessful, then as a final alternative, denaturing conditions of 4 M guanidine hydrochloride or 6 M urea could be attempted.

D. ELECTRON MICROSCOPY AND IMMUNOBIOLOGICAL STUDIES

Earlier studies on the M-band of skeletal muscle had indicated that the M-band could be selectively removed by low ionic strength buffers. Pepe and Huxley (130) had shown that "M-protein" appeared to be loosely attached to the thick filaments and became released with vigorous homogenization. Samosudova (73), using electron microscopy techniques, was able to demonstrate that removal of the M-band from myofibrils did not result in any effect on the myosin filaments. The buffer system they chose was 5 mM Tris, pH 8, after the procedure of Perry and Corsi (131). Samosudova (73), Stromer et al. (132) and Kundrat and Pepe (74) were able to demonstrate that the extracted material could also be added

back to re-form the M-band structure. These results suggested that reconstitution studies could prove highly informative. Subsequent investigations in this field by Morimoto and Harrington (75), Trinick and Lowey (89) and Eppenberger and his colleagues (24, 79, 96, 124) involved antibody and ultrastructure techniques. These studies on the skeletal system encouraged the initiation of a similar investigation of the bovine cardiac system. Cardiac myofibrils, like their skeletal counterparts, possess many similar features. It is known that in skeletal muscle, CPK appears bound to the myofibrillar structure (57, 62, 64) and seems involved in M-band structure (97). It therefore was of interest to examine, and possibly determine if a parallel situation exists in cardiac muscle. With this end in view, localization studies of the myofibrillar creatine kinase from bovine cardiac muscle were initiated.

1. Antibody Studies

Extraction of bovine cardiac myofibrils with 5 mM Tris, pH 8, selective removal of the M-band when sections were examined in the electron microscope. Prolonged overnight extraction showed a remarkably intact structure minus the M-band, and this result encouraged further investigation of the origin of the myofibrillar creatine kinase. The results of Morimoto and Harrington (75) have since been confirmed many times by Eppenberger and his colleagues (24, 79, 96, 124). Hence when the localization studies of the bovine myofibrillar CPK antibodies showed no binding to the myofibril, the result was unexpected. It seemed to indicate a complete disparity with skeletal results. The possibility that the myofibrillar creatine kinase did not have its origin in the M-band came reluctantly to mind. A second possibility was that antibody binding was being sterically hindered. Due to the similarity

in phylogeneticity between beef and rabbit, antibodies to bovine cardiac CPK had to be produced in chickens rather than the preferred rabbit source. Chicken antibodies possess a characteristic aggregation property which could conceivably hamper the infiltration of antibodies into the myofibril. To overcome this aggregation phenomenon, myofibril sections that were already stained and fixed were incubated with antibody which had been sonicated to disrupt any aggregates. Examination of these sections in the electron microscope showed no evidence of antibody binding. It seemed, therefore, most likely that creatine kinase bound in the myofibril may have its antibody-antigen binding sites blocked. Alternatively, an attempt was made to try and reconstitute the myofibril after it had undergone M-band extraction.

2. Reconstitution Studies

Reconstitution studies have been reported by Stromer et al. (132) and Kundrat and Pepe (74) who found that M-bands that have been extracted can be reconstituted by treatment with the extracted solution. Stromer et al. (132) had further reported that the extract, minus creatine kinase, seemed unable to reconstitute the M-band. Eppenberger et al. (124) have attempted reconstitution of both skeletal and heart muscle M-bands in chicken and found that incubation of extracted myofibrils with MM-CPK alone did not lead to re-forming a normal M-band, although an increase in diffuse electron dense material in the M-band could be detected. They found preferential binding of MM-CPK to the M-band, and of BB-CPK to the Z lines, although both enzymes were able to bind at both M and Z bands. They noted that glycerination prior to extraction, and the duration of the extraction, influenced the subsequent binding.

In view of these results, the clarity of the reconstitution

micrographs in this study is in itself a remarkable feature. Perhaps this can be attributed to species difference, or more likely to the method of reconstitution. The choice of phosphate buffered saline was prompted by the feeling that the myofibril should be incubated in a system as close to its natural state as possible. Perhaps this buffer system, combined with a quick preparative technique, facilitated reconstitution of the definitive M-band structure by both the creatine kinase and the 165,000 dalton component from rabbit skeletal muscle.

Observations of particular interest were noted in the electron micrographs of reconstitution studies in both the cardiac and skeletal myofibrils. When one compares the Z to M-band relative intensities, both the CPK and the 165,000 dalton proteins appear to bind with equal electron density at the M-band. What is more puzzling is that reconstitution micrographs of the sequential studies with both components present do not seem to show an additional electron density at the M-band. One would expect that binding of both components at two different sites would double, or at the least increase, this density. The fact that this is not the case demands an explanation and further study. One possibility could be that binding of the first component may alter the configuration of the M-band structure so that the second is sterically hampered for binding. Perhaps, some or all of the first component molecules become displaced; if so, it would be of interest to determine which is preferred. Alternatively the two components have been shown to interact with each other (91). They thus could possibly affect the subsequent binding such that both could be present in specific amounts.

One approach to study the roles of these components could be applied to the skeletal system since both CPK and the 165,000 dalton

protein are likely to produce antibodies with application of the methods of Trinick and Lowey (89) and Eppenberger (124). Taking the case of the 165,000 dalton component as an example, one could reconstitute the M-band with only the 165,000 dalton protein, and following with binding by antibodies to the 165,000 dalton component. In a second experiment one could add first CPK and then follow with the 165,000 dalton component to reconstitute the extracted myofibril. Myofibrils from this sequential addition could be incubated with antibody to the 165,000 dalton protein and the relative intensity of binding at the M-band could be compared with the micrographs from the first experiment. If CPK does sterically prevent binding of the 165,000 dalton protein, no antibody binding should be evident in the sequential study micrograph. If a decreased intensity of antibody binding occurs, this indicates possibly partial binding of the 165,000 dalton protein after the initial binding of CPK. In a like manner, the reverse experiment using CPK alone, and in sequential additions, could be performed to hopefully show if this equal density is due to one or both components being present.

The results of the reconstitution studies have certainly fulfilled the main goal of this project, namely to localize the myofibrillar creatine kinase from bovine cardiac muscle. The ability of the bovine creatine kinase to re-form the overall M-band structure not only suggests its origin is within the M-band region, but also unequivocably demonstrates it has a structural role. Furthermore, the ability of the rabbit skeletal 165,000 dalton protein to substitute in reconstituting the M-band of bovine cardiac myofibrils argues for the probable presence of a bovine analogue in the M-band of cardiac muscle.

The results of these reconstitution studies, together with the

biochemical data, may now be integrated with the Knappeis-Carlsen model (19). The ability of both creatine kinase and the 165,000 dalton protein to re-form the M-bands independently of each other has some interesting implications. It suggests that their binding sites within the M-band are probably different. This is consistent with the findings from the ATPase studies that creatine kinase interacts with the head region of myosin (82, 83) while the 165,000 dalton protein interacts with the subfragment-2 portion of the molecule (92). In the Knappeis-Carlsen model, myosin interacts with creatine kinase and not the M-filament, presumably the 165,000 dalton protein. The fact that the 165,000 dalton component can independently re-form the M-band suggests that it too is an integral structural element within the M-band. This discrepancy with the model, i.e., the binding of the 165,000 dalton component to myosin, may be an indication that the model suffers from a basic inadequacy because it could reflect only one static contractile state. In dynamic contraction, undoubtedly many enzymatic functions come into play, and perhaps the interactions noted in this study, along with those from other laboratories, are only a small part of the total features involved in the M-band.

One aspect of interest in the M-band is related to changes in sarcomere length. The distance between thick filaments is known to increase as the sarcomere length shortens during contraction (134), yet the M-band patterns observed in longitudinal and transverse sections in the electron microscope are the same at all sarcomere lengths. In order to explain this, the M-band structure must expand reversibly to accommodate the variable interfilament distance. One way this could be accomplished is if the M-bridges possess an ability to "stretch" or slip

past each other. Two observations have been reported which suggest the M-band structure is different at different sarcomere lengths. Kundrat and Pepe (74) found that the M-band could be removed completely in 15 min extractions only from fibrils with sarcomere length greater than 2.1 μ. Fibrils at less than 1.9 μ retained their structure for up to 3 hours. At short lengths, the I filaments penetrate the M-band region and it seems unlikely they inhibit extraction since the increased space between thick filaments would compensate for interference to extraction by the thin filaments. This result suggested solubility characteristics of the longer M-bridges are different from that of the shorter bridges. A second observation by Frank et al. (135) indicates a structural variation in the M-band. These workers found muscle fixed in formaldehyde and stained with fluorescent antibodies showed binding only at longer sarcomere lengths. Those myofibrils which had contracted by 40% of the resting length were unstained. This result implied that the longer M-bridges found at short sarcomere length were antigenically altered by the formaldehyde, while the shorter M-bridges at long sarcomere length remained unaffected. It has been speculated by Eaton and Pepe (94) that some type of complex-subunit transition is involved as the M-bridge lengthens or shortens during contraction.

The possibility that the MM-CPK at the M-band is important in establishing the proper register of myosin molecules for construction of the thick filaments seems unlikely in view of the finding of Etlinger and Fischman (136) that in developing muscle, M-bands are not visible at early stages of myofibril assembly. However, the appearance of the M-band later on is in accord with a stabilizing role in maintaining the integrity of the constructed thick filament. Perhaps the MM-CPK acts

along with other M-band proteins as stabilizing crosslinks between the thick filaments and M-filaments.

In conclusion, this study has succeeded in displaying the parallel features of the M-band in both cardiac and skeletal muscle systems. The main contribution rests with the reconstitution studies, which for both cardiac and skeletal M-bands have proven that creatine kinase and the 165,000 dalton component possess structural roles. The interaction studies of bovine cardiac creatine kinase have been shown, in general, to parallel findings of similar studies in the skeletal systems. These studies for the most part fit in with the Knappeis-Carlsen model, although it appears that the model still falls short of explaining all the data available. Nonetheless, it serves as a preliminary prototype which has proven to be useful in formulating some general principles of M-band structure. It remains to be seen if it can be improved upon in the future. Certainly, this study has not produced evidence to discard the model as it now stands.

E. SUGGESTIONS FOR FURTHER RESEARCH

The present investigation has produced questions that could conceivably form the basis of future experimentation. At this point, it would be appropriate to summarize some suggestions for further study. The following objectives are submitted for future research projects in both the skeletal and cardiac systems where appropriately suited.

The use of MB and BB isozymes for reconstitution studies may reveal if the M-band is spetific for a particular enzyme. Particularly in the cardiac case, perhaps antibody binding to the BB form when it is present in the myofibril may be more successful than has been demonstrated

in the MM-CPK case. Should this be so, then the use of antibody methods may serve as a viable technique in detecting this isozyme in reconstitution studies.

The isolation of light chains from myosin and subsequent interaction studies with creatine kinase may prove of interest, in that a comparison of both the skeletal and cardiac systems may show some inherent differences.

The isolation, purification and characterization of the 165,000 dalton protein from bovine cardiac muscle should be realized.

Another objective would be the formation, in vitro, of a synthetic M-band using myosin, CPK and the 165,000 dalton component with subsequent examination of this formation in the electron microscope. Such an experiment, if successful, may prove of benefit in formulating ideas of M-band assembly.

The reconstitution studies with both the CPK and the 165,000 dalton proteins added sequentially should be clarified.

These are but a few of the possibilities that have arisen from this project. This study has served as a threshold for further research in the M-band of cardiac and skeletal systems, and at this stage it seems apparent that the bulk of future experiments in this area will likely involve the techniques of electron and light microscopy in conjunction with immunobiology.

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