

STUDIES ON THE M-SUBUNITS OF CREATINE KINASE OF RABBIT SKELETAL
MUSCLE

by

ELIZABETH THOMAS

A Thesis Submitted to the University of Manitoba in Partial
Fulfillment of the Requirements for the Degree of Master of Science

Department of Biochemistry

University of Manitoba



1986

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STUDIES ON THE M-SUBUNITS OF CREATINE KINASE OF RABBIT
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ABSTRACT

Rabbit skeletal muscle creatine kinase (ATP: creatine-phosphoryltransferase) catalyzes the reversible transfer of phosphate to creatine to form the compound creatine phosphate, important for replenishing the supply of ATP needed for muscle contraction. Creatine kinase, therefore, has an important enzymatic function and recent studies have indicated a structural function, as well, in the M-line of the sarcomere.

Studies on rabbit skeletal muscle creatine kinase have detected 21 dimeric CK-MM subforms. This confirms an earlier report which also detected 21 CK-MM subforms from rabbit skeletal muscle creatine kinase.

Creatine kinase from rabbit skeletal muscle has been separated into 6 M-subunit bands which have been shown to consist of one main creatine kinase M-subunit type with minor amounts of the other five M-subunit types. M-subunit bands 1 and 6 have been detected as pure bands from primary preparative Sephadex-urea gels. M-subunit 2 has been purified to electrophoretic homogeneity by a two-step procedure. Renaturation studies on purified M-subunit 2 detected only one CK-MM subform (CK-M2M2).

Apparent pI values in 7 M urea have been assigned to the M-subunit bands 1-4: M-subunit band 1 (pI=8.42), M-subunit band 2 (pI=8.28), M-subunit Band 3 (pI=8.08), M-subunit 4 (pI=8.00). The molecular weight for M-subunit 1 has been shown to be 43,000 by SDS-PAGE.

M-subunit bands 1-6 from rabbit skeletal muscle creatine kinase preparations have been renatured into the active or dimeric form and have been shown to have different enzymatic activity. The most active M-subunit bands were M-subunit bands 6 and 2; the least active was M-subunit band 1. Boehringer M-subunit band 1 has been shown to contain a protein contaminant which inhibits the enzymatic activity of renatured M-subunit band 1 material.

A model has been proposed to explain how 6 different M-subunits could associate into 21 CK-MM subforms. The heterogeneity seen for the 6 M-subunits is not caused by any artifact or known post-translational modification. This heterogeneity seems to be a reflection of either multiple genes and/or alternative processing of such genes.

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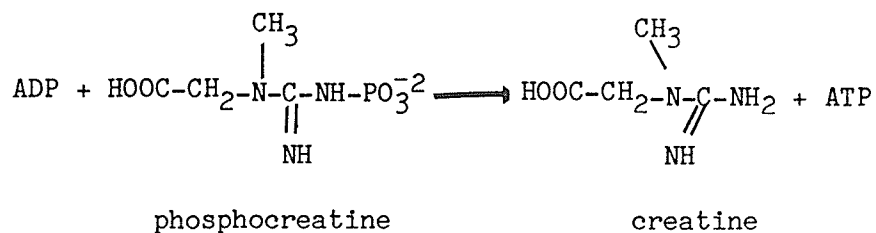
LIST OF ABBREVIATIONS

ADP, ATP	-	Adenosine 5' di-and tri-phosphate
Bis	-	Bisacrylamide
BSA	-	Bovine serum albumin
cDNA	-	Complementary DNA
CK	-	Creatine kinase
CK-BB	-	Brain creatine kinase
CK-MM	-	Muscle creatine kinase
CP	-	Creatine phosphate
DDW	-	Double distilled water
DTE	-	Dithiothreitol
EDTA	-	Ethylenediamine tetraacetic acid
IEF	-	Isoelectric focusing
IU	-	International units
MeOH	-	Methanol
M _r	-	Relative molecular weight
mRNA	-	Messenger RNA
NTCB	-	2-nitro-5-thiocyanobenzoic acid
PAGE	-	Polyacrylamide gel electrophoresis
pI	-	Isoelectric point
PMSF	-	Phenylmethylsulfonyl fluoride
PTH	-	Phenylthiohydantoin
SDS	-	Sodium dodecyl sulfate
S.F.	-	Super fine

TCA	- Trichloroacetic acid
TNB	- 2-mercapto-5-nitrobenzoic acid
TRIS	- Tris(hydroxymethyl) aminoethane
TPCK	- N-tosyl-L-phenylalanylchloromethyl ketone

I. INTRODUCTION

Creatine kinase(ATP:creatine-phosphoryltransferase) is an enzyme first described in the 1930's by Lohmann (1). It is found in many cell types and is associated with ATP regeneration in contractile or transport systems (2). It is normally found in the cytoplasm but forms of creatine kinase have also been isolated from the M-line of the sarcomere, from the inner membrane of the mitochondria, and from serum (3). Lehmann (4) is credited with deriving the equation for the reaction that creatine kinase catalyzes:



As researchers continued to study creatine kinase, they soon found that creatine kinase was not one enzyme but a family of isozymes containing subunits M or B. This was determined in 1964 by Burger et al. (5): CK-MM (the predominant form in skeletal muscle), CK-MB (the predominant form in developing skeletal and cardiac muscles and adult hearts of many species), and CK-BB (the predominant form in brain). A fourth form of creatine kinase was discovered in mitochondria in 1964 by Jacobs et al. (6). The dimeric structure of

creatine kinase was confirmed in 1965 by Dawson et al. (7), who reported that creatine kinases from brain, muscle, and heart were each composed of two dissociable subunits, which are called "M" and "B", after muscle and brain, respectively.

If we concentrate on CK-MM, a few researchers in the last five years have investigated the question of possible heterogeneity for this enzyme. Of special interest is work done by Guslits and Jacobs (8) who published a paper reporting the separation of human, rabbit, and bovine CK-MM by isoelectric focusing into 21 subforms. In the years 1984-1986, several laboratories published sequences for the M-subunit of creatine kinase from various species: rat (9), chick (10), *Torpedo californica* (11), *Torpedo marmorata* (12), rabbit (13, 14), rhesus monkey (14), and dog (15). Two of these groups (11, 15) found heterogeneity for the M-subunit after analyzing the results of two-dimensional electrophoresis patterns. Recently, Hershenson et al. (16), reported on their attempts to crystallize CK-MM from rabbit muscle. The crystals proved to be of poor quality unless they continued purifying the supposedly "pure" preparation of CK-MM. After these efforts, their crystals improved in quality. All these papers would suggest that there is more than one form of CK-MM and this is due to the existence of more than one M-subunit.

Microheterogeneity has recently been described for several proteins. For example, microtubules, which are found in many plant and animal cells, are composed of recurring molecules of tubulin. Tubulin has been shown to exist as a complex of two non-identical

polypeptide chains, alpha and beta. Recently, both polypeptide chains have been reported to show microheterogeneity using the technique of isoelectric focusing and tryptic peptide mapping (17-18). Therefore, it is not unreasonable that other dimeric proteins such as creatine kinase might also consist of different subunits. Williamson et al. (19) have set out four categories which can be used to explain any observed heterogeneity for a protein. These explanations include: 1) gene duplication; 2) alleles; 3) random association of different subunits; 4) substitution of side chains such as acetylation, phosphorylation, or sulfation. These categories will be considered with reference to the literature review in Section II.

A special interest in multiple forms of CK-MM lies in its application as a diagnostic tool in the detection of various disease conditions. In polymyositis, for example, it has already been suggested that different proportions of MM-subforms may reflect different stages of the disease (20). Knowing which subforms are found in normal tissue, it is possible then to study changes associated with disease. It might be of even more clinical use if the subunits that make up CK-MM are studied for changes between the normal state and the disease condition.

Because there are few published reports that have specifically looked at the existence of subunit heterogeneity for muscle creatine kinase, it should prove an interesting project to investigate this question of different M-subunits for rabbit muscle

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creatine kinase and, further, to isolate and purify these M-subunits.

II. REVIEW OF LITERATURE

CK-MM has been studied for heterogeneity either by maintaining the enzyme in its native or dimeric state or by dissociating the dimeric molecule into its subunits. Since much of the literature concerns CK-MM native heterogeneity, this aspect will be considered first.

Heterogeneity of Native CK-MM in Serum

In 1972, Smith (21), using polyacrylamide electrophoresis, resolved human serum creatine kinase from patients with myocardial infarction into three CK-MM and two CK-MB subforms. In 1978, Wevers et al. (22), using agarose gel electrophoresis, reported on the resolution of CK-MM into three subforms in both muscle and serum. He called these three subforms MM_3 , MM_2 , MM_1 with MM_1 having the most anodal electrophoretic mobility. This work was confirmed by several investigators: Yasmineh et al. (23), Falter et al. (24), Edwards and Watts (25). Subsequently, Wevers et al. (26) attributed this heterogeneity to the conversion of MM_3 into MM_2 and subsequently into MM_1 by a thermolabile factor of about 192,000 Daltons present in serum. Wevers et al. (22, 26) reported that they were unable to show heterogeneity for muscle tissue CK-MM unless they pre-incubated the tissue with blood. This is in contrast to more recent investigations (8, 30) which did not require serum to show microheterogeneity in tissue creatine kinase.

George et al. (27) attributed multiple forms of CK-MM in plasma to hydrolysis of the COOH-terminal lysine residue by a carboxypeptidase leading to the formation of two new electrophoretic forms of CK-MM. This Conversion Factor (C) was presumed to be carboxypeptidase-N (27). EGTA was used as an inhibiting agent, preventing the conversion of MM₃ in plasma into the other two subforms (27). CaCl₂ reversed this inhibition. It is known that chelating agents inhibit carboxypeptidase-N and this was one piece of evidence this group presented for considering carboxypeptidase-N as the conversion factor. Perryman et al. (28) continued to investigate this conversion of CK-MM into additional forms. They used carboxypeptidase-N and carboxypeptidase-B on human, rabbit and canine CK-MM and found that two additional subforms of CK-MM were formed from the MM₃ form of creatine kinase found in these tissues.

In 1984, Edwards and Watts (29) reported that the creatine kinase conversion factor was an exopeptidase active towards the C-terminal basic amino acid residues of proteins such as lysine. The creatine kinase conversion factor was shown to be larger than carboxypeptidase-A or carboxypeptidase-B but smaller than carboxypeptidase-N. They concluded that the creatine kinase conversion factor could be a hitherto undescribed carboxypeptidase but could not entirely exclude the possible role of carboxypeptidase-N.

Therefore, it seems that there is a thermolabile factor in serum which may be carboxypeptidase-N that can convert CK-MM into

other forms. However, researchers using protease inhibitors have found heterogeneity for creatine kinase inside muscle that cannot be explained by the action of any serum carboxypeptidase.

Heterogeneity of Native CK-MM in Muscle

Chapelle et al. (30) working with polyacrylamide gel electrophoresis found one CK-MM form (pI 6.88) in human muscle extract. When Chapelle's group switched to the technique of isoelectric focusing, they found an additional band (pI 7.10) that also showed creatine kinase activity. Incubation experiments indicated that of the two CK-MM subforms from muscle extracts, the subform with pI 7.10 was more rapidly inactivated by normal human serum at 37° C.

In 1983, Guslits and Jacobs (8) studied rabbit, bovine and human skeletal muscle for CK-MM heterogeneity. Using polyacrylamide gel isoelectric focusing, they resolved CK-MM from human, rabbit, and bovine muscle into twenty-one subforms. The pI values of the 21 bands from human skeletal muscle were between 6.60 and 7.79 which was in the same pI range that Chapelle's group (30) found for their two CK-MM subforms (pI values of 6.88 and 7.10). Guslits and Jacobs (8) attributed the high degree of resolution for the multiple forms of CK-MM mainly to the use of enzyme preparations of relatively high activity as well as to a fast, easily stopped, staining procedure. It should be noted that Chapelle et al. (30) used extracts of muscle obtained 12 hours after death while Guslits and Jacobs (8) performed

their isoelectric focusing experiments 15 minutes after removal of muscle samples from patients undergoing surgery.

Heinbokel et al. (31) examined CK-MM from human skeletal muscle (m. pectorals) using agarose gel isoelectric focusing. Five bands were resolved on their gels and tested positive with anti-CK-MM anti-serum. Heinbokel et al.(31) cited Wevers' papers (22, 26) and questioned why Wevers' group could only see multiple bands for skeletal muscle extracts after incubation with human serum. If Wever's methods are reviewed (22,26), it is noted that gel electrophoresis was used to resolve the subforms of CK-MM while Heinbokel et al. (31) used the more sensitive technique of isoelectric focusing.

Vaidya et al. (32) published a report on the purification of four CK-MM variants or subforms from human skeletal muscle. This muscle was obtained at an autopsy and preserved by freezing at -70° C. The subforms were separated by chromatofocusing and pI values were determined by agarose gel isoelectric focusing. The pI values for human CK-MM I-IV were 7.2, 6.9, 6.7, and 6.4, respectively. Purification of human skeletal muscle creatine kinase was carried out in the absence or presence of a wide range of proteinase inhibitors, such as pepstatin, PMSF, TPCK, leupeptin, bestatin. It was found that the presence of these inhibitors did not alter the number, ratio, or elution pH of the CK-MM subforms from human skeletal muscle. Vaidya et al. (32) believed, therefore, that the subforms were present in the homogenate and were not formed by proteolysis

during purification. EDTA was also used in all purification schemes in order to prevent any conversion of CK-MM to other forms by the carboxypeptidase naturally found in serum. This was also done by George et al. (27) who used EDTA to prevent the conversion of CK-MM₃ into MM₂, and further, into MM₁ in serum. Vaidya et al.(32) tested their four CK-MM subforms for differences in specific activity. They found that the most active was CK-MM I with a specific activity of 720 KU/g protein.

Mickelson et al. (33) separated CK-MM from canine heart into four subforms. Two techniques were used: chromatofocusing or agarose gel isoelectric focusing of tissue supernatant. The pI values for MM₃, MM₂, MM₁, and MM_x were 6.90, 6.70, 6.45, and 6.25 respectively if the CK-MM from canine heart was resolved by the isoelectric focusing method. It was found that if the chromatofocusing method was used, pI values of all subforms were higher and were close to the values determined by Vaidya et al. (32) for human skeletal muscle subforms. Mickelson et al. (33) have analyzed the four CK-MM subforms on SDS/PAGE and found that each subform had a mass of about 43,000 which is the mass of an M-CK subunit. Each subform was then analyzed for specific activity. Each of the four subforms was found to have a slightly different specific activity. MM₃ had the highest 272 (IU/g protein) $\times 10^3$ while the lowest was MM_x with 175 (IU/g protein) $\times 10^3$. In the discussion, Mickelson et al. (33) present an argument for the existence of different subforms (subspecies). They suggest that different

subforms could play important roles in transferring energy between mitochondria, cytosol, and myofibrils.

Another group of researchers, Lebherz et al. (34), noticed heterogeneity in purified preparations of chicken CK-MM. They were using CK-MM to investigate the effect of Proteinase K and Pronase E on the abilities of creatine kinase subunits to establish precise subunit-subunit recognition in vitro. Even though CK-MM was isolated from chicken breast muscle using 1mM EDTA in the homogenizing buffers, CK-MM still showed microheterogeneity. This is one report in which researchers were not planning to investigate heterogeneity of CK-MM but discovered it as they were investigating another problem.

The next source of information on heterogeneity for muscle creatine kinase comes from work reported by Hershenson et al. (16). They encountered difficulty when they attempted to crystallize creatine kinase from rabbit skeletal muscle in order to study its molecular structure by X-ray diffraction. After purification of creatine kinase using ion-exchange and gel filtration steps, the creatine kinase material was focused in a pH gradient of 3-10. The authors detected heterogeneity in a sample that was shown to be pure on the basis of SDS-polyacrylamide gel electrophoresis and of its high specific activity (1170 units/mg protein). Hershenson et al. (16) then used Sephadex flat-bed isoelectric focusing to reduce the heterogeneity of the creatine kinase preparation. They found that there were five individual bands of protein with pH values of 6.09,

6.31, 6.55, 6.73, and 6.90. When these bands were individually harvested and the material used to prepare diffraction quality crystals, the number of crystals suitable for X-ray diffraction analysis increased from one in five crystals to one in two crystals.

Heterogeneity of CK M-Subunit

The muscle form of creatine kinase has also been studied for heterogeneity using denaturing conditions. The first investigations were by Traugott and Massaro (35), who in 1972, resolved rabbit skeletal muscle creatine kinase using urea starch gel electrophoresis into 6 subunits, which they named MM₋₁, MM, MM₁, MM₂, MM₃, MM₄. They dissolved rabbit skeletal muscle creatine kinase purchased from Boehringer in a solution of 8 M urea, 1% mercaptoethanol and incubated the preparation for 24 hours at 4° C. The six bands identified exhibited approximately equal staining intensity for protein but band MM had the greatest activity compared to the other five. This band was the second from the cathode. They dialyzed the urea-dissociated CK-MM sample and then ran the dialysand on Tris-citrate starch gel electrophoresis to determine the native or reassociated subbanding pattern. Six CK-MM subbands were resolved using this electrophoresis method. They did electrophoresis experiments in the absence or presence of mercaptoethanol and found identical patterns. This would appear to rule out sulfhydryl group interactions as the cause of rabbit CK-MM isozyme subbanding. On SDS-PAGE electrophoresis, only a single sharp homogeneous band with a molecular weight in the neighbourhood of 43,000 was found. This

eliminated the possibility that partial hydrolysis of the M-subunit was responsible for the heterogeneity observed. The authors were concerned that only six dimers (CK-MM) could be resolved using starch gel electrophoresis since in theory 6 subunits should reassociate into more than just 6 dimers. However, it could be that even if more dimers were present, starch gel electrophoresis is not sensitive enough a technique to resolve closely spaced bands.

Rosenberg et al. (36), using two-dimensional electrophoresis techniques and dissociating conditions (9.5 M urea), resolved purified chicken CK-MM into two subunits with apparent pI values of 7.5 and 7.4. The subfractions were tested for enzymatic activity by the removal of urea and ampholytes and were shown to have creatine kinase activity using a histochemical staining technique. Purified rat CK-MM was also resolved on two-dimensional gels and shown to consist of two different subunits. Polysomal RNA from 18-day embryos or 72-hour differentiated myogenic cell cultures was translated in a reticulocyte-derived cell-free translation system. The creatine kinase subunits were isolated by immunoprecipitation and then resolved on a two-dimensional system. In both cases, two closely spaced M-subunits were observed. Rosenberg et al. (36) concluded that polymorphism might be best explained by the existence of multiple but closely related genes for the creatine kinase subunits.

Rosenberg et al. (37) then prepared isolated poly(A)⁺RNA from breast or leg muscle from 19 to 20 day-old-chicken embryos or from adult chicken. Double-stranded cDNA was synthesized from the muscle

poly(A)⁺RNA, inserted into plasmid pBr322 and then transfected into Ca²⁺-treated *Escherichia coli* cells. A clone designated pMCK1 was translated and yielded one protein band with the same mobility as M-CK subunit on an SDS-gel. The translation products were further analyzed on two-dimensional gels and found to consist of two subspecies of the M-CK subunit. From this work, Rosenberg et al. (37) conclude that differential transcription of mRNA likely governs creatine kinase heterogeneity.

To summarize, Rosenberg et al. (36, 37) presented evidence for M-CK subunit heterogeneity. They showed that there are at least two subunits which may be the result of a) multiple but closely related genes or b) differential transcription of DNA to form mRNA.

Noting Rosenberg's work (36) on the M-subunit of creatine kinase, Schweinfest et al. (38) explored the heterogeneity of the M-subunit from chick muscle. Poly(A)⁺mRNA from chick breast and thigh muscle was used to prepare complementary DNA. This cDNA was inserted in pBr322 and transfected into bacterial cells. Two clones yielding mRNA were selected that were then translated in a wheat germ translation system. Two-dimensional electrophoresis (SDS/urea) electrophoresis followed by isoelectric focusing) showed two subunit forms of M-CK which had pI values of 6.85 and 6.50. The colonies containing the plasmids were not likely to be "siblings" because the plasmids had different restriction digestion patterns as noted by Schweinfest et al. (38).

Witzemann (39) analyzed CK-MM from *Torpedo marmorata* muscle

tissue. Proteins with an apparent M_r of 43,000 on SDS/polyacrylamide gels had been found to co-purify with the acetylcholine receptor (ACHR)-enriched membranes. It had been shown that a subset of proteins called v_2 polypeptides (pI less than or equal to 6.5) occurred in several isoelectric forms and were mainly of cytoplasmic origin (40-41). It was previously shown that a polypeptide of an M_r of 43,000 would specifically phosphorylate some of the acetylcholine receptor subunits (42). Witzemann's main objective was to compare the v_2 polypeptides with purified creatine kinase from the electric organ of *Torpedo marmorata*. What he did show was that: 1) both react with anti-CK antibodies which were raised from creatine kinase purified from the electric organ; 2) these anti-CK antibodies react with a 43,000 M_r -polypeptide found in *Torpedo* back and heart muscle but not brain tissue thus showing both v_2 polypeptides and purified creatine kinase from *Torpedo* electric organ represent the MM-type creatine kinase; 3) There are three different subunits for v_2 polypeptides and purified CK. Two-dimensional gel electrophoresis reveals two major forms of apparent pI at pH 6.5 and 6.3 and a minor form at pH 6.2.

If the major 43,000- M_r proteins of the electric organ are synthesized in a cell-free translation system, the three polypeptides synthesized show identical isoelectric properties compared to purified creatine kinase and v_2 polypeptides. This would support the view that these polypeptides (pI 6.5, 6.3, and 6.2) are not greatly modified by post-translational events and the heterogeneity is

probably not due to a creatine kinase precursor polypeptide.

Witzemann has presented some intriguing ideas about creatine kinase subunit heterogeneity. It does not seem to arise at the post-translational level. Second, it might be that variant forms of creatine kinase or different amounts of variant forms of creatine kinase are associated with definite areas or structures in organs.

Degani and Degani (43) studied the effect of subunit-selective modifications on creatine kinase. They showed that the two reactive thiol groups in the dimeric creatine kinase react non-identically with the cyanylating reagent, 2-nitro-5-thiocyanobenzoic acid (NTCB). The thiol group in one subunit becomes carbamylated while the other thiol group forms a mercaptonitrobenzoate (TNB) mixed disulfide. The absorption spectrum of a NTCB-modified enzyme showed a band at 330 nm characteristic of the TNB mixed disulfide derivative of the enzyme. If the dimeric creatine kinase was denatured before treatment with NTCB, the subunits underwent essentially quantitative S-carbamylation. Therefore, the authors feel that the abnormal reaction of NTCB with native creatine kinase appeared to be due to a specific chemical environment existing at one thiol group in the native enzyme which leads to S-arylthiolation in place of S-carbamylation. Degani and Degani cite other authors (44-46) from the sixties who concluded, at that time, that the two subunits were identical. However, the Deganis' work suggests that: 1) the subunits of the dimeric creatine kinase are inherently and initially non-equivalent; 2) the subunits

of creatine kinase are associated asymmetrically for reasons still to be explained.

Sequencing of CK-MM from Different Tissues

In the last few years, there have been several reports in the literature on CK-MM sequencing. The first report indicating sequence heterogeneity is that of Takasawa et al. (47). In 1983, they reported that they could resolve pig CK-MM into 3 subforms, FI, FII, FIII. Using amino-terminal analysis, the authors found that the 3 subforms had different primary structures. In addition, FI and FII were both heterogeneous at residue 1 (proline and serine) and at residue 23 in FI (serine and leucine). No sequence heterogeneity was found for FIII. Another early report came from West et al. (11). They obtained cDNA clones encoding muscle creatine kinase sequences using mRNA from the electric organ of *Torpedo californica*. One clone, CKABh9, was ^{32}P -labelled by nick translation and used to screen 2600 other clones. CKABh9 and a second clone, CK52g8, both arrested in vitro synthesis of proteins of an M_r of 40,000-43,000 after translation of electric organ mRNA. When CK52g8 was sequenced and the protein sequence encoded by CK52g8 was inferred, it was found that the protein sequence was 68% homologous to a known partial sequence of rabbit muscle creatine kinase. West looked at possible heterogeneity at the messenger RNA level. The experimental data showed that all of the cDNAs that had hybridized to ^{32}P -labelled CKABh9 had the same restriction sites as CKABh9 and that only a

single band appeared in the RNA blot hybridizations with CK52g8 and CKABh9 insert probes. From this evidence, West concluded that there is only a single 1600 bp mRNA species.

Giraudat et al. (12) constructed a cDNA library from electric organ mRNA from *Torpedo marmorata*. The amino acid sequence of muscle-specific creatine kinase was deduced and analyzed. When they compared their sequence to protein from *Torpedo californica* electric organ (48), they found that the sequences were almost identical. On two-dimensional polyacrylamide gels, proteins from electric organ homogenates were separated and stained using anti-chicken M-CK antibody. Two spots stained positive. Thus it would appear from Giraudat's work (12) that there are at least 2 slightly different M-subunits for *Torpedo marmorata* electric organ creatine kinase. Both West et al. (11) and Giraudat et al. (12) indicate one mRNA for creatine kinase. This is in contrast to the paper of Benfield et al. (9).

Benfield et al. (9) reported that for rat muscle creatine kinase, isoelectric microheterogeneity is reproduced upon cell-free translation of mRNA and, therefore, was unlikely to be the result of post-translational modifications. The identity of the translation product was confirmed by immune precipitation with goat anti-rat muscle creatine kinase-specific antiserum. It should be noted that their total amino acid sequence was a composite deduced from overlapping but independent cDNAs. Therefore, they could not be certain that the sequence was derived from a single mRNA species

encoded by a single-copy gene. If there were more than one mRNA, these microheterogeneous forms would be difficult to detect. They suggested that the only way to solve this problem would be to isolate and sequence full length cDNAs.

Putney et al. (13) constructed a cDNA library from rabbit muscle poly(A)RNA. Using two deoxyoligonucleotide probes complementary to a creatine kinase cDNA sequence, they isolated two clones whose cDNA segments partially overlap and which together encode the entire protein. The primary protein structure was deduced from the sequence of these two cDNA clones. They then looked at the primary structure and detection of human analogues to cDNA clones from rabbit skeletal muscle. They obtained genomic DNA from eleven unrelated humans and digested the DNA with the restriction enzymes EcoRI, HindIII, MspI or TaqI. The digested DNA was then probed with either a 0.18 Kb PstI fragment or a 0.8 Kb probe from pCK19 (a clone which contains part of the entire creatine kinase coding sequence). With either probe, they observed between three and eight hybridizing fragments. In general, the fragments observed with the 0.18Kb probe were a subset of the 0.8 Kb probe. It is their suggestion that there are at least three loci in the human genome homologous to rabbit muscle creatine kinase. In another of their studies using TaqI digested DNA from four people, they found all four had a common 3.5 Kb fragment, whereas two of the four people had unique 4.1 Kb fragments. Putney et al. (13) suggested that the 3.5 and 4.1 Kb fragments are allelic and that these creatine kinase polymorphisms

might be useful as genetic markers.

Ordal et al. (10) prepared cDNA clones from chick muscle creatine kinase mRNA. They used these clones to derive the amino acid sequence for the protein product. During their sequence analysis, they noted that there were several inverted repeats which could potentially form base-paired hair-pin loops of the mRNA. If these were to base-pair, they would loop out segments of muscle creatine kinase mRNA which could lead to different forms of M-subunit.

Roman et al. (15) determined the complete nucleotide sequence of dog heart creatine kinase mRNA. The amino acid sequence of the myocardial enzyme was deduced and compared to the CK-M subunits of other species. A high degree of similarity exists for CK-M subunits from dog, rabbit, rat, and chick. This suggested to the authors that much of the creatine kinase molecule is required for one or more functions such as enzymatic activity or possibly structural requirements. When hybridization analysis of dog heart RNA was performed with an 835-bp fragment of the M-subunit of creatine kinase, a single species of mRNA (1.6 Kb) was seen migrating. But when southern blot analysis of dog spleen DNA was performed with the same probe after digestion of the genomic DNA with EcoRI, HindIII, or BamHI, multiple hybridization bands were detected in all three digests. There are three possible explanations for these results: 1) incomplete digestion of the DNA by the restriction enzymes; 2) presence of many introns within the dog M-CK gene,

containing EcoRI and BamHI restriction enzyme sites; or 3) hybridization of the probe to multiple creatine kinase genes. The first possibility was unlikely since a fatty acid binding protein was used as a control to probe the DNA and it was found that this probe bound to only one fragment of genomic DNA. The second explanation was also discounted since no sites for these enzymes were present in the coding region of dog M-CK. Thus Roman et al. (15) favoured the explanation that the hybridization probe was binding to more than one creatine kinase gene, possibly the M and B CK genes.

Chegwidden et al. (14) have also noticed heterogeneity during their analysis of the N-terminal sequence of creatine kinase from skeletal muscle of rabbit and Rhesus monkey. They found that the N-terminal residue of rabbit muscle creatine kinase was proline but that there were several PTH derivatives for the N-terminal residue of Rhesus monkey creatine kinase. They favour the hypothesis that muscle proteins with blocked N-terminals are the normal in vivo products of biosynthesis and several stages of post-translational modifications may well occur (49).

It is apparent from the information obtained from sequencing that many researchers have noticed some heterogeneity for the M-CK subunit whether at the genetic (13) level, the mRNA level (9), or the protein level (12, 14). No one has, as yet, clearly defined where the source of this heterogeneity lies.

Evidence for Heterogeneity for CK-BB

A second form of creatine kinase, the brain form (CK-BB) has also been shown by some researchers to be heterogeneous. One of these groups was McBride and Rodgerson (50) who isolated creatine kinase BB from human brain tissue obtained within four hours of death. After disc electrophoresis in 7.5% polyacrylamide gels, the final creatine kinase material clearly showed three protein bands with the anodic band being the most intense. After storage for eight months at -80° C, the three bands showed different changes in mobility on disc electrophoresis. These three bands could not be reduced to one band by the addition of a larger amount of mercaptoethanol (500mM). When the final CK-BB material was analyzed by two-dimensional electrophoresis studies, more than one spot was visible indicating microheterogeneity.

In 1985, McBride and Rodgerson published another paper in which they again reported heterogeneity for CK-BB (51). They assigned a pI value of 4.5 for the most intense protein band. It follows that if there are three subforms of CK-BB, then there must be two slightly different B-subunits making up these three dimeric molecules.

From these two papers on the brain form of creatine kinase, it is apparent that microheterogeneity is not unique to the muscle form of creatine kinase.

Recent Evidence for Heterogeneity for Human Mitochondrial CK

A paper was recently published by SiragEldin et al. (77) confirming heterogeneity for human CK-MM as well as reporting for the first time on heterogeneity for mitochondrial creatine kinase. SiragEldin et al. (77) analyzed tissue extracts from human heart and papillary muscle by analytical and preparative agarose isoelectric focusing. They were able to detect multiple CK-MM subforms that focused roughly between pH 6 to 7. Guslits and Jacobs (8) reported slightly higher pI values for their 21 CK-MM subforms from human psoas muscle (6.60 to 7.79). An activity stain was used to show CK activity. Additional testing was performed to confirm that these bands were not adenylate kinase bands.

III. OBJECTIVE

From the literature review, it has been shown that there is some evidence already for heterogeneity of the M-subunit of creatine kinase and the dimeric form, MM-CK. Some questions still need to be answered:

- (1) How many M-subunits are there for rabbit muscle creatine kinase and how do their biochemical properties differ?
- (2) Are these M-subunits the result of artifacts due to protein-medium interaction, protein-ampholyte interaction, or carbamylation products?
- (3) Are these pure M-subunits? Can they be isolated from one medium and do they show stable properties if re-run on the same or on a different medium?

These questions have been answered as fully as possible in the time allotted. The work described in this thesis can be divided as follows:

- I. Comparison of heterogeneity for rabbit muscle creatine kinase on different media.
- II. Investigation of possible heterogeneity due to artifact formation.
- III. Properties of the different M-subunits.

IV. Materials

Acetic Acid	Fisher Scientific Company
Acrylamide	Bio-Rad Laboratories
Adenosine 5' Diphosphate	Sigma Chemical Company
Agarose	Sigma Chemical Company
Agarose, IEF	Pharmacia Fine Chemicals
Albumin, Bovine	Sigma Chemical Company
Alpha-Chymotrypsin	Worthington Biochemical Corporation
Ammonium Bicarbonate	Fisher Scientific Company
Ammonium Persulfate	Bio-Rad Laboratories
Ampholine, pH 7-9	LKB
Bio-Lyte 3/10	Bio-Rad Laboratories
Bromophenol Blue	Fisher Scientific Company
C K Buffer	LKB
Coomassie Brilliant Blue R	Sigma Chemical Company
Creatine Kinase(Rabbit Skeletal Muscle)	Sigma Chemical Company and Boehringer Mannheim GmbH
Creatine Phosphate	Boehringer Mannheim GmbH
Cytochrome C	Sigma Chemical Company
Dithiothreitol	Bio-Rad Laboratories
Dye Reagent Concentrate	Bio-Rad Laboratories
Ethanol	Fisher Scientific Company

Ethylenediamine Tetraacetic Acid	Sigma Chemical Company
Formaldehyde	Fisher Scientific Company
B-D(+) Glucose	Sigma Chemical Company
Glucose-6-Phosphate Dehydrogenase	Boehringer Mannheim GmbH
Glycerin	Fisher Scientific Company
Glycine	Sigma Chemical Company
Hexokinase	Boehringer Mannheim GmbH
Imidazole	Sigma Chemical Company
Immobilines	LKB
Lauryl Sulfate	Sigma Chemical Company
Magnesium Acetate	J.T. Baker Chemical Company
Methanol	Fisher Scientific Comany
2-Mercaptoethanol	Fisher Scientific Company
Nicotinamide Adenine Dinucleotide	Boehringer Mannheim GmbH
Nitroblue Tetrazolium	Sigma Chemical Company
Nonidet P40	Particle Data Laboratories
N,N'-Methylene Bisacrylamide	Aldrich Chemical Company, Inc.
N,N,N',N'-Tetra Methylene Diamine	Bio-Rad Laboratories
Pharmalyte, 6-8	Pharmacia Fine Chemicals
Phenazine Methosulfate	Sigma Chemical Company
Phospho(enol)Pyruvate	Sigma Chemical Company

Polybuffer Exchanger, 94	Pharmacia Fine Chemicals
Polybuffer, 96	Pharmacia Fine Chemicals
Potassium Cyanate	Aldrich Chemical Company, Inc.
Potassium Phosphate	Fisher Scientific Company
Repelsilane	LKB
Rexyn 300(H-OH)	Fisher Scientific Company
Silver Nitrate	Engelhard Industries of Canada Ltd
Sephadex G75(SF),G50(SF)	Pharmacia Fine Chemicals
Servalyte, 6-7	Serva
D-Sorbitol	Sigma Chemical Company
Sulfosalicylic Acid	Fisher Scientific Company
N,N,N',N'-Tetramethylethylenediamine	Bio-Rad Laboratories
Trichloroacetic Acid	Fisher Scientific Company
Tris(hydroxymethyl)aminomethane	Sigma Chemical Company
Trypsin	Sigma Chemical Company
Trypsin Inhibitor	Boehringer-Mannheim GmbH
Urea	Fisher Scientific Company

IV. Methods

A. Chromatofocusing

1) Chromatofocusing, first described by Sluyterman et al. (52-53), was the initial method chosen to explore heterogeneity of creatine kinase subunits. A 30 cm column was packed at a flow rate of 1.5 cm/min with a slurry of Polybuffer exchanger, PBE 94, and a starting buffer of 0.025 M imidazole acetate. The column was packed using a pump for a minimum of 10 bed volumes. The column was washed with 3 bed volumes of 0.025 M imidazole acetate, pH 7.2, 8 M urea. Then 56 ml of Polybuffer 96 (1/12 dilution), pH 6.0, containing 8 M urea, were pumped through the column and collected. Boehringer rabbit skeletal muscle creatine kinase (10 mg) was applied to the top of the column. The pump was reconnected and a further 144 ml were collected in 2 ml fractions. The fractions were read on a Radiometer pH M63 Meter and the readings plotted on a graph.

All peaks were dialyzed against 25 mM potassium phosphate, pH 7, 10 mM mercaptoethanol for 24 hours. The buffer was changed and collected peaks were dialyzed for a further 24 hours. The peak samples were then dialyzed against DDW for 12 hours and lyophilized.

2) Isoelectric focusing was used to check for band purity. Polyacrylamide slab gels (13x12.5x0.2cm) contained 4.9% (w/v) acrylamide, 0.15% (w/v) N,N'-methylenebisacrylamide, 10% (v/v) glycerol, 0.025% (w/v) ammonium persulfate, 2% (v/v) ampholytes (1% pH 5-7 and 1% pH 7-9). Experiments were run at 4° C, 6 watts

constant power, for 3.5 hours using an Isco Model 492 Power Supply and an LKB Model 2117 Multiphor. Catholyte was 0.5 mol/l ethanolamine and anolyte was 0.2 mol/l citric acid.

Activity stain as described by Harris and Hopkins (54) and modified by Guslits and Jacobs (8) was used to test for creatine kinase activity and also to check the chromatofocusing technique's efficiency in separating CK-MM into subunits.

<u>Reagents</u>	<u>Final Concentration</u>
Tris acetate buffer(pH 7.5, 37° C)	50 mmol/l
ADP	1.5 mmol/l
Creatine phosphate	22 mmol/l
Glucose	17 mmol/l
NADP	2.5 mmol/l
Magnesium acetate	8 mmol/l
Glucose-6-phosphate dehydrogenase	1000 units/l
Hexokinase	1000 units/l
Nitrotetrazolium-blue	0.3 g/l
Phenazine methosulphate	0.3 g/l
Agarose	0.8%

Agarose was dissolved in 85% of the total volume of Tris buffer and cooled to 50° C. All other substrates, except for creatine phosphate, were dissolved in 15% of the total volume of Tris buffer. Creatine phosphate was then dissolved in the buffer containing substrates and immediately added to the agarose solution. The

agarose and substrate mix was poured over the gel and the reaction monitored at 37° C. The reaction was stopped by immersing the gel in 7% acetic acid for 30 minutes.

B. Isoelectric Focusing in Polyacrylamide (8 M Urea)

1) Isoelectric focusing studies on CK-MM were carried out in a denaturing system using 8 M urea. Polyacrylamide slab gels (13x12.5x0.12 cm) were composed of 4.9% (w/v) acrylamide, 0.15% (w/v) N,N'-methylenebisacrylamide, 10% (w/v) glycerol, 0.025% (w/v) ammonium persulfate, 8 M urea, 5% (v/v) ampholytes (2.5% pH 5-7 and 2.5% pH 7-9).

CK-MM standard and bands were dissolved in Buffer 1: 8 M urea, 1 mM EDTA, 100 mM mercaptoethanol, and 2% (v/v) ampholytes (1% pH 5-7 and 1% pH 7-9). Per application pad, 100 µg of CK-MM were applied. Various amounts of lyophilized material were applied to the gels.

All experiments were carried out at 12-15° C using 0.5 mol/l ethanolamine as the catholyte and 0.2 mol/l citric acid as the anolyte. Gels were focused for various times (3.5-4 hrs) using a constant power of 6 watts with a maximum voltage of 1100 V. Gels were fixed overnight in 3.5% sulfosalicylic acid, 10.0% TCA, and 30% MeOH. Gels were then washed in 25% ethanol, 8% acetic acid for 3 hours, stained in 0.2% Coomassie Brilliant Blue, 10% acetic acid, 50% ethanol for 3-6 hours, and then destained in 40% methanol, 8% acetic acid until the background was clear. After staining and destaining, the gels were scanned using a Bio-Rad densitometer with a 600 nm

filter.

2) Protein was determined using the method described by Bradford (55). Protein standard used was BSA (1 mg/ml). Bio-Rad Dye Reagent Concentrate (20%) was added to tubes containing various amounts of protein and the optical density measured at 595 nm on a Beckman DB-G spectrophotometer.

3) Isoelectric focusing studies on CK-MM were also done in a nondenaturing polyacrylamide system. Gels consisted of 4.9% (w/v) acrylamide, 0.15% (w/v) glycerol, 0.25% (w/v) ammonium persulfate, 5% ampholytes (pH 3-10). Samples of CK-MM were dissolved in 0.4% (w/v) EDTA, 1% (v/v) N-P-40 and 0.07% DTE. Isoelectric focusing (IEF) standards from Bio-Rad were used as references for determining the pI values of the CK-MM bands.

C. Carbamylation Experiments

1) Pre-incubation of CK-MM

CK-MM (1 mg/0.1 ml) was dissolved in three different buffers: A, B, and C. Buffer A contained 8 M urea, 1 mM EDTA, 100 mM mercaptoethanol, and 1% ampholytes. Buffer B was the same as Buffer A except that the urea was filtered through a mixed bed ion-exchanger (Rexyn-300) before EDTA, mercaptoethanol, and ampholytes were added. Buffer C contained 4% (w/v) KCNO, 0.04% (w/v) EDTA, 0.07% (v/v) mercaptoethanol, and 1% (v/v) ampholytes which was added just before

sample was applied to the gel. The first pH range used was 5-9. CK-MM was first run on polyacrylamide gels with no pre-incubation in the three buffers. Next, CK-MM was incubated in Buffer C for 24 hours at 30° C and then run on a polyacrylamide gel. In the second set of experiments the pH range was expanded to 3.5-10. Buffers B and C were the same as above but the third buffer contained 0.04% (w/v) KCNO, 0.04% (w/v) EDTA, 0.07% (v/v) mercaptoethanol. Ampholytes (1%) were added just before samples were applied to the gel.

2) Isoelectric focusing on polyacrylamide gels (8 M urea)

The CK-MM samples were run on polyacrylamide slab gels (13x12.5x0.2cm) containing 4.9% (w/v) acrylamide, 0.15% (w/v) N,N'-methylenebisacrylamide, 10% (v/v) glycerol, 0.025% (w/v) ammonium persulfate, 5% ampholytes (pH 5-9 or pH 3.5-10), and 8 M urea. The gels were focused for 4.5 hours, 13.5° C, 6 W (constant power). They were fixed and stained as previously described. The gels then were scanned with a Bio-Rad densitometer using a 600 nm filter.

D. IEF in Agarose, 7 M Urea

1) Agarose slab gels were prepared by the method of Olsson and Laas (56). Gels (12.5x12.5x.2cm) contained 2% (w/v) agarose (IEF), 12% (w/v) sorbitol, 7 M urea, 6% (v/v) ampholyte, 1% (v/v) mercaptoethanol. Focusing experiments were conducted at 14° C using 0.5 mol/l ethanolamine as catholyte and 0.2 mol/l citric acid as the

anolyte. Gels were prefocused for 45 minutes before CK-MM samples(100 µg/LKB application pad) were applied. CK-MM was dissolved in Buffer 1 as previously described in Section B.1 of Methods. Gels were run for 3.5 hours at 6 W, constant power. After focusing, the bands were visualized in two ways.

- a) Gels were fixed in 10% (w/v) TCA for 30 minutes, washed in 30% ethanol, 10% acetic acid for 2x15 minutes, stained in .2% Coomassie Brilliant Blue, 10% acetic acid for 3-6 hours, and destained in 30% methanol, 10% acetic acid.
- b) A nitrocellulose blotting method, described by Takehana et al. (57) for bands on polyacrylamide gels was adapted to visualize the bands on agarose-urea gels. The agarose was covered with a nitrocellulose membrane (Bio-Rad) and three sheets of thick filter paper (Whatman 3M) which had been presoaked in 0.25 M Tris buffer (pH 8.3), containing 0.192 M glycine. A thick glass plate was placed on top and left for 2.5 hours at 13.5° C. After transblotting, the nitrocellulose membrane was immersed in 0.05% Coomassie Brilliant Blue, 10% acetic acid for 10 minutes. The membrane was rinsed with DDW and placed in destaining solution (7% ethanol, 5% acetic acid) for about 15 minutes. The membrane was air-dried on a sheet of filter paper. Last, the membrane was placed under the gel using the method of Takehana et al. (57) and the bands of protein cut out.

2) pH measurements were made on an agarose-7 M urea gel containing 2% (w/v) agarose-IEF (a specially prepared grade of

agarose), 7 M urea, 12% (w/v) sorbitol, 6% (v/v) ampholytes. No mercaptoethanol was added to the gel. The gel was prefocused for 45 minutes, 13.5° C, 5 W (constant power). Samples dissolved in Buffer I were applied to filter paper pads and the gels were then run for 3 hours at 5 W, constant power. The pH measurements were made with an Ingold surface electrode and Radiometer pH meter, Model 28. The pI values for the first 4 bands were calculated from the pH graph and distance of the bands from the cathode.

3) Strips of gel containing a single band were placed into test-tubes and macerated. One ml of glycine buffer (0.1 M), pH 9.0 was added. Samples were frozen and then thawed out. The tubes were centrifuged at 40 K for 15 minutes in a 65 Ti Rotor. The supernatant was removed and the procedure repeated 2x. Supernatants were combined and dialyzed against DDW, 1 mM mercaptoethanol or 10 mM NH_4HCO_3 , for 72 hours. Protein estimates were made using the Bradford protein assay. The dialyzing buffer was changed every 24 hours. Extracts were then lyophilized.

E. IEF in Immobilized pH Gradients

a) Preparation of Gels

Gels were prepared using the method of Gasparic et al. (58). Stock solutions for gels were: 29.1%/0.9% (w/v) acrylamide/bisacrylamide, 10% (w/v) ammonium persulfate, 10% (v/v) TEMED, 87% (w/w) glycerol, respectively.

<u>Acidic, dense mixture</u>			<u>Basic, light mixture</u>		
Immobilines	pH 3.6	277.6 μ l	pH 3.6	69.1 μ l	
(LKB)	pH 6.2	99.4 μ l	pH 6.2	55.1 μ l	
	pH 7.0	66.6 μ l	pH 7.0	100.1 μ l	
	pH 8.5	193.7 μ l	pH 8.5	130.3 μ l	
Acrylamide/Bis		3.0 ml		3.0 ml	
Glycerol		1.51 ml		0	

DDW was added to a total of 5.4 ml for both the acidic mixture and the basic mixture. The next additions were 7.2 μ l ammonium persulfate solution and 7.2 μ l TEMED solution to each mixture. Before acrylamide/bis solution was added, the pH of each mix was measured with a Radiometer PHM 26 Standard pH Meter.

Gels were 12.5x12.5x0.2 cm in size and cast on templates that had been coated with Repelsilane. Gel solutions were not degassed as the gradient mixer was used with both chambers open. Acidic and basic mixtures were added to separate chambers of a Hoefer Gradient Mixer. The catalysts, TEMED and ammonium persulfate, were added and the mould was allowed to fill. After pouring, the gel was allowed to polymerize at 50° C in an oven for 1 hour and then weighed. The gel was washed for 1 hour in DDW. It was allowed to return to original weight. If CK-MM was to be focused under denaturing conditions, the immobiline gel was soaked in an 8 M urea solution overnight. Finally, the gel was air-dried to about 5% of its original weight.

b) Running Conditions for Immobiline Gels

CK-MM (1 mg/100 μ l) was dissolved in a buffer of 8 M urea and 1 mM EDTA. The gels were pre-run for 30 minutes. CK-MM (30 μ g) was pipetted onto LKB application pads and the gels focused using a LKB 2297 Microdrive 5 Power Supply and LKB 2217 Ultrophor for 4-5 hours at 13° C, 2 mA, 4000 Volts. The catholyte was 0.5 mol/l ethanolamine and the anolyte was 0.2 mol/l citric acid. The gels were fixed in a solution of 11.5% (w/v) TCA, 3.5% (w/v) sulfosalicylic acid for 1 hour. Gels were stained for 10 minutes at 60° C in 0.1% (w/v) Coomassie Brilliant Blue, 25% (v/v) ethanol, and 4% (v/v) acetic acid. Gels were destained in 25% ethanol and 4% acetic acid. Immobiline gels were scanned with a Bio-Rad densitometer at 600 nm.

F. Purification of Subunits of CK-MM

a) IEF in Sephadex G-75, S.F. (Primary Run)

The gels were prepared by making a slurry of 5% (w/v) Sephadex G-75, Super fine (SF), 8 M urea, 0.7% (v/v) mercaptoethanol, and 5% (v/v) ampholytes and poured into a mold. Water content was reduced with an air stream to 80% of the original weight. A solution of 8 mg CK-MM dissolved in 2-3 ml DDW, 5% (v/v) ampholytes was applied directly to the surface of the gel or applied by a trough method. Gels were run overnight at 13° C, 8 W (constant power). A blotting method using Whatman Grade #1 filter paper was used to visualize the bands. The filter paper was placed on the surface of the gel for 2.5 minutes, removed, dried in an oven at 100° C for 15 minutes, fixed in

10% TCA for 3x15 minutes, and then stained in a 0.2% (w/v) Coomassie Brilliant Blue solution of methanol, acetic acid, DDW (50:10:50) for 1 hour. The blot was then removed and placed in destaining solution until the background was clear. The blot was dried and then used as a template to cut out bands.

b) Secondary Run

After bands were isolated, they were either eluted through a gel filtration column and concentrated or they were run on a secondary Sephadex G-75, SF, gel of the same or a narrower pH range. The conditions for the secondary run were the same as a primary focusing run except that the slab gel was separated into two so that two bands could be run at the same time. The bands were isolated, chromatographed, and concentrated as described below.

c) Gel Filtration and Concentration of CK-MM Subunits

A 1.5x50 cm column was packed with Sephadex G-50, S.F., and a buffer of 0.1 M ammonium bicarbonate. Total bed volume was 80 ml. Bands in Sephadex G-75 were applied to the top of the column which had been pre-equilibrated with 0.1 M ammonium bicarbonate and 100 mM mercaptoethanol and eluted with the same buffer. Protein peaks were monitored on an Isco UA-5 Absorbance Monitor. Fractions were collected, assayed for activity, and concentrated to about 400 μ l using nitrogen gas and an Amicon Concentrator with an Amicon PM 10 membrane. Samples were then brought into 50%(v/v) glycerol and

stored at -10° C.

d) Activity Check for Renatured CK-MM

Activity of the bands was tested using the luciferin-luciferase reaction and a reagent kit described by Scheuerbrandt et al. (59). Creatine phosphate and ADP (25 μ l each) were added to a test-tube containing 200 μ l of LKB CK Reagent. Slopes were recorded using an LKB 1250 Luminometer and a Coleman 165 Recorder. Various amounts of sample were then added to the mix and the change in slope recorded in mV units. Conversion of mV units to IU can be made using the formula of Scheuerbrandt et al. (59). Two sampling methods were used to assay for activity: (1) renaturation of CK by dilution to 0.1 M urea using the method by Zhou and Tsou (60). Samples of Sephadex G-75 (8 M urea) containing M-subunit bands 1-6 were diluted in 0.1 M glycine (pH 9), 1 mM EDTA, and incubated at room temperature for 1 hour. Aliquots of 10 μ l of renatured band material were then added to the reaction mixture described above. (2) After gel filtration and concentration of the sample, an aliquot containing 1.5 μ g was taken and added to the test-tube containing ADP, CP, and CK Reagent mix.

e) Purity of Subunits of CK-MM

Purity of bands isolated from the Sephadex G-75, S.F. gels was checked by running the bands on agarose(IEF) gels (12.5x12.5x0.2cm) containing 2% (w/v) agarose, 12% (w/v) sorbitol, 5 M urea, 5% (v/v) ampholytes, and 0.9% (v/v) mercaptoethanol. Agarose was poured on the hydrophilic side of Gel-Bond plastic film. Agarose gels were then either allowed to age for 2 hours at 4° C or overnight at room

temperature. Agarose gels were prefocused for 30-45 minutes at 3 W, constant power. Gels were fixed and stained as previously described for agarose-urea gels. Purity of bands after gel filtration and concentration was checked by re-running bands on a nondenaturing agarose gel (12.5x12.5x0.2cm) containing 1% (w/v) agarose, 12% (w/v) sorbitol, and 6% (v/v) ampholytes. Gels were pre-focused and run as above. Nondenaturing gels were stained with the activity stain described under Chromatofocusing or using the staining method originally described by Saravis and Zamcheck (61) with some modifications. Gels were placed in a fixing solution of 10% (w/v) TCA, 5% (w/v) sulphosalicylic acid for 30 minutes. Gels were then washed 2x15 minutes in 95% ethanol. Filter paper (Whatman #3) soaked in ethanol was placed on top of the gel with four dry filter papers on top. A glass plate was placed on top and left for 20 minutes. The gel was air dried and then stained with a solution of 0.2% (w/v) Coomassie Blue R-250, 50% ethanol (w/v), and 10% acetic acid (v/v) for 10-15 minutes at 60° C. Gels were then destained in 50% methanol, 10% acetic acid, 40% DDW until the background was clear. The gel was then soaked in destaining solution with 4% glycerol for 30 minutes, air dried and stored in plastic.

G. Electrophoresis of Band 1 on SDS-PAGE gel

a) SDS-PAGE gels using O'Farrell's method (59) were prepared. Gels consisted of a stacking gel (4.5% acrylamide/bis, 0.009% ammonium persulfate,) and a resolving gel (12.5% (w/v)

acrylamide/bis, 0.009% (w/v) ammonium persulfate). Samples were prepared by heating in 25 μ l buffer (1.5% (w/v) Tris/HCl, pH 6.8, 0.5% SDS (w/v), 0.05% Bromophenol Blue (w/v)) for 2 minutes at 100° C and then cooling to 37° C. Mercaptoethanol was then added to a final concentration of 10% (v/v), SDS to a final concentration of 2% (w/v), and glycerol to a final concentration of 30% (v/v). The samples were applied to the top of the stacking gel. Gels were run at 30 ma, constant current for 6 hours. Gels were stained overnight in a solution of 0.2% Coomassie Brilliant Blue (w/v) and 10% acetic acid (v/v). Then gels were destained in a solution of 50% methanol and 10% acetic acid until the background was clear.

b) Protein was determined by the Bradford Protein assay as described previously.

VI. RESULTS

A. Chromatofocusing

The initial technique chosen to separate the subunits of rabbit skeletal muscle creatine kinase was chromatofocusing. Other investigators have used chromatofocusing to purify mixtures of proteins according to differences in isoelectric points (53, 63). The first buffer used to equilibrate the column was 0.025 M Tris/acetate (8 M urea), but this buffer produced poor M-subunit resolution. The next buffer used to equilibrate the column was 0.025 M imidazole/acetate (8 M urea) and this produced somewhat improved resolution of the M-subunits of muscle creatine kinase.

1) CK-MM resolution on chromatofocusing column using denaturing conditions

Rabbit skeletal muscle creatine kinase (CK-MM) from Boehringer was chosen for the chromatofocusing experiments. Sigma rabbit skeletal muscle creatine kinase was also tested and did not seem to give any better resolution of M-subunits for the chromatofocusing experiments. Therefore, Boehringer CK-MM was used exclusively for all further experiments with one exception. This one exception was an experiment to check for possible protein contamination of CK-MM starting material. Both Boehringer and Sigma CK-MM were used as the starting material in this experiment. The results of that one experiment will be discussed in Section G.

After equilibrating the column with 0.025 M imidazole acetate, pH

7.2, 8 M urea, and then titrating with Polybuffer 96, pH 6.0, 8 M urea, a linear pH gradient was generated from pH 7.68 to pH 6.40. This gradient was produced over a range of 200 ml (100 x 2 ml fractions). Four separate peaks were detected at 280 nm (see Figure 1). The pH values at which the peaks eluted are given in Table I. For each of six runs, a peak was detected after Polybuffer 96 (8 M urea) was pumped into the column and before the sample of creatine kinase was applied (see Figure 1). This seems to be an artifact of the polybuffer medium as the column was washed extensively between runs or repacked with new polybuffer exchanger material. Chromatofocusing runs (4 out of 6) did not always produce a linear pH gradient over the entire 200 ml of collected polybuffer fractions. A linear pH gradient is required for good resolution of protein material (53) but generating a reproducible linear pH gradient in this alkaline range proved to be a problem regardless of which buffer was used.

2) Analysis of peaks from the chromatofocusing column using IEF

After each peak from the chromatofocusing column was collected, dialyzed to reduce the urea concentration, and lyophilized, peak material was then applied to a 5% polyacrylamide IEF gel that was focused for 3.5 hours. After dialysis, the peak material from peaks 1-4 should be in dimeric form (CK-MM) which is the active form of the enzyme. All chromatofocused peaks did show creatine kinase activity but the activity pattern was similar although not identical to

unchromatofocused CK-MM (see Figure 2). Band patterns appeared within 15 minutes of applying the activity stain for both unchromatofocused CK-MM and lyophilized peak material. At this stage, no attempt was made to quantify the amount of chromatofocused material applied to the polyacrylamide gels.

It was decided from these results that the chromatofocusing technique was not sensitive enough for demonstrating M-subunit heterogeneity. Other researchers have used isoelectric focusing to show microheterogeneity (64, 65) and therefore, this was the next method selected to explore M-subunit heterogeneity for CK-MM.

B. IEF in polyacrylamide gels

1) Nondenaturing system

Isoelectric focusing on polyacrylamide gels of rabbit skeletal muscle CK showed the pattern described by Guslits and Jacobs (8) (see Figure 3). Bio-Rad pI markers (4.65-9.60) were used as pI indicators for the 21 subforms of CK-MM. CK-MM subforms were estimated to focus between pI values of 6.50 and 7.70 at 4° C in close agreement with Guslits and Jacobs (8). They reported the apparent pI values of rabbit CK-MM subforms to be between 6.43 and 7.67 at 4° C.

2) Denaturing system

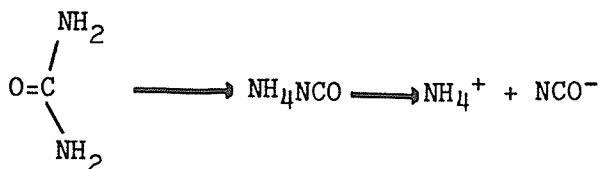
CK-MM was denatured into subunits in an 8 M urea buffer and applied to a polyacrylamide-urea isoelectric focusing gel. Coomassie staining detected a 6 M-subunit band pattern (see Figure 4), that was

reproducible and stable with time. Bands were named 1-6 starting with the band closest to the cathode. A time study showed that the M-subunit bands were stable whether the gels were focused for 3.5 hours, 4.5 hours, or 5.5 hours (see Figure 5). The only observed difference was an increased sharpness in the individual bands when isoelectric focusing times were increased.

A densitometer scan at 600 nm of the gel in Figure 4 detected a total of six distinct bands (see Figure 6). There were 4 major protein bands and 2 minor protein bands. This M-subunit band pattern for creatine kinase was reproducible but although various methods such as electroelution or freezing and thawing were tried to elute the bands from the polyacrylamide gel, none were successful in producing large amounts of purified M-subunit bands sufficient for further study.

C. Carbamylation Experiments

All experiments to denature the CK-MM required 8 M urea buffers. It is known that aqueous solutions of urea tend to hydrolyze into NH_4^+ and cyanate. Eventually, an equilibrium pair is formed according to the following equation:



At equilibrium, an 8 M urea solution contains 0.02 M cyanate (66). The cyanate can react with NH_4^+ and SH groups particularly at high temperatures to form carbamylation products. Precautions were taken to prevent such reactions.

- a) All urea was deionized just before using for sample buffers and polyacrylamide gels by passing the urea solution through a column of Rexyn 300 mixed bed ion-exchanger.
- b) The temperature was kept as low as possible during the running of the gel, viz. 13.5 ° C. This temperature was just high enough to prevent crystallization of urea in the polyacrylamide gel.
- c) CK-MM was left in the urea buffer for the minimum time required for dissociation into M-subunits and the minimum time required to focus the M-subunits.
- d) Mercaptoethanol was added to all sample buffers since it is known that cyanate will combine preferentially with the SH groups of mercaptoethanol. Although all these precautions were taken, a series of experiments was conducted to test whether the M-subunit pattern seen in isoelectric focusing experiments could be due to carbamylation of the amino or sulfhydryl groups of the M-subunits of CK.

1) Analysis at pH 5-9

In the first set of experiments to test for the possibility that the 8 M urea solutions were modifying the M-subunits of creatine kinase, CK-MM was solubilized in 0.5 M KCNO with no pre-incubation

period and then focused on a polyacrylamide-8 M urea gel. CK-MM, dissociated into subunits in an 8 M urea buffer or deionized 8 M urea buffer, was also run on the same gel for comparison. Isoelectric focusing of creatine kinase solubilized in 0.5 M KCNO (see Figure 7.C) produced bands that were modified when compared to the isoelectric focusing results of creatine kinase dissociated in 8 M urea buffer (see Figure 7.A) or deionized 8 M urea buffer (see Figure 7.B). KCNO-treated M-subunit bands are wavy in appearance and displaced slightly in position towards the anode with extra bands near the anode. It should be noted that dissociating muscle creatine kinase (CK-MM) in either 8 M urea or deionized 8 M urea buffers produced similar band patterns on the polyacrylamide-8 M urea gels. When a densitometric scan of bands from Figure 7.C (CK-MM treated with 0.5 M KCNO) was compared to a scan of bands from Figure 7.B (CK-MM treated with deionized 8 M urea), a shift of bands was clearly indicated (see Figure 8).

It was then decided to test for any further effects, when creatine kinase was pre-incubated with 0.5 M KCNO for a period of 24 hours at 30°C before isoelectric focusing on a polyacrylamide-8 M urea gel (see Figure 9.a). There appeared to be only one set of closely spaced bands near the anode for the KCNO-treated creatine kinase. The other two sets of bands (Figure 9.b and 9.c) were the result of isoelectric focusing of creatine kinase that had been solubilized in 8 M urea and deionized 8 M urea buffers, respectively. A densitometric scan also showed that the normal M-subunit pattern for

urea-treated creatine kinase is absent for the KCNO-treated creatine kinase (see Figure 10). It is possible that because of the restricted pH range (5-9) that there might actually be more than one band for the KCNO-treated creatine kinase. Therefore, an expanded pH range was chosen for the next experiment. Since it has been reported that an 8 M urea aqueous solution contains 0.02 M cyanate at equilibrium (67), it was decided to use a concentration that was slightly below the equilibrium concentration. CK-MM was, therefore, preincubated with 0.005 M KCNO.

2) Analysis at pH 3.5-10

CK-MM was incubated in 0.5 M KCNO and 0.005 M KCNO for 24 hours at 30°C before isoelectric focusing on a polyacrylamide-8 M urea gel. CK-MM solubilized in an 8 M urea buffer was also run on the same gel and produced a pattern of M-subunit bands near the middle of the gel (see Figure 11.a). CK-MM solubilized in 0.005 M KCNO produced a band pattern that was slightly displaced towards the anode (see Figure 11.b). CK-MM solubilized in 0.5 M KCNO produced a multi-band pattern that could only be seen on a gel with an expanded pH of 3.5 to 10.0 (see Figure 11.c). Band patterns were exceptionally sharp and distinct. All experiments with KCNO (0.5 M and 0.005 M) demonstrated that treatment of CK-MM in KCNO and subsequent isoelectric focusing on polyacrylamide-8 M urea gels did not produce the same bands as those produced by the 8 M urea buffers.

Since it was thus confirmed that the heterogeneity for the

M-subunit band pattern for denatured creatine kinase was not due to carbamylation of side groups (NH_2 and SH), the experiments to confirm heterogeneity for the M-subunits were continued. Acrylamide can react with free amino groups, thiol residues and the hydroxyl groups of tyrosine if polymerization is not completed before samples are focused (67). To test for the possibility that the bands visualized by Coomassie stain were only artifacts due to unpolymerized acrylamide-protein reaction, denatured muscle creatine kinase was isoelectrically focused on an agarose-7 M urea gel. As a continued precaution against any possible carbamylation of M-subunits, mercaptoethanol was added to all buffer and gel solutions.

D. Isoelectric Focusing in Agarose-7 M Urea Media

1) Detection of M-subunits of CK-MM

After isoelectric focusing on an agarose-7 M urea gel, denatured creatine kinase was resolved into a 5 M-subunit band pattern that in intensity and spacing were similar to M-subunit band patterns of denatured creatine kinase that was isoelectrically focused on polyacrylamide-urea. It was found that at this concentration of urea (7 M), the agarose gels were extremely fragile and therefore could not be fixed, stained, or destained as usual. Therefore, Takehana's nitrocellulose blotting method for polyacrylamide (57) was adapted as a detection method for M-subunit bands on agarose-urea gels. The nitrocellulose blotting method was not as sensitive compared to direct staining of the gel. However, the blotting method had the

advantages of not damaging the gel and also allowing the gel to be used as a preparative medium. The nitrocellulose blot of muscle creatine kinase focused under denaturing conditions detected the first four major M-subunit bands only (see Figure 12): M-subunit bands 1, 2, 3, and 4 using the numbering order first described in Section B.2 of Results.

2) Recovery of M-subunits

Per gel, an average of 15 lanes were each loaded with 100 ug of denatured creatine kinase. Attempts were made to recover the protein from each of the four major bands from the agarose gel by an extraction procedure using freezing and thawing. Table II shows the results of one extraction of bands from an agarose-7 M urea gel. The total protein applied to the gel was 1.5 mg (100 μ g x 15 lanes). Recovery of protein from the agarose-urea gel was fairly good (approximately 57%).

Table II

Distribution of M-subunit bands 1-4 after isoelectric focusing on agarose-urea gels.

<u>M-subunit</u>	<u>Total Protein After Dialysis</u>	<u>Estimated % Recovery</u>
1	0.253 mg	17.0%
2	0.251 mg	16.9%
3	0.188 mg	12.5%
4	0.156 mg	10.4%

3) Change in Mobility

After lyophilization, the M-subunit bands were solubilized in an 8 M urea buffer and isoelectrically focused on either an agarose-7 M urea or a polyacrylamide-8 M urea gel. It was found that after freezing, thawing, and lyophilization, the mobility of the M-subunit bands had changed. The samples, after being isoelectrically focused the second time, stained for protein only on the application pads. No M-subunit pattern could be detected on either type of gel. Creatine kinase solubilized in an 8 M urea buffer for the first time and run on the same gel produced the M-subunit pattern normally seen for the polyacrylamide-urea or agarose-urea gel.

It was decided that although isoelectric focusing on agarose-urea gels produced M-subunit bands that could be recovered in fairly high yield (57%), the extraction or lyophilization procedure had a detrimental effect on the mobility of the isolated M-subunit bands. Therefore, an agarose medium was deemed not suitable as a preparative medium for isolating M-subunit bands.

4) Apparent pI values for M-subunit bands 1-4 in urea

An agarose-7 M urea gel (pH 5-9) was measured at regular intervals along its surface for values of pH as described in Methods. The pH values were plotted against distances from the cathode (see Figure 13). From the graph, the apparent pI values for the first 4 M-subunit bands were calculated (see Table III).

Table III

Apparent pI values for M-subunit bands 1-4 under denaturing conditions

<u>Band</u>	<u>Apparent Isoelectric Point in 7 M Urea</u>
M-subunit 1	8.42
M-subunit 2	8.28
M-subunit 3	8.08
M-subunit 4	8.00

E. IEF in Immobilized pH gradients

So far, it has been shown that the M-subunit band pattern for muscle creatine kinase isoelectrically focused under denaturing conditions was not due to carbamylation of side groups of CK-MM by cyanate formed from the decomposition of urea or to an acrylamide-protein artifact. There is the possibility of ampholytes reacting with the proteins and producing false bands (70). Therefore, studies using the immobililine-polyacrylamide system were next conducted. Immobililines differ from free ampholytes in that the immobililines have covalent links between ampholytes and acrylamides and cannot move when current is applied (58). Two types of gels were prepared: nondenaturing and denaturing (urea).

1) Studies on native CK-MM Using Immobiline pH Gradients

Rabbit CK-MM was focused in its native state on a polyacrylamide-immobiline gel. After staining of the gel with an activity stain (results not shown) or a Coomassie stain (see Figure 14), the same multiple subform pattern was visible. This CK-MM subform pattern was similar to the CK-MM subform pattern seen on previous nondenaturing agarose and polyacrylamide gels using ampholytes (see Figure 2). The use of immobilines to produce a fixed pH gradient did not interfere with the activity of the subforms as judged by the activity stain. Band resolution, however, was not improved using the Immobiline pH gradient system when the CK-MM bands on the immobiline gel were compared to CK-MM bands on an ampholyte-polyacrylamide gel (see Figure 2).

2) Denaturing Studies on CK-MM using 8 M Urea

CK-MM was dissociated into subunits in an 8 M urea, 1 mM EDTA buffer (with no added ampholytes or immobilines) and isoelectrically focused on a polyacrylamide-immobiline-8 M urea gel. A 5 M-subunit band pattern was detected using the Coomassie stain described in Methods (see Figure 15). Bio-Rad marker proteins were run as pI indicators but it was found that the isoelectric focusing pattern for the markers in 8 M urea was entirely different from that for the native system. Thus these marker proteins could not be used for reference pI values for the five M-subunits.

A densitometric scan (see Figure 16) showed improved resolution

for the first five M-subunits as compared to the densitometric scan for the M-subunits detected on an 8 M urea-polyacrylamide gel (see Figure 6). The return to baseline is improved for the M-subunits resolved on polyacrylamide-immobiline-urea gels. From these experiments, it appeared that the isoelectric focusing pattern obtained for the M-subunits denatured with 8 M urea was not an artifact due to ampholyte-protein interaction.

This was the final experiment conducted to demonstrate that the heterogeneity seen for the M-subunit pattern obtained after treatment with 8 M urea and isoelectric focusing was not due to artifact formation from the urea buffer or the gel medium.

F. Purification of Boehringer Rabbit Skeletal Muscle M-subunits

The next objective was to purify the M-subunits of muscle creatine kinase and to run these purified M-subunits on a second isoelectric focusing gel. Sephadex G-75, Super Fine (S.F.), was chosen as the preparative medium to produce M-subunits bands and then to purify the individual M-subunits 1-6.

1) IEF on Primary Sephadex G-75, S.F.-8 M Urea gels

a) Resolution of CK-MM into M-subunits

After 8-10 mg of Boehringer rabbit CK-MM was dissociated in an 8 M urea buffer and applied to the surface of a Sephadex G-75, S.F.-8 M urea gel, the gel was focused for 16 hours. The blotting method detected a 6 M-subunit band pattern (see Figure 17). Bands were

numbered in the usual manner: 1-6 beginning with the major band closest to the cathode. Faint bands (x, y) which stained for protein were detected running ahead of M-subunit band #1. These extra bands were detected on all primary isoelectric focusing runs of Boehringer rabbit skeletal muscle creatine kinase. Extra bands were also detected for Sigma rabbit skeletal muscle creatine kinase but these extra bands had focused closer to the cathode.

b) Different Activities for Bands 1-6

All bands from primary Sephadex G-75, S.F.-8 M urea gels were renatured into their active forms using the renaturing technique described by Zhou and Tsou (60). CK activity was measured using the method of Scheuerbrandt et al. (59). M-subunit bands 1-6 showed a reproducible pattern of enzymatic activity (see Figure 18). Activity was measured by the angle generated after addition of creatine phosphate. The larger the angle, the greater the creatine kinase activity. Samples (50 mg)^{of gel} were taken from the center of each band detected on the Sephadex-urea gels. The most active bands were M-subunit bands 2 and 6. Boehringer M-subunit band 1 was a major protein band but consistently showed very little creatine kinase activity.

The bands running ahead of M-subunit band 1 (x, y) did not show any activity after renaturation by the surface sampling technique. This indicated that these bands are contaminants of the Boehringer rabbit skeletal muscle creatine kinase preparation and not true

M-subunits. Since the major 6 M-subunit bands did show creatine kinase activity after renaturation, it was decided to isolate and run each band separately on a second Sephadex G-75, S.F.-8 M urea gel as a check on its purity and stability.

2) IEF of M-subunits on a Secondary Sephadex G-75, S.F.-8 M Urea Gel

M-subunit band material that had been isolated from a primary preparative Sephadex G-75, S.F.-8 M urea gel was purified by refocusing each M-subunit band on a second Sephadex-urea gel. After secondary isoelectric focusing, the M-subunit bands were visualized by a blotting method and showed enrichment for one main M-subunit type with various amounts of contamination from neighbouring M-subunit bands (see Figure 19). Two points should be emphasized: 1) The six band pattern visualized in the primary Sephadex-urea gel was not repeated for any isolated M-subunit band refocused on a second Sephadex-urea gel. 2) When the M-subunit bands were isolated from a primary gel for refocusing on a second gel, it was felt that as much of each band should be taken as possible. If there was any contamination from neighbouring bands, this contamination would be separated from the main M-subunit band after the second isoelectric focusing run.

Band 2 (see Figure 19.a) and Band 3 (see Figure 19.b) were refocused 4 days after isolation from a primary isoelectric focusing run on a second Sephadex G-75-8 M urea gel, using a 7:3 mixture of Ampholines pH 6-7 and 3.5-10, respectively. M-subunit Band 2

material seemed to be almost pure with only a trace of M-subunit Band 3 material. Band 3 material (see Figure 19.b) was isolated from two primary IEF Sephadex/urea gels and although composed mainly of band 3 material, there was minor contamination with band 2 material.

The bands were harvested and next applied to a gel filtration column. Various grades of Sephadex were tried such as G-25 and G-75, but G-50 produced the best separation of M-subunit band protein from urea and ampholytes.

3) Gel Filtration and Concentration of M-subunit Bands

Isolated M-subunit bands from primary or secondary Sephadex G-75, S.F.-8 M urea gels were renatured by a gel filtration step. M-subunit band material was applied to the top of a Sephadex G-75, S.F. gel filtration column and eluted with a 0.1 M ammonium bicarbonate, pH 7.0, 100 mM mercaptoethanol buffer. There was good separation of protein (renatured CK-MM) from ampholytes and urea using this method (see Figure 20). Protein fractions were then concentrated under nitrogen gas using an Amicon concentrator. Aliquots of the renatured CK-MM fractions were assayed for protein using the Bradford protein assay (see Table 1V).

Table IV

Recovery of M-subunit Bands 1-3 from Sephadex G-75-urea gels.

<u>M-subunit Band #</u>	<u>Times Band Focused</u>	<u>Protein Recovered</u>
1	2x	0.50 mg
2	1x	0.30 mg
3	2x	0.13 mg

Recovery of protein was low considering that usually 8 mg of CK-MM were loaded onto the primary Sephadex G-75-8 M urea gels.

4) Results of M-subunit bands from Sephadex G-75, S.F.-8 M urea gels refocused on agarose-5 M urea gels

Aliquots of M-subunit bands 1-6 from primary (preparative) Sephadex/urea gels were applied to LKB pads and isoelectrically focused on agarose-5 M urea gels. Coomassie staining of the gel detected different patterns for Band 1 and Band 2 (see Figure 21). Band 1 consisted mainly of M-subunit 1 with only a trace of M-subunit 2. Band 2 was composed of almost equal amounts of M-subunit 1 and M-subunit 2. For both Bands 1 and 2, the extra contamination was caused partly by the technique used to harvest bands. It was considered better to obtain as much material as possible from each band from the primary (preparative) Sephadex G-75, S.F.-8 M urea gel than to isolate only very narrow strips of band material. However, it should be noted that even with this isolation technique there was

no evidence of M-subunits 3, 4, 5, or 6 in Band 1 or Band 2 material.

Bands 3, 4, 5, and 6 material from a primary (preparative) Sephadex-urea gel were applied to separate lanes of an agarose-5 M urea gel which was then isoelectrically focused for five hours (see Figure 22). Again, there was contamination from other M-subunits but it should be noted that not one lane showed the 6 band pattern for muscle creatine kinase that had been first detected on the primary (preparative) Sephadex G-75, S.F.-8 M urea gel. When M-subunit bands 1 and 6 were isolated from preparative gels and focused on agarose-urea gels, they appeared to be composed of only one M-subunit type.

Band 1 (isolated from a primary Sephadex-urea gel) and band 2 (isolated from a secondary Sephadex-urea gel) were renatured by gel filtration and concentrated as described previously. Aliquots of concentrated Bands 1 and 2 were then isoelectrically focused on native agarose gels. Half of the gel was stained with an activity stain (see Figure 23). Band 1 material had been isolated from a primary Sephadex-urea gel. After renaturation by gel filtration, Band 1 produced on the native agarose gel, a pattern of one major CK-MM subform with a second minor CK-MM subform closer to the cathode (-). This Band 1 material had previously been shown to consist of 2 M-subunits (refer to Figure 21). Band 2 material, however, was first isolated from a primary Sephadex-urea gel, refocused on a second Sephadex-urea gel, and then isolated and applied to a gel filtration column. Band 2 material, after renaturation and concentration,

produced on the native agarose gel only one native CK-MM subform. This CK-MM subform for Band 2 material was detected at the same position as the minor CK-MM subform for Band 1.

G. Contamination of Boehringer rabbit skeletal muscle CK

Because of problems, initially, in refocusing bands, it was decided to check for possible protein contamination in the Boehringer preparation of rabbit skeletal muscle creatine kinase that had been used for all experiments.

Boehringer M-subunit band 1 material was isolated from a Sephadex G-75-urea gel and eluted through a Sephadex G-50 gel filtration column. A minor protein peak (p) was recorded eluting just before the main protein peak for Boehringer Band 1 (see Figure 24.a). This minor protein peak (p) was reproducible for Boehringer M-subunit band 1 material. It was not seen for the other five bands (Bands 2-6) also resolved from Boehringer rabbit skeletal muscle creatine kinase.

When Sigma M-subunit Band 1 material was isolated from a Sephadex G-75-urea gel and eluted through a Sephadex G-50, S.F. gel filtration column, no protein peak was detected eluting before the main protein peak for Sigma Band 1 (see Figure 24.b).

Both Sigma and Boehringer Band 1 material, after gel filtration and concentration, were tested for activity using Scheuerbrandt's method (59). Renatured Sigma Band 1 material had significantly higher activity than renatured Boehringer Band 1 material (see Figure 25). It should be pointed out that the renatured Boehringer Band 1

material contained protein peak p.

H. Analysis of Sigma Band 1 on SDS-PAGE

Sigma Band 1 material was isolated from a Sephadex G-75-urea gel, eluted through a Sephadex G-50 gel filtration column, and concentrated. An aliquot of 50 μ l of concentrated Band 1 material was then electrophoresed on SDS-PAGE (see Figure 26). Sigma Band 1 material migrates with a molecular weight of 43,000. Boehringer M-subunit band 2 and 3 material were also run on the same gel but Coomassie staining did not detect any bands running at the same level as the Sigma muscle creatine kinase standard. However, faint lines were detected at the top of the stacking gel, perhaps indicating that aggregation had occurred inspite of the 100 mM mercaptoethanol that was regularly added to all band preparations.

VII. DISCUSSION

1. Purification

Rabbit skeletal muscle creatine kinase has been dissociated in 8 M urea into 6 M-subunits. These CK M-subunits have been shown, by isoelectric focusing on different media, to focus initially into 6 CK M-subunit bands. A two-step purification technique has been developed: first to resolve these 6 CK M-subunit bands in sufficient quantities that can be easily isolated; and second to purify each isolated CK M-subunit band into one pure M-subunit. Sephadex was chosen as the preparative medium for the first and second purification steps because of its excellent band separation properties and the ease in isolating CK M-subunit bands and pure CK M-subunits.

(a) Preparative Sephadex-urea gels - The first step in purification is the separation of CK-MM into 6 CK M-subunit bands by isoelectric focusing on primary preparative Sephadex-urea gels. This 6 CK M-subunit band pattern was first demonstrated by Traugott and Massaro (35) who separated rabbit skeletal muscle CK into 6 CK M-subunit bands using urea-starch electrophoresis. The results of the present work confirm the existence of 6 CK M-subunit bands but indicate that these 6 CK M-subunit bands are not pure. They consist of one main CK M-subunit type with varying amounts of contamination from the other 5 CK M-subunit types. However, Boehringer CK M-subunit band 6 and Sigma CK M-subunit band 1 have been shown to be

electrophoretically homogeneous on analytical agarose-urea gels after only one purification step on Sephadex-urea gels.

The second step in the purification of CK M-subunits is the isolation of individual CK M-subunit bands and refocusing on a second preparative Sephadex-urea gel. It was found that after the second purification step, each initial CK M-subunit band consisted of one main CK M-subunit with none to fair amounts of contamination from the other five CK M-subunits (Refer to Figure 19).

CK M-subunit 2 (M2) was isolated from a second preparative Sephadex-urea gel and, after isolation and renaturation, was shown to focus as one CK-M2M2 homodimer subform (Refer to Figure 23). CK M-subunit band 1, isolated from a primary preparative gel, was analyzed on an agarose(IEF)-urea gel. This band was shown to be composed mainly of CK M-subunit 1 (M1) with trace amounts of CK M-subunit 2 (M2). The CK M-subunit band 1 material was then renatured without further purification and isoelectrically focused on the same gel as CK M-subunit 2. The lane for renatured CK M-subunit band 1 material showed one main CK-MM subform (CK-M1M1) and a second minor CK-MM subform at the same position as the CK-M2M2 subform. These were the only subforms detected for renatured CK M-subunit band 1 material. These results were somewhat surprising since 2 CK M-subunits (M1 and M2) can renature in theory, if there are no restrictions, into 3 CK-MM subforms, ie. M1M1, M1M2, and M2M2. Since CK-M2 was the major CK M-subunit making up CK M-subunit band 2 material and CK-M1 was only a minor contaminant, it would be logical

to expect that the major CK-MM subform should be CK-M2M2 and the minor CK-MM subforms should be M1M2 and M1M1. However, only two CK-MM subforms could be detected by either an activity stain or Coomassie protein stain. This is possibly due to a favoured renaturation into dimers of the same CK M-subunit type, M1M1, M2M2, etc.

This could explain results seen by Traugott and Massaro (37) who found that after rabbit skeletal muscle creatine kinase was dissociated into 6 CK M-subunit bands and then renatured into CK-MM subforms, only 6 CK-MM subforms could be detected on urea-starch gels.

(b) Chromatofocusing - Although Ende et al. (63) used a chromatofocusing technique to separate staphylococcal enterotoxins B and C1, this technique was not sensitive enough to separate 21 subforms with pI values ranging from 6.43 to 7.67 (8) or to separate 6 CK M-subunit bands which have been shown in the present work to focus with apparent pI values (in urea) of 8.43 to 8.00 (first 4 CK M-subunits). Ende et al. (63) did note that for staphylococcal enterotoxin B, their third peak (pI 8.25) appeared to be inhomogeneous, consisting of 2 components with very close isoelectric points. It should be noted that when a pH gradient is measured in urea, the apparent pH is not the same as that obtained in normal aqueous solution (80). There is a conformational change in the structure of the protein which could change the surface charge and

therefore, the pI of the protein. Errors can also be introduced by the primary medium effect and by the pH measuring cell effect (81). The primary medium effect is the change in pH caused by the urea acting on both the carrier ampholyte and water by changing the ionization constant of individual groups and therefore affecting the pI of the protein. The pH measuring cell effect concerns the effect of the urea upon the glass electrode.

Analysis of the renatured 4 peaks of CK-MM subforms showed that peaks 1-4 were not homogeneous and consisted of more than one CK M-subunit type. The CK-MM subform patterns for peaks 1-4 were similar to the standard CK-MM subform pattern, although it would appear that there was some slight purification using the chromatofocusing technique. Figure 2 indicates some change in CK-MM subforms densities for renatured peaks 3 and 4 material and some CK-MM subforms appear to be missing. This could be due to partial separation of CK M-subunits 1-6 in peaks 1-4 or perhaps to a tendency for CK M-subunits to renature into homodimers. It is possible that if a two-step chromatofocusing purification technique were used, there would be better resolution of CK M-subunits. However, the preparative Sephadex-urea gels are a more efficient purification method for the reasons previously stated.

(c) Ampholyte-polyacrylamide-urea gels - Although good resolution of the 6 CK M-subunit bands was obtained using a polyacrylamide medium, it was not possible to easily recover the band

material. An electro-elution technique was used for this purpose with only limited success. Separation of CK M-subunit bands was reproducible but not as good as on the immobililine-polyacrylamide-urea gels or the preparative Sephadex-urea gels.

One suggestion for better separation of CK M-subunit bands would have been to focus the gels overnight. This was tried but it was found that the CK M-subunit band pattern showed slight signs of carbamylation that had not been seen previously (data not shown). Therefore, it was decided not to use ampholyte-polyacrylamide-urea gels as a preparative medium.

(d) Agarose(IEF)-urea gels - Four to five CK M-subunit bands could be detected on the agarose-urea gels depending on the visualization technique used. Resolution or separation between the CK M-subunit bands was inferior to ampholyte-polyacrylamide-urea, immobililine-polyacrylamide-urea, or Sephadex-urea gels. Recovery of the CK M-subunit bands 1-4 was fairly good - 57% of the total muscle creatine kinase sample applied. After renaturation, however, it was found that the mobility of the renatured bands had changed. It is not certain whether the problem was due to the repeated freezing/thawing extraction procedure or the subsequent lyophilization procedure. Because the renatured CK-MM subforms would not enter 1% agarose(IEF) or 5% polyacrylamide gels, it appeared that aggregation of the subforms had occurred. Mercaptoethanol and urea or DTE and urea treatment of the material was tried but neither

combination could disrupt the aggregation of CK-MM subforms.

(e) Immobiline-polyacrylamide-urea gels - The immobiline system of isoelectric focusing has been said to be superior to the ampholyte-polyacrylamide-urea system (76). The advantages of immobiline pH gradients are:

a) stability with time; b) very narrow pH gradients may be formed; c) higher resolution and higher loading capacity; d) uniform conductivity and buffering capacity; e) cathode drift is completely abolished.

Superior resolution was noted for CK M-subunit bands 1-5. Return to baseline was better for CK M-subunits from immobiline gels compared to ampholyte gels. The densitometric scan for the immobiline gels detected multiple peaks for the CK M-subunit bands 1 and 2. This can now be explained using the results from the preparative Sephadex-urea gels. The sample of CK-MM was applied near the cathode on the immobiline gels. The CK M-subunits would leave the cathode and migrate to the anode in order of isoelectric points. Therefore, the bands nearest to the anode should be more homogeneous than the bands closest to the cathode. The densitometric scan (refer to Figure 16) of the immobiline gel confirms that CK M-subunit bands 3 to 5 are more homogeneous than CK M-subunit bands 1 and 2.

The immobiline medium was used to study possible artifact formation and not for any purification studies because of the difficulty previously experienced in extracting the CK M-subunit

bands from the ampholyte-polyacrylamide-urea gels.

2. Investigations for heterogeneity due to artifact formation

(a) Carbamylation - Initial experiments were conducted to eliminate the possibility that the CK M-subunit bands were due to carbamylation. It has been reported that for 8 M urea, the equilibrium contains 0.02 M cyanate (66). Takasawa and Shiokawa (47) dissociated CK-MM into CK M-subunits in 8 M urea using an incubation time of 2.5 hours at 20 ° C. and did not report any problems with carbamylation products. Our procedure to dissociate CK-MM into CK M-subunits was to incubate CK-MM for no more than 30 minutes at 20 ° C.

Results from the KCNO pre-treated CK-MM would indicate that the CK M-subunit bands are not due to carbamylation of any NH_2 or SH groups on the CK M-subunits M1 to M6. Even the 0.005 M KCNO incubation buffer produced a difference in isoelectric focusing pattern when compared to the CK M-subunit band pattern seen for 8 M urea dissociated CK M-subunits.

(b) Isoelectric focusing media - CK-MM was dissociated and focused on different media to eliminate the possibility that the CK M-subunit bands were artifacts due to protein reacting with gel media. Bosisio et al. (67) reported that acrylamide can react with free amino groups, thiol residues, and the hydroxyl groups of

tyrosine if polymerization of the acrylamide was not complete. This would not appear to be a problem with our procedures since the same CK M-subunit pattern was detected on agarose as well as acrylamide and Sephadex. The only difference is better separation of the CK M-subunit bands on preparative Sephadex-urea gels.

(c) Charge - Righetti and Marcelloni (61) reported on the possibility of ampholyte-protein reactions. The immobililine system was used to check for this possible cause of artifact formation producing false microheterogeneity for CK M-subunits. Immobilines are fixed into the gel matrix and cannot move when current is applied. CK M-subunit bands 1-5 were separated after isoelectric focusing on immobililine-polyacrylamide-urea gels. The improved resolution on immobililine gels has already been discussed. To prove that the observed heterogeneity for the multiple CK-MM subforms from human heart and papillary muscle were not artifacts, SiragEldin et al. (76) used different commercial ampholytes with different pH ranges in agarose or polyacrylamide gels. This present work also used different commercial ampholytes (LKB, Pharmacia, Serva, Bio-Rad) without affecting the 6 CK M-subunit band pattern. Various pI ranges were used with no new CK M-subunit bands appearing. The only difference was the degree of resolution of the bands depending on the pI range used.

3. CK M-subunit stability

(a) Ampholyte-polyacrylamide-urea gels - One experiment on CK M-subunit band stability was a time study. The CK M-subunit band pattern was stable after 3.5 hours, 4.5 hours, or 5.5 hours of isoelectric focusing. There was better return to baseline for the CK M-subunit bands at 5.5 hours but no drastic change in CK M-subunit band pattern was detected as the isoelectric focusing time was increased (refer to Figure 5).

A second experiment on stability was the overnight focusing of CK M-subunit bands on an ampholyte-polyacrylamide-urea gel. The CK M-subunit pattern was similar to the 5.5 hour band pattern but signs of slight carbamylation were evident in the wavy appearance of CK M-subunit bands.

(b) Sephadex-urea gels - The preparative Sephadex-urea gels used to purify CK M-subunits also indicated CK M-subunit stability. The CK M-subunit bands could be isolated from a primary preparative Sephadex-urea gel and then refocused on a second preparative Sephadex-urea gel. Purified CK M-subunit 2 (M2) could be isolated and isoelectrically focused on an agarose-urea gel with no change in CK M-subunit position or disintegration into multiple band patterns.

4. Aggregation problem

When attempts were made to isolate CK M-subunit bands from agarose(IEF)-urea gels, it was noted that after extraction by freezing/thawing, dialysis to remove urea, and lyophilization,

aggregation of CK-MM subforms had occurred. Lyophilization was performed in the presence of mercaptoethanol or DTE but the problem of aggregation persisted. CK M-subunit bands from Sephadex-urea gels were renatured by gel filtration and then lyophilized. A problem was also noticed with aggregation of CK-MM subforms after attempts were made to isoelectrically focus this material. Thus it would appear that after dissociation into CK M-subunits and reassociation into CK-MM subforms, lyophilization causes aggregation that cannot be easily disrupted.

5. pI values of CK M-subunit bands 1-4 on agarose-urea gels

Traugott and Massaro (35) separated CK-MM into 6 CK M-subunit bands but did not assign pI values to them. Rosenberg et al. (36) resolved chick muscle CK into 2 CK M-subunits with apparent pI values of 7.5 and 7.4 in urea. Schweinfest et al. (38) resolved chick muscle CK by two-dimensional electrophoresis into 2 CK M-subunits with apparent pI values of 6.85 and 6.50 in urea. These results indicate the difficulty in comparing pI values from different laboratories even when the same tissue is studied.

Studies in this laboratory have detected four CK M-subunit bands on an agarose(IEF)-urea medium. These bands, as judged by the results from Sephadex-urea gels, are not homogeneous. The CK M-subunit bands 1-4 probably contain more than one type of CK M-subunit. Apparent pI values have been assigned to the CK M-subunit bands: CK M-subunit band 1 (pI=8.42), CK M-subunit band 2 (pI=8.28),

CK M-subunit band 3 ($pI=8.08$), CK M-subunit band 4 ($pI=8.00$). It must be remembered that these pI values were measured in the presence of 7 M urea, thereby leading to a change (an alkaline shift) of pI values. Theoretically, pI values of subunits and native dimers should be similar.

6. CK-MM subform activity

It has been shown on a secondary preparative Sephadex-urea gel that the 6 CK M-subunit bands are composed of one main CK M-subunit type with varying amounts of other CK M-subunit types. When the activity was tested for each of the renatured CK M-subunit bands 1-6, there was a reproducible pattern of activity. For Boehringer renatured CK M-subunit bands, the order of activity from most to least enzymatically active was CK M-subunit bands 2, 6, 3, 5, 4, and 1. However, when the bands were visualized by Coomassie protein stain, the order was different: major to minor bands - CK M-subunit bands 1, 2, 3, 4, 5 and 6. There could be two explanations for these observations: 1) each CK-MM homodimer could have different enzymatic activity; 2) the CK M-subunit bands could contain an inhibitor.

CK M-subunit band 6 was only a minor protein band on primary preparative Sephadex-urea gels. However, it consistently showed high creatine kinase activity. Therefore, it might naturally have high enzymatic activity in the native state.

Protein contamination

The second possibility would explain Boehringer renatured CK M-subunit Band 1's position in the experimental order of enzymatic activity detected. Gel filtration of Sigma and Boehringer CK M-subunit band 1 material detected a protein contaminant for only Boehringer CK M-subunit band 1 material. Sigma CK M-subunit band 1, after renaturation into dimers, generated greater enzymatic activity than renatured Boehringer CK M-subunit band 1 material. The activity for renatured Sigma CK M-subunit band 1 material was even greater than for renatured Boehringer CK M-subunit band 2 material. These observations would suggest that the protein contamination found in Boehringer CK M-subunit band 1 inhibits enzymatic activity. As reported in the Results, Sigma rabbit skeletal muscle creatine kinase does not contain this protein contamination. Guslits and Jacobs (8) used Sigma rabbit skeletal muscle creatine kinase in their isoelectric focusing experiments and detected the same 21 CK-MM subform band pattern shown in Figure 2. In the present work, when Sigma rabbit skeletal muscle creatine kinase was isoelectrically focused in Sephadex-urea, a five M-subunit band pattern was detected similar to the M-subunit band pattern visualized for Boehringer rabbit skeletal muscle creatine kinase. The protein contamination found in the Boehringer preparation does not seem to affect the band pattern either in the native or denatured state but affects only the activity of renatured M-subunit band 1 material.

7. Molecular weight

The molecular weight of Sigma CK M-subunit 1 (M1) has been shown to be 43,000 which is the reported molecular weight of a CK M-subunit (2). It is, therefore, unlikely that CK M-subunit 1 has been drastically altered by the purification procedure developed in our laboratory. Using two-dimensional electrophoresis, Giometti et al. (82) also detected microheterogeneity for rabbit muscle creatine kinase M-subunits. They detected six closely spaced subunits with molecular weights of 43,000. Four of these six subunits were positively identified as CK M-subunits.

8. Model for the association of 6 CK M-subunits into 21 CK-MM subforms

Researchers have demonstrated polymorphic subforms for muscle specific proteins such as myosin heavy chains and light chains (69, 70, 71), actin (72, 73) and troponin (74). These subforms are expressed in a constant pattern and not in a tissue specific manner (36). CK-MM is a muscle specific protein and therefore, it would be reasonable to find that it existed in polymorphic form.

Other researchers such as Heinbokel et al. (31) have also investigated muscle CK for CK-MM subforms. They used an agarose isoelectric focusing technique to investigate human skeletal muscle CK for CK-MM subforms. They were able to detect only five CK-MM subforms using this medium. Where very high resolution is required, polyacrylamide is the medium of choice (75). Polyacrylamide might

have increased the number of CK-MM subforms that could have been resolved.

It was noticed in the present work that for CK M-subunit band or CK-MM subform detection, polyacrylamide gels were superior to agarose gels. A second reason why Heinbokel et al. (31) saw limited heterogeneity might have been due to the pH range that they used for their isoelectric focusing experiments. They used a wide pH gradient of 3.5-10 for their isoelectric focusing gels. The pH range used in our experiments to detect CK-MM subforms was 5-9. This narrower pH gradient would allow for better separation and resolution of CK-MM subforms.

Model

For 21 CK-MM subforms to exist, 6 slightly different CK M-subunits are required. These 6 CK M-subunits could combine in the following manner:

M1, M2, M3, M4, M5, M6

M1M1	M2M2	M3M3	M4M4	M5M5	M6M6
M1M2	M2M3	M3M4	M4M5	M5M6	
M1M3	M2M4	M3M5	M4M6		
M1M4	M2M5	M3M6			
M1M5	M2M6				
M1M6					

The model proposed would explain the separation of rabbit skeletal muscle creatine kinase into the 21 CK-MM subforms which have been observed in our laboratory. Recently, SiragEldin et al (79) have observed multiple CK-MM subforms for human heart muscle and papillary muscle. They suggested two reasons why other researchers could not detect this microheterogeneity for CK-MM. The first reason is insensitivity of the methods used and the second is inactivation of CK-MM subforms, since CK-MM subforms can undergo reversible and irreversible inactivation, in response to oxidation and temperature, respectively (78, 79). There are at least two technical procedures described in the paper by SiragEldin et al. (76) that would further explain their success in demonstrating CK-MM subform heterogeneity: 1) the pH range that they chose for their native agarose gels was 5-9 which was the same as the pH range that we selected for our native agarose gels. 2) SiragEldin et al. (76) recognized the need for quick processing of samples for isoelectric focusing and obtained their samples within 4 hours of death. Guslits and Jacobs (8) obtained specimens of human psoas muscle within 5 hours of death and also obtained samples of vastus lateralis muscle removed at the time of operation for total hip replacement. These samples were analyzed by IEF within 15 minutes of removal from the patient.

It has been shown in this present work, that there are 6 CK M-subunits for rabbit skeletal muscle. They have slightly different pI values in urea and after renaturation into dimers have different specific activities. The 6 M-subunits that we have detected are not

the result of artifacts or common protein modifications. They could possibly be the products of multiple CK-M genes and/or alternative processing of such genes.

VIII. CONCLUSIONS

1. Rabbit skeletal muscle creatine kinase has been separated into 6 CK M-subunit bands which are composed mainly of one M-subunit type but may contain varying amounts of contamination from the other 5 M-subunit types.
2. CK M-subunit 2 (M2) has been purified by a two-step procedure developed in this present work. After homogeneous CK M2-subunit was renatured into the active or dimeric form, only one CK-MM homodimer subform (CK-M2M2) was detected with either an activity stain or a protein stain.
3. Boehringer CK M-subunit band 6 and Sigma CK M-subunit band 1 have been separated on primary preparative Sephadex-urea gels into pure CK M-subunits. After isolation and refocusing, only one CK M-subunit type was detected for both Boehringer CK M-subunit band 6 (M6) and for Sigma CK M-subunit band 1 (M1).
4. All CK M-subunits bands (1-6) can be renatured into the active or dimeric form (CK-MM). Boehringer CK M-subunit bands 1-6, after renaturation, show different enzymatic activity. In order of decreasing enzymatic activity they were: CK M-subunit band 2, CK M-subunit band 6, CK M-subunit band 3, CK M-subunit band 5, CK M-subunit band 4, and CK M-subunit band 1.

5. Sigma CK M-subunit band 1, after renaturation, had greater enzymatic activity compared to Boehringer CK M-subunit band 1.

6. Boehringer rabbit skeletal muscle CK preparation contains a protein contaminant which migrates with CK M-subunit band 1. Sigma rabbit skeletal muscle CK preparation does not contain this protein contaminant.

7. Sigma CK M-subunit 1 has a molecular weight of 43,000 daltons.

8. Apparent isoelectric points for CK M-subunit bands 1-4 on agarose(IEF)-7 M urea gels at 13.5⁰ C. are: CK M-subunit band 1 (pI=8.42), CK M-subunit band 2 (pI=8.28), CK M-subunit band 3 (pI=8.08), CK M-subunit band 4 (8.00).

9. The 6 CK M-subunit bands are not the result of modifications due to carbamylation, acrylamide-protein reaction, or ampholyte-protein reaction.

10. After Boehringer rabbit CK-MM is dissociated into subunits and then renatured, lyophilization causes CK-MM subforms to aggregate into high molecular weight compounds that cannot be disrupted by treatment with SDS, mercaptoethanol-urea or DTE-urea.

11. The foregoing results show conclusively that the heterogeneity of native CK observed in intact muscle tissue has its basis in the heterogeneity of the constituent CK M-subunits. The phenomenon is not related to any known process of post-translational modifications. The heterogeneity of CK M-subunit seems a reflection of multiple CK-M genes and/or alternative processing of such genes.

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Table I

pH Range for Peaks 1-4 from Chromatofocusing Column

Peak #	Starting pH ¹	End pH ²	pH range ³
1	7.40	7.38	0.02
2	7.36	7.28	0.08
3	7.22	7.00	0.22
4	6.92	6.40	0.52

1. pH at which peak began eluting from chromatofocusing column and was detected by absorption at 280 nm.

2. pH at which peak finished eluting.

3. pH range = starting pH - end pH.

Figure - 1

Elution profile of M-subunits of rabbit skeletal muscle creatine
kinase from PBE chromatofocusing column.

Boehringer rabbit CK-MM (8 mg) was dissociated in 0.5 ml of an 8 M urea, 0.025 M imidazole acetate buffer, pH 7.4, and applied to the top of a PBE 94 chromatofocusing column.

Bed volume - 19.1 ml

Flow rate - 30 cm/hour

Gradient - Linear consisting of 57 ml of a starting buffer (8 M urea and 0.025 M imidazole acetate, pH 7.4), followed by 200 ml of the eluting buffer (8 M urea, Polybuffer 96 (8 % v/v), pH 6.4).

FIGURE - 1

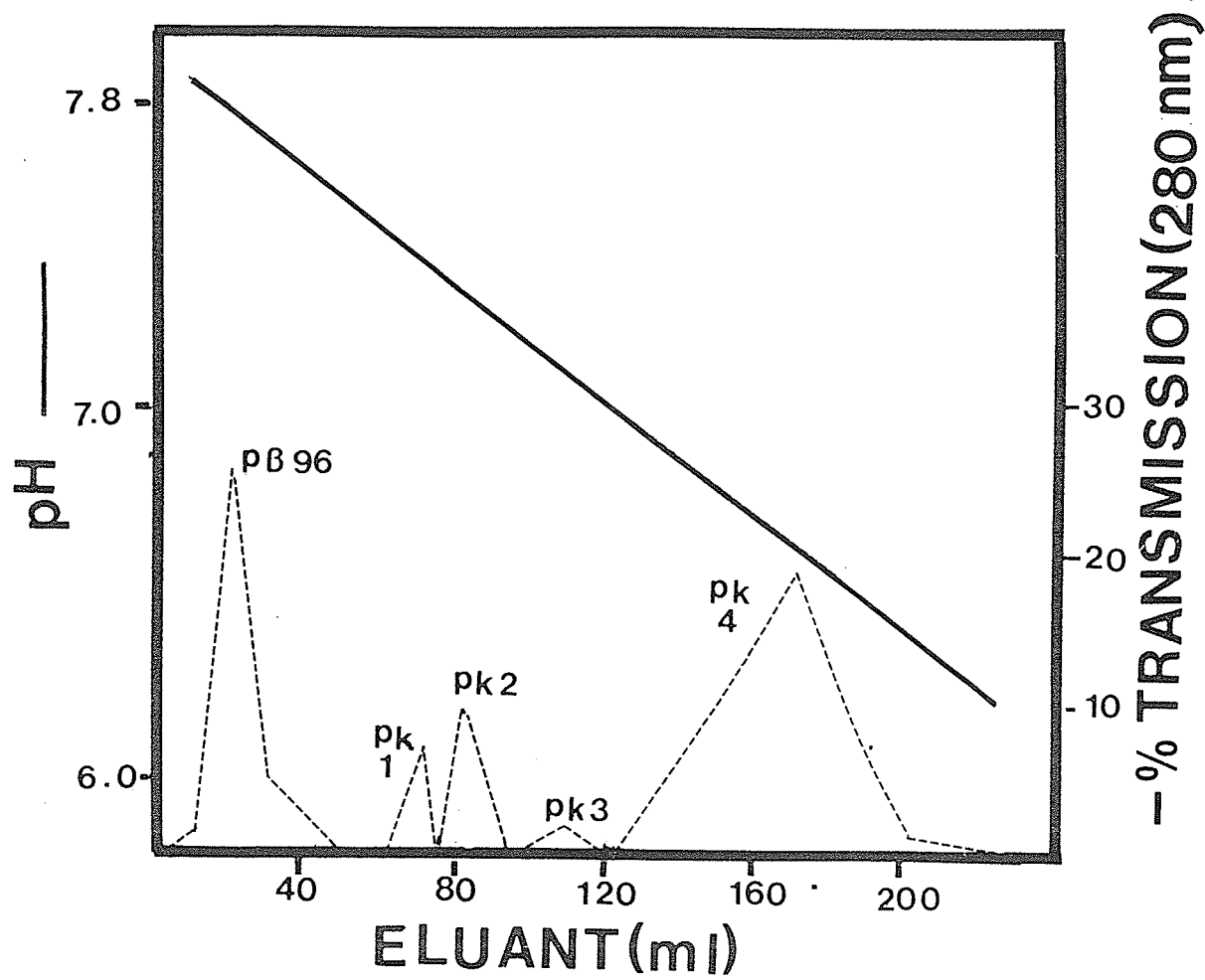


Figure - 2

Results from IEF on native polyacrylamide gels of rabbit skeletal
muscle creatine kinase and peaks 3 and 4 from
chromatofocusing experiment.

Figure 2.A - Boehringer CK-MM was solubilized in DDW and applied to a 5% polyacrylamide gel that was isoelectrically focused at 4° C., 6 W., constant power for 3.5 hours. The catholyte was 0.5 M ethanolamine and the anolyte was 0.2 M citric acid. Activity stain as described in methods was used to visualize the bands. Major CK-MM subforms 5, 10, 11, 13, and 16 are indicated for Boehringer CK-MM.

Figure 2.B - Peak 3 and 4 material from a chromatofocusing column was dialyzed to remove urea and lyophilized as described in methods. Renatured peak materials (3 and 4) were then isoelectric focused as described above. Major CK-MM subforms are indicated for Peak 4 material.

Intensity of staining of CK-MM subform bands is indicated by different sizes of circles.

FIGURE - 2

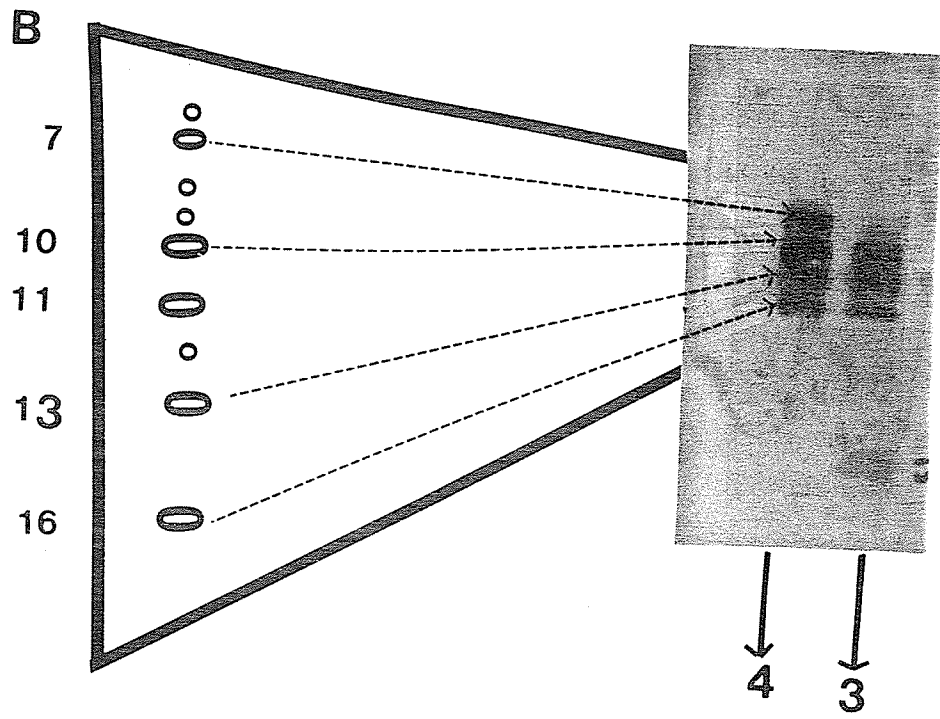
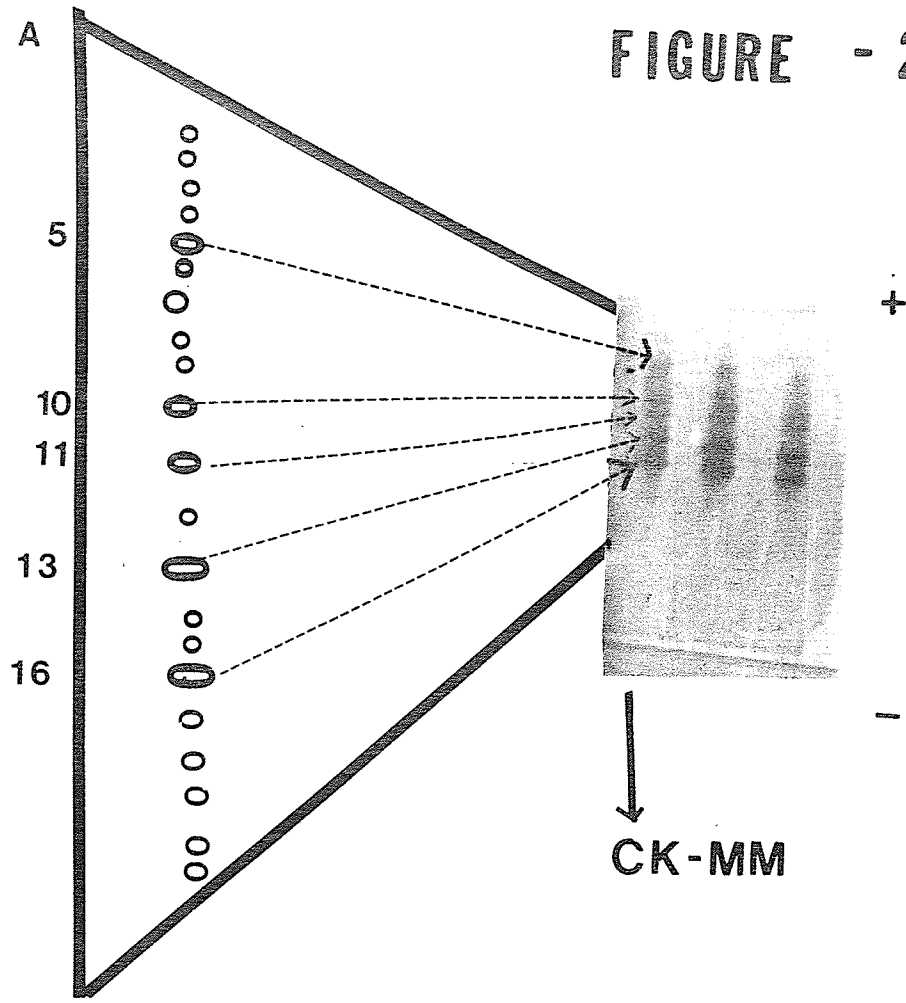


Figure - 3

Detection of band patterns for rabbit CK-MM subforms and pI markers
after IEF on a 5% polyacrylamide gel

Boehringer rabbit CK-MM was solubilized in a buffer of 1 mM EDTA, 100 mM DTE, and 1% N-P-40. An aliquot of 100 µg of solubilized CK-MM was applied to a 5% polyacrylamide gel. Bio-Rad pI markers (5 µg) were applied directly to the gel. The gel was isoelectric focused for 3.5 hours, at 4° C., 6 W., constant power. The catholyte was 0.5 M ethanolamine and the anolyte was 0.2 M citric acid. After focusing, the gel was fixed in a solution of 3.5% sulfosalicylic acid, 10% TCA, and 30% methanol. A 0.2% Coomassie stain was used to visualize the protein bands.

<u>pI markers</u>	<u>pI</u>
1. Phycocyanin	4.65
2. B-lactoglobulin B	5.10
3. Bovine Carbonic Anhydrase	6.00
4. Human Carbonic Anhydrase	6.50
5. Equine Myoglobin, minor band	
6. Equine Myoglobin	7.00
8. Whale Myoglobin	8.05
9. Chymotrypsin	8.80
10. Cytochrome C	9.60

FIGURE - 3

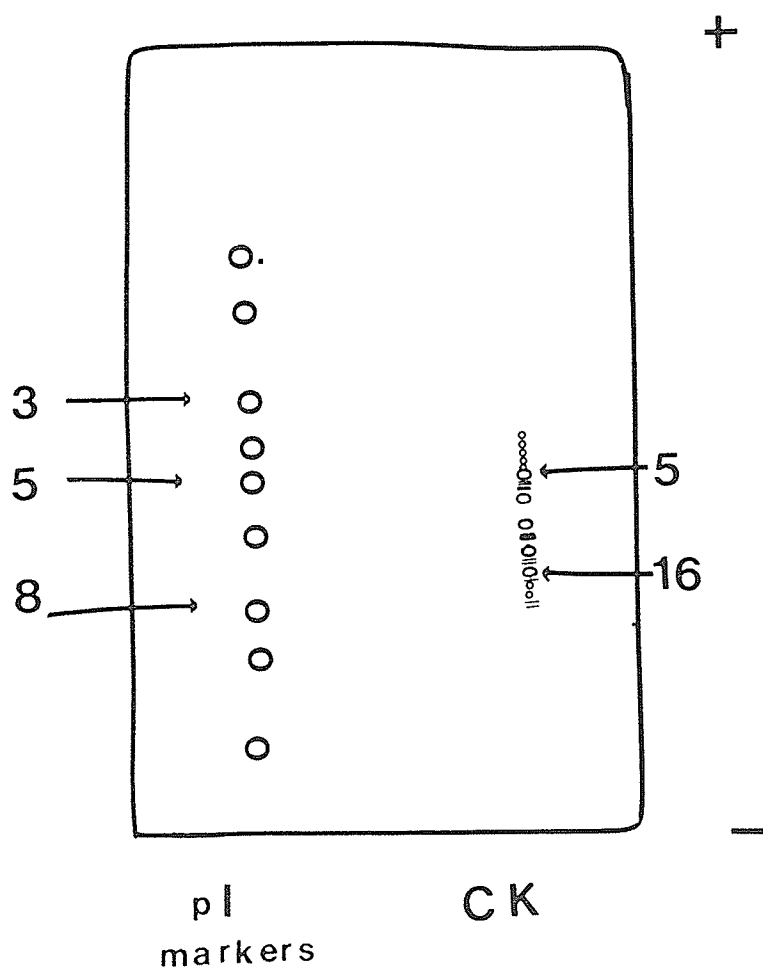


Figure - 4

Detection of band pattern for M-subunits after IEF
in a denaturing system.

CK-MM (1 mg/ml) was dissociated into subunits in an 8 M urea, 1 mM EDTA, 50 mM mercaptoethanol, 1% ampholytes buffer before isoelectric focusing on a 5% polyacrylamide gel at 13° C., at 6 W., for 3.5 hours. The gel was fixed and stained as in Figure 3.

Six bands are visible and are numbered from 1 to 6 beginning with the band closest to the cathode (-).

FIGURE - 4

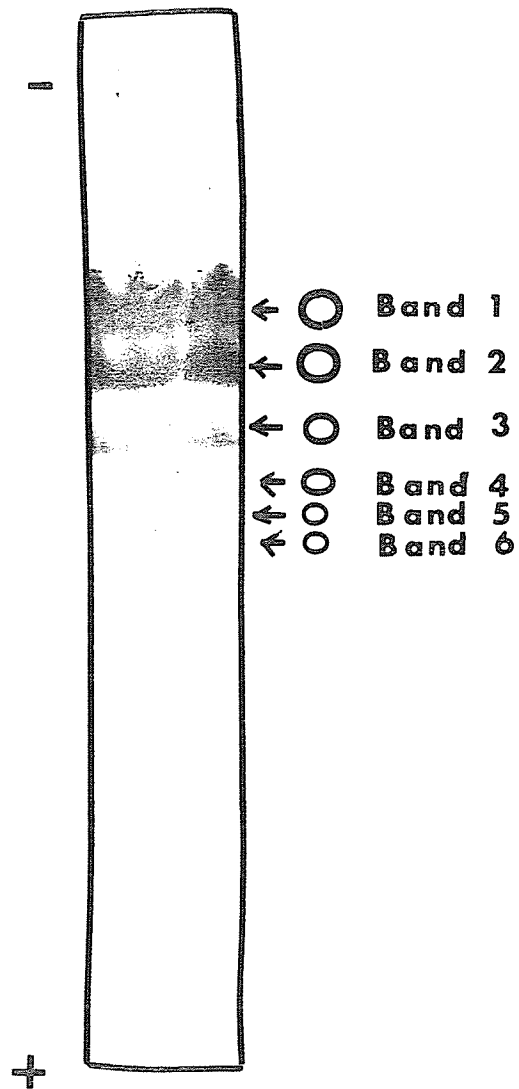


Figure - 5

Time study showing the effect of IEF for various times
on the M-subunits of creatine kinase.

Rabbit CK-MM was solubilized as described in Figure - 4. 100 μ g was pipetted per LKB application pads and isoelectrically focused on a 6% polyacrylamide-8 M urea gel. Samples were focused for various times at 13.5 °C., 6 W., constant power. Gels were cut at appropriate times, removed, fixed and stained as described in Figure - 3. Gels were then scanned at 600 nm for protein.

5.A - Isoelectric focusing time was 3.5 hours.

5.B - Isoelectric focusing time was 4.5 hours.

5.C - Isoelectric focusing time was 5.5 hours.

FIGURE - 5

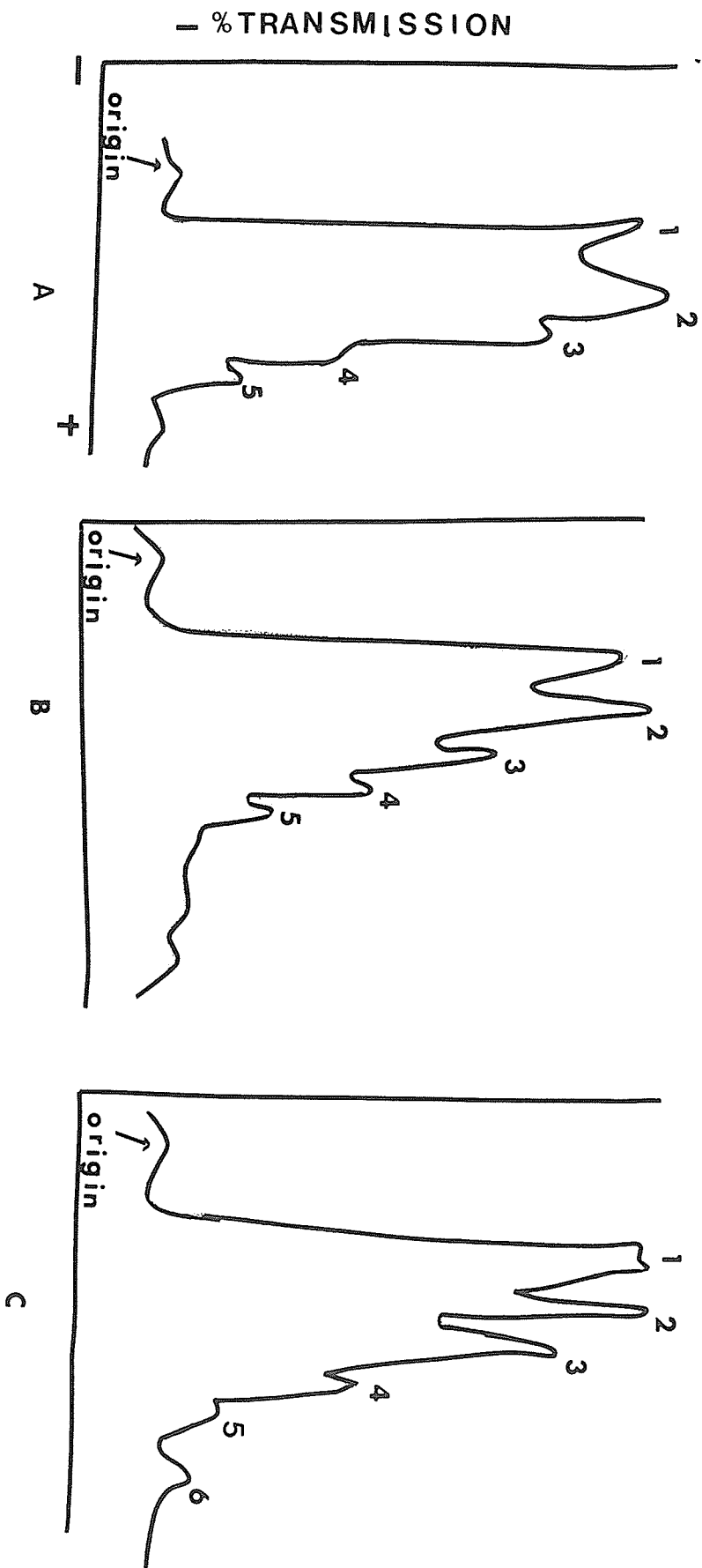


Figure - 6

Densitometric tracings of M-subunit bands of creatine kinase
after isoelectric focusing on 5% polyacrylamide-8 M urea gels.

Samples of rabbit skeletal muscle creatine kinase were dissociated into subunits and applied to a 5% polyacrylamide -8 M urea gel as described in Figure - 4. Isoelectric focusing time for the gel was 5.5 hours at 13.5°C., 6 W., constant power.

The gel was fixed and stained for protein with a 0.2% Coomassie blue stain as described in Figure - 3. Lanes on the gel were scanned for protein at 600 nm. Six bands were detected.

FIGURE - 6

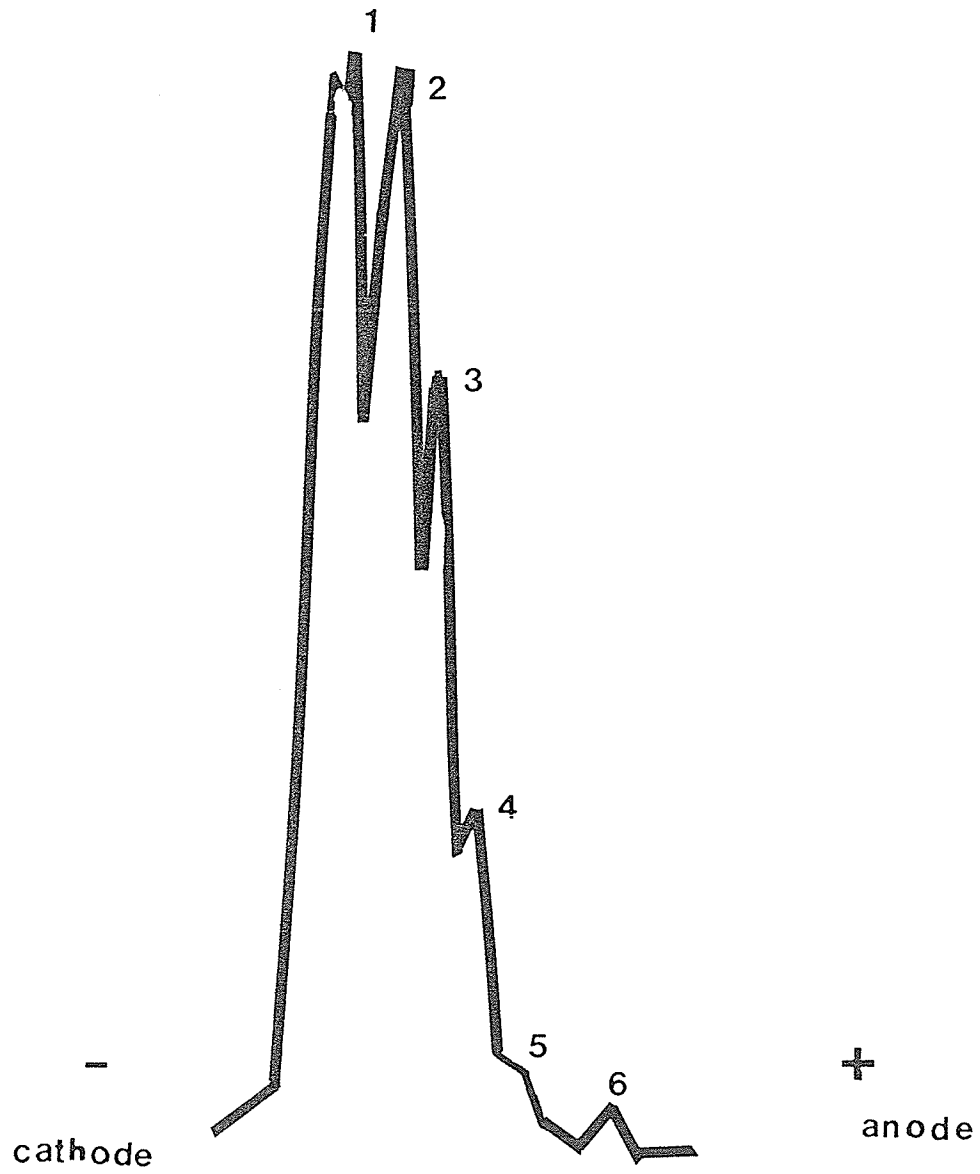


Figure - 7

Detection of band patterns for M-subunits of creatine kinase
using various denaturing agents.

CK-MM was dissociated into subunits in Buffer A, B, or C. The protein concentration was 1 mg/100 ml of buffer.

A - 8 M urea, 1 mM EDTA, 50 mM mercaptoethanol, 1% ampholytes

B - same as Buffer A, but the urea was deionized in a

Rexyn 300 mixed-bed ion exchange column.

C - 0.5 M KCNO, 1 mM EDTA, 50 mM mercaptoethanol,

1% ampholytes

Aliquots of creatine kinase (100 ug) in Buffers A, B, or C, were applied to a polyacrylamide-8 M urea gel and isoelectrically focused for 4.5 hours at 13.5° C., 6 W., constant power. Gels were fixed and stained as in Figure - 3.

FIGURE -7

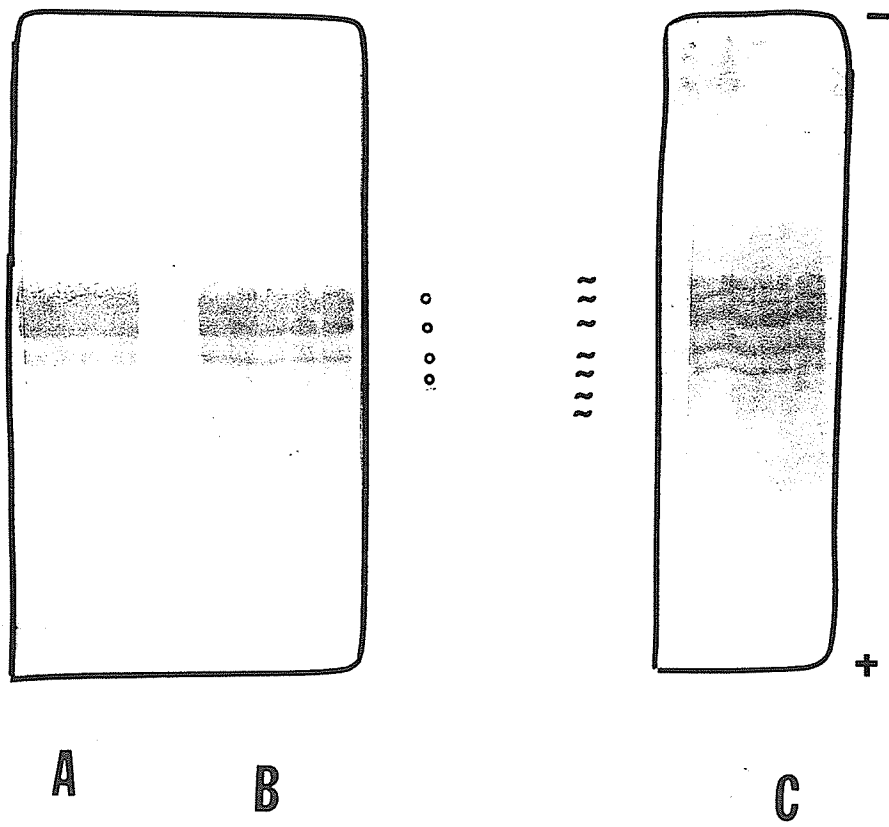


Figure - 8

Comparison of densitometric tracings of bands from Figure 7 (B)
and Figure 7 (C).

CK-MM was solubilized in 0.5 M KCNO buffer (Fig. 7.C) or deionized 8 M urea buffer (Fig. 7.B). Isoelectric focusing was performed as described in Figure 7. Proteins were fixed in 3.5% sulfosalicylic acid, 10% TCA, 30 % methanol and then stained with a 0.2% solution of Coomassie.

Bands were scanned at 600 nm.

FIGURE - 8

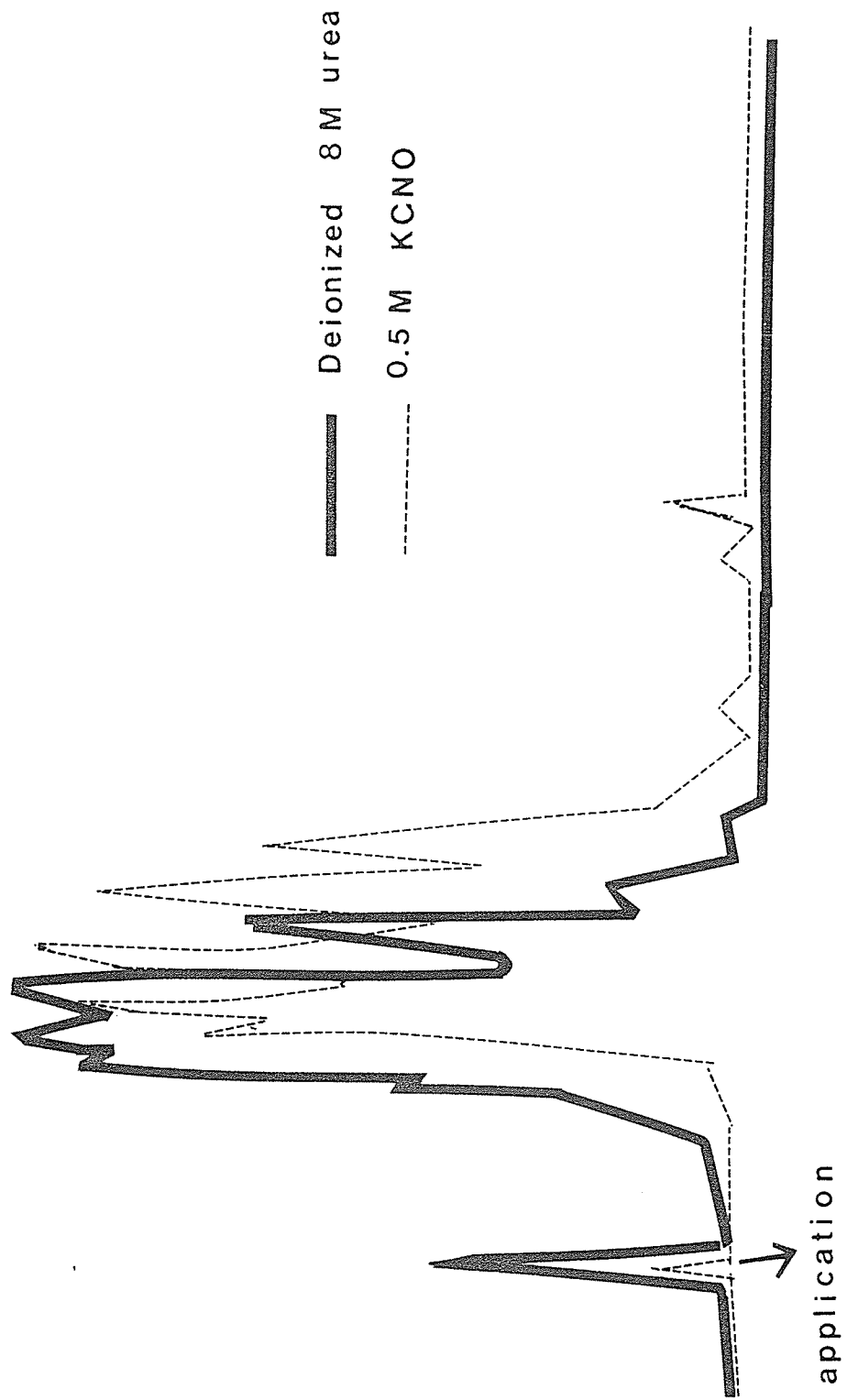


Figure - 9

Band patterns for CK-MM pre-incubated with 0.5 M KCNO and CK-MM
dissociated in 8 M urea or deionized 8 M urea.

CK-MM was pre-incubated with 0.5 M KCNO, 1 mM EDTA, 50 mM mercaptoethanol for 24 hours at 30°C. 1% ampholytes were added just before samples were applied to a polyacrylamide-8 M urea gel. Samples of CK-MM were solubilized in Buffer A and B as described in Figure 7 and applied to the gel with no pre-incubation.

Gels were isoelectrically focused for 4.5 hours at 13.5°C., 6 W., constant power. Gels were fixed and stained as in Figure 3.

(a) CK-MM preincubated with 0.5 M KCNO, 1 mM EDTA, 50 mM mercaptoethanol; 1% ampholytes were added just before isoelectric focusing.

(b) 8 M urea, 1 mM EDTA, 50 mM mercaptoethanol, 1% ampholytes.

(c) Same as (b) but the urea was deionized in a Rexyn 300 mixed bed ion exchange column.

FIGURE - 9

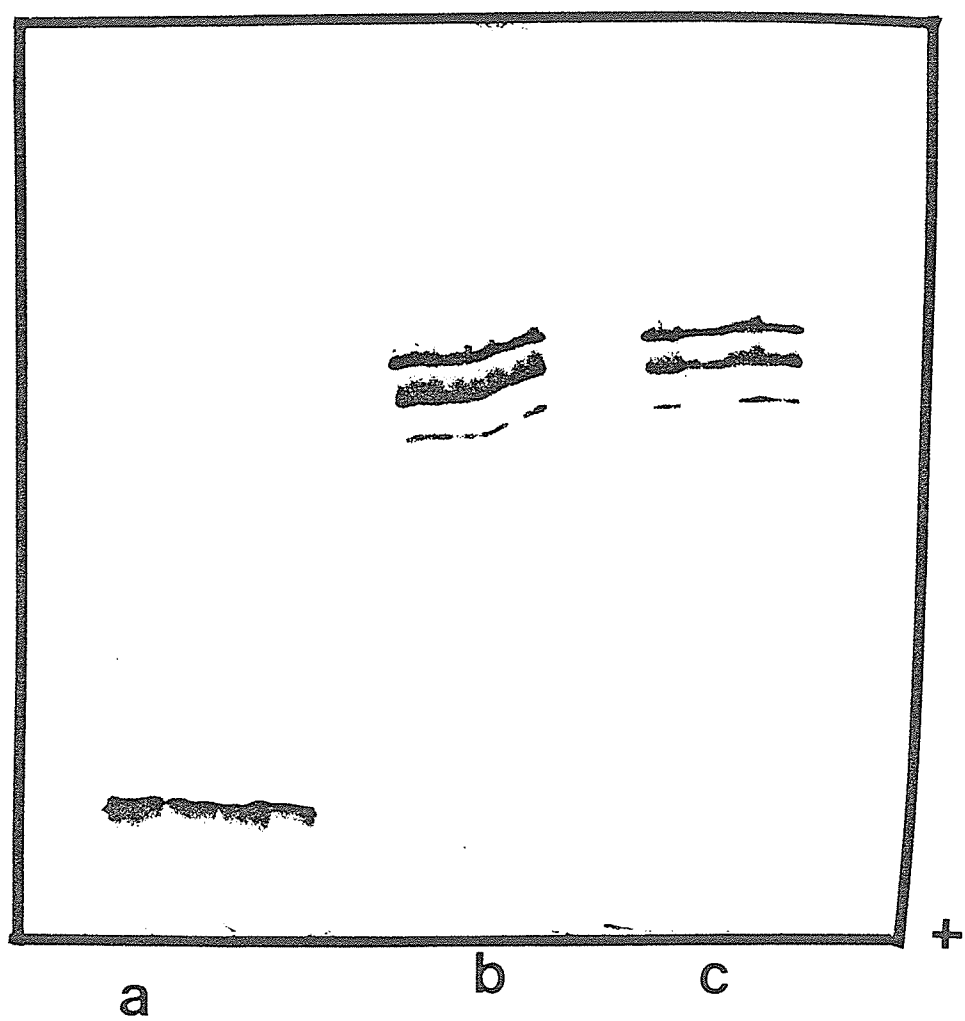


Figure - 10

Densitometric Tracing of creatine kinase preincubated with 0.5 M KCNO

After CK-MM was pre-incubated with 0.5 M KCNO buffer and isoelectric focused on a polyacrylamide-8 M urea gel as described in Figure 9, the gel was fixed and stained as described in Methods.

A densitometric scan at 600 nm of the M-subunit band patterns was recorded.

FIGURE-10

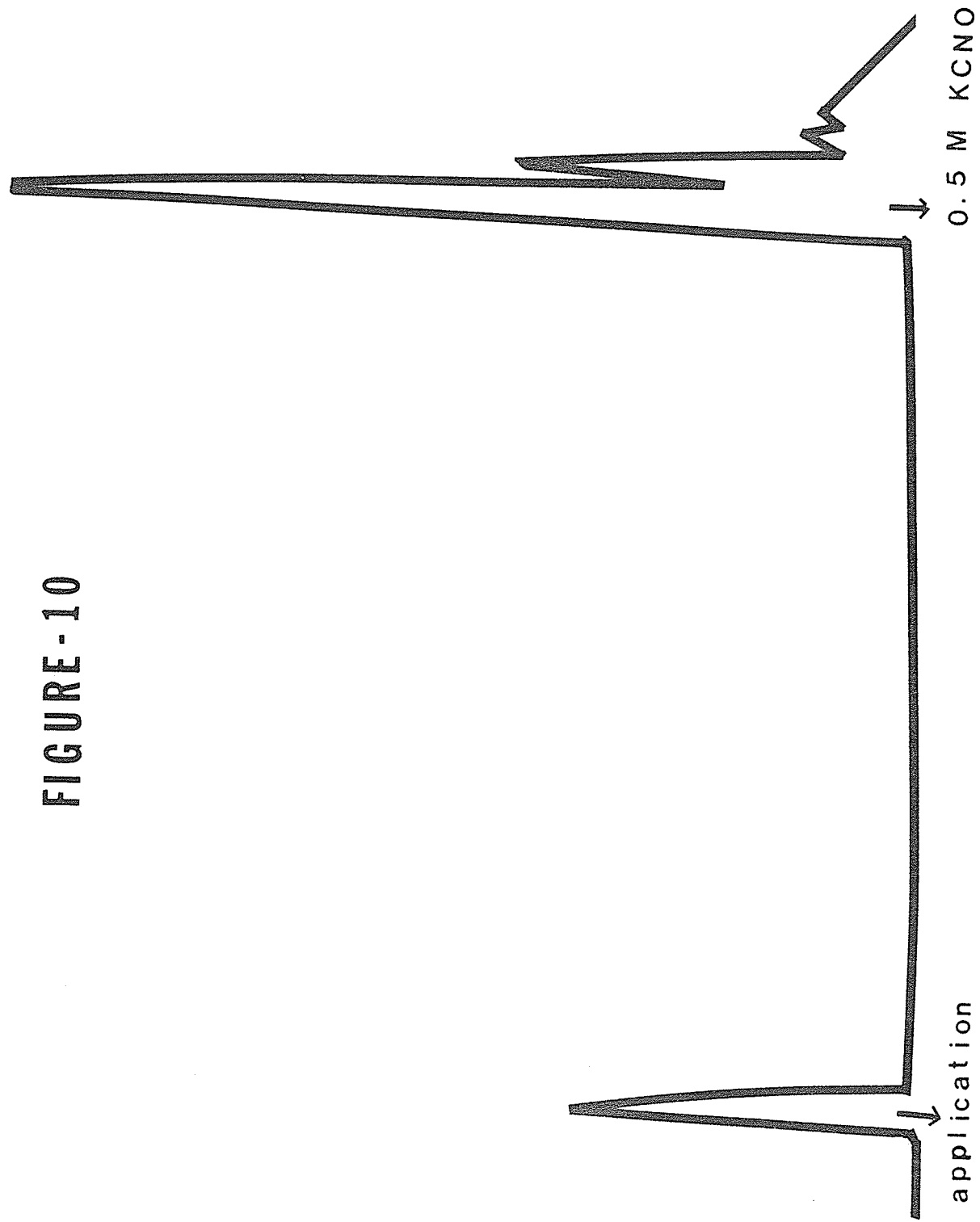


Figure - 11

pH 3-10

Band pattern for creatine kinase after pre-incubation
with 0.5 M KCNO or 0.005 M KCNO buffers.

CK-MM was pre-incubated for 24 hours at 30°C. with two concentrations of KCNO (b, c) or dissociated into subunits in an 8 M urea buffer (a) before being isoelectrically focused on a polyacrylamide-8 M urea gel.

a) 8 M urea, 1 mM EDTA, 50 mM mercaptoethanol, 1% ampholytes

b) 0.005 M KCNO, 1 mM EDTA, 50 mM mercaptoethanol

- 1% ampholytes added just before isoelectric focusing

c) 0.5 M KCNO, 1 mM EDTA, 50 mM mercaptoethanol

- 1% ampholytes added just before isoelectric focusing

Gels were focused for 4.5 hours at 13° C., 6 W., constant power. Fixing and staining of the gel was described as in Figure 3.

A - Photocopy of gel after isoelectric focusing.

B - Polaroid photograph of gel after isoelectric focusing.

FIGURE - II

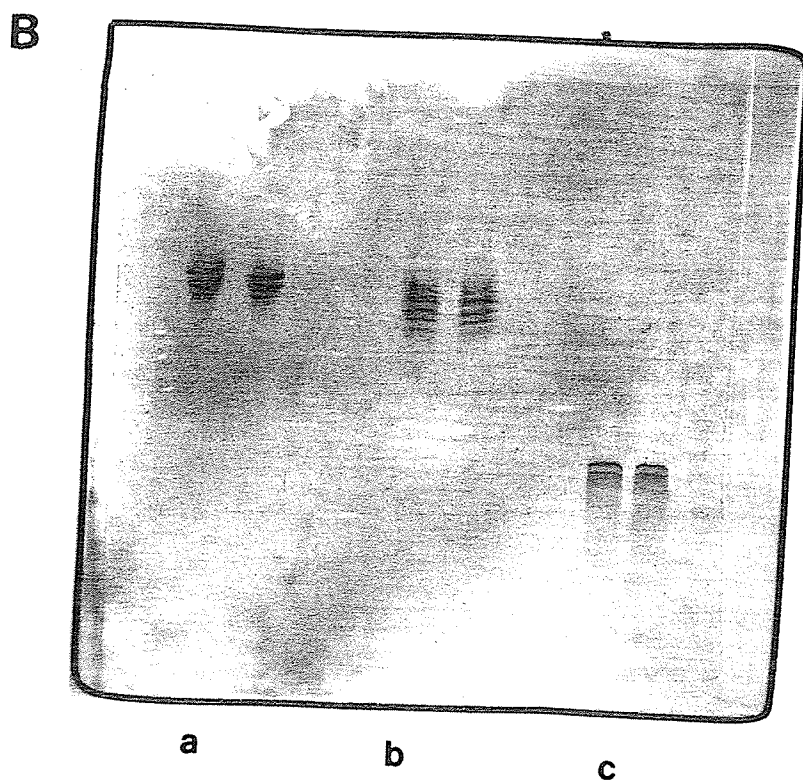
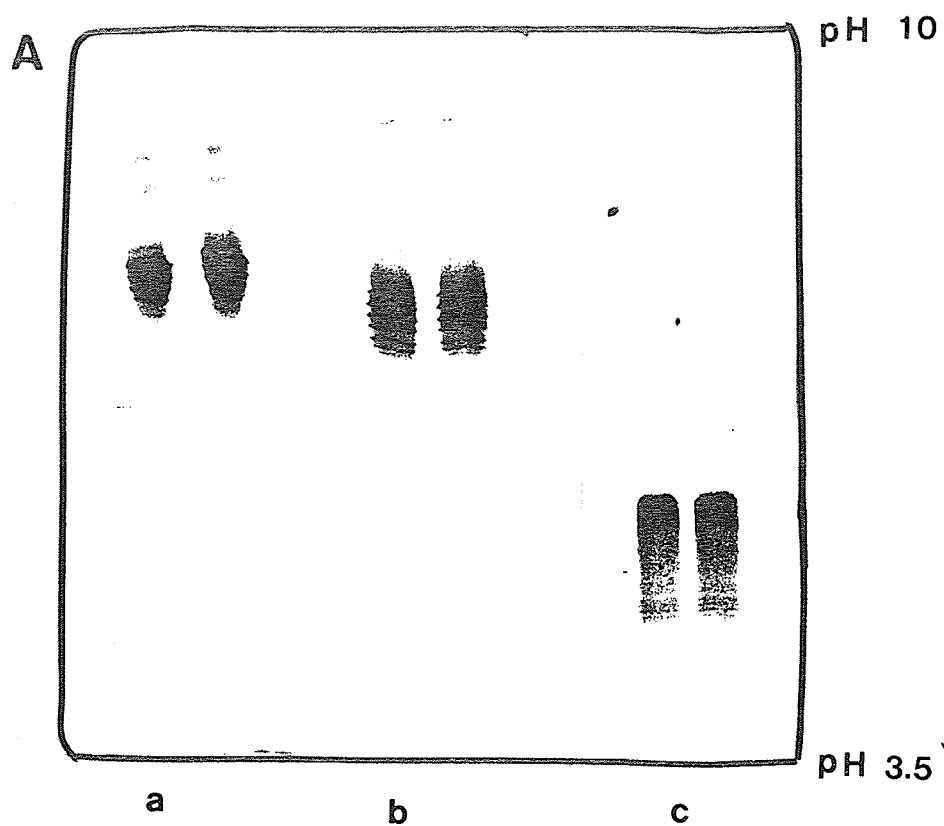


Figure - 12

Nitrocellulose blot of M-subunit bands of creatine kinase
after isoelectric focusing on an agarose-7 M urea gel.

CK-MM was dissociated into subunits in an 8 M urea buffer, 1 mM EDTA, 50 mM mercaptoethanol, 1% ampholyte buffer for 30 minutes. Aliquots (100 µg/lane) were isoelectric focused on agarose-7 M urea gels for 3 hours, 4 W., at 13.5° C. All lanes have identical sample loads. A nitrocellulose blot was then made of the gel as described in Methods. The intensity of the stain is indicated by circles (major bands) or lines (minor bands).

FIGURE-12

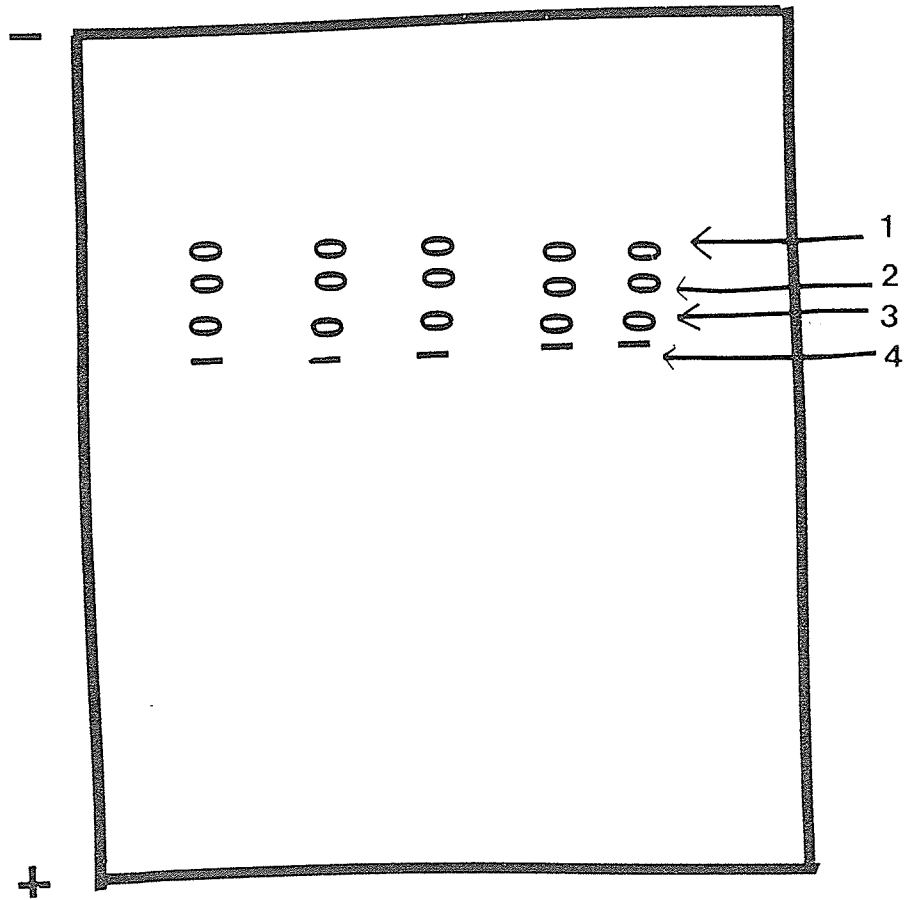


Figure - 13

pI values for M-subunit bands 1-4 in 7 M urea

pH measurements were made on an agarose-7 M urea gel at 10 mm intervals beginning at the cathode as described in Methods. From the graph, pI values for the M-subunit bands 1, 2, 3, and 4, at 13.5⁰ C., were calculated to be 8.42, 8.28, 8.08, and 8.00, respectively.

FIGURE -13

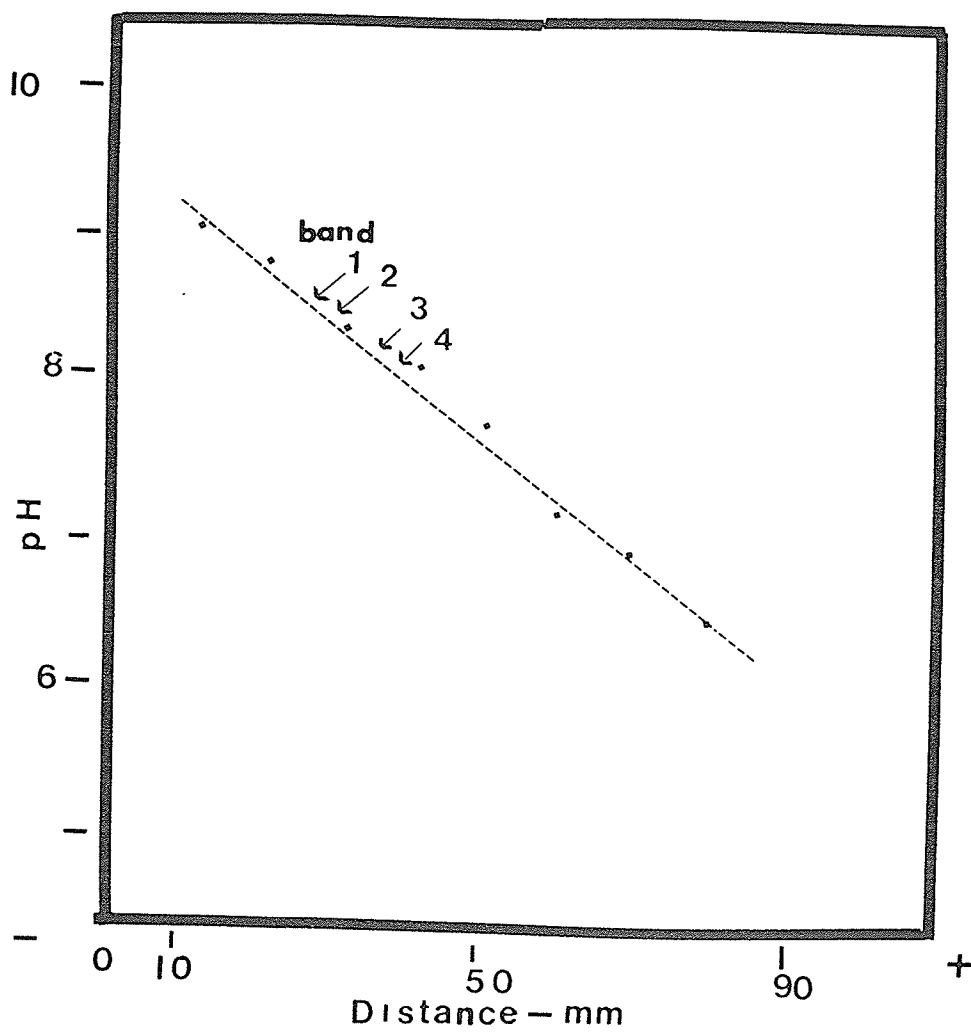


Figure - 14

Detection of CK-MM subform band pattern on a nondenaturing
polyacrylamide-immobiline gel (pH 5.78 - 8.23).

CK-MM was solubilized in DDW and applied (80 ug/lane) to the surface of a native polyacrylamide-immobiline gel. The gel was isoelectrically focused for 3.5 hours, 4° C., 3 W., 2 m.a., 4000 V.

The gel was then stained with a Coomassie stain as described in Methods. Circles indicate bands that were visualized on the gel.

FIGURE - 14

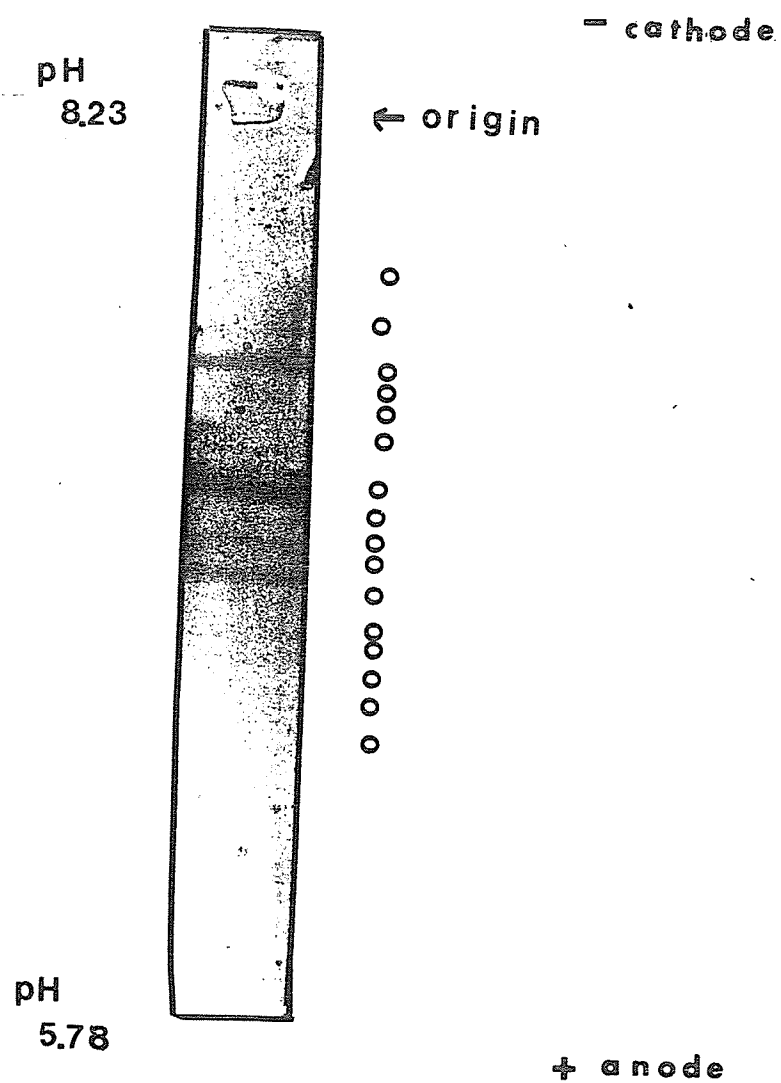


Figure - 15

M-subunit band pattern for creatine kinase obtained after isoelectric
focusing on an urea-polyacrylamide-immobiline gel

CK-MM was solubilized in an 8 M urea, 1 mM EDTA buffer and applied (30 μ g/lane) to the surface of a polyacrylamide-urea-immobiline gel, pH 5.49 - 8.29. The gel was then isoelectrically focused for 4.5 hours at 13.5° C., 2 m.A., 4000 V. The gel was then fixed and destained as described in Methods.

Lane 1 - Bio-Rad marker proteins

Lane 2 - Creatine kinase M-subunits (30 μ g/lane)

FIGURE-15

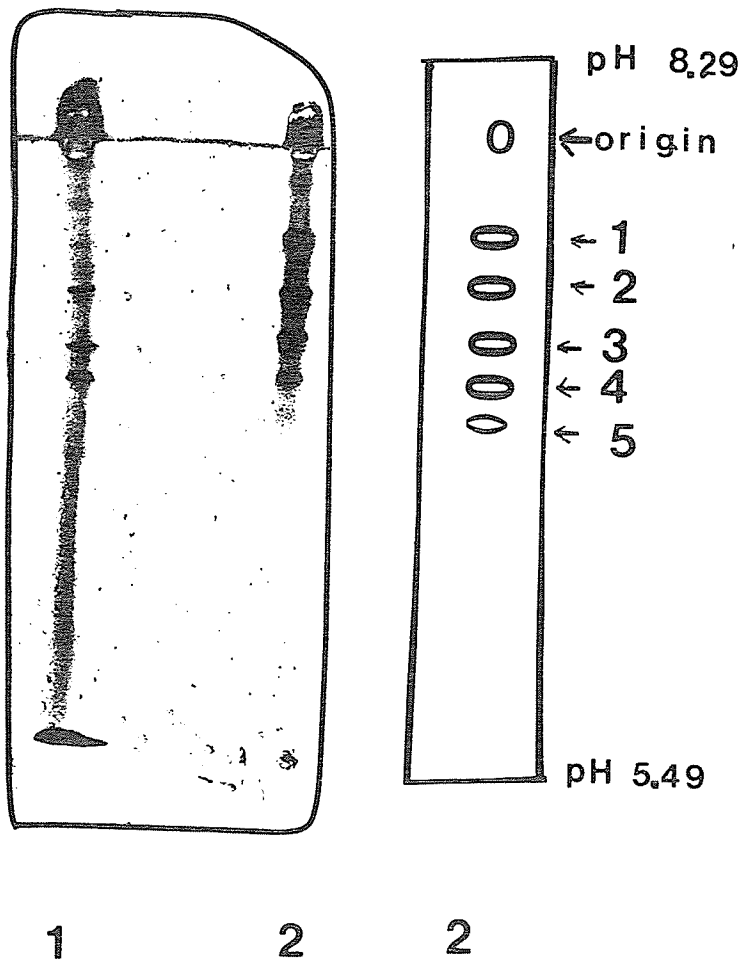


Figure - 16

Densitometric scan of M-subunit bands of creatine kinase resolved on
a urea-polyacrylamide-immobiline gel.

Rabbit skeletal muscle creatine kinase was denatured as described in Figure 15 and applied to a polyacrylamide-8 M urea-immobiline gel. Conditions for isoelectric focusing and staining procedures were as described in Figure 15.

Lanes on the gel were then scanned for protein at 600 nm.

Five peaks were detected.

Peak 1 = M-subunit band 1

Peak 2 = M-subunit band 2

Peak 3 = M-subunit band 3

Peak 4 = M-subunit band 4

Peak 5 = M-subunit band 5

FIGURE - 16

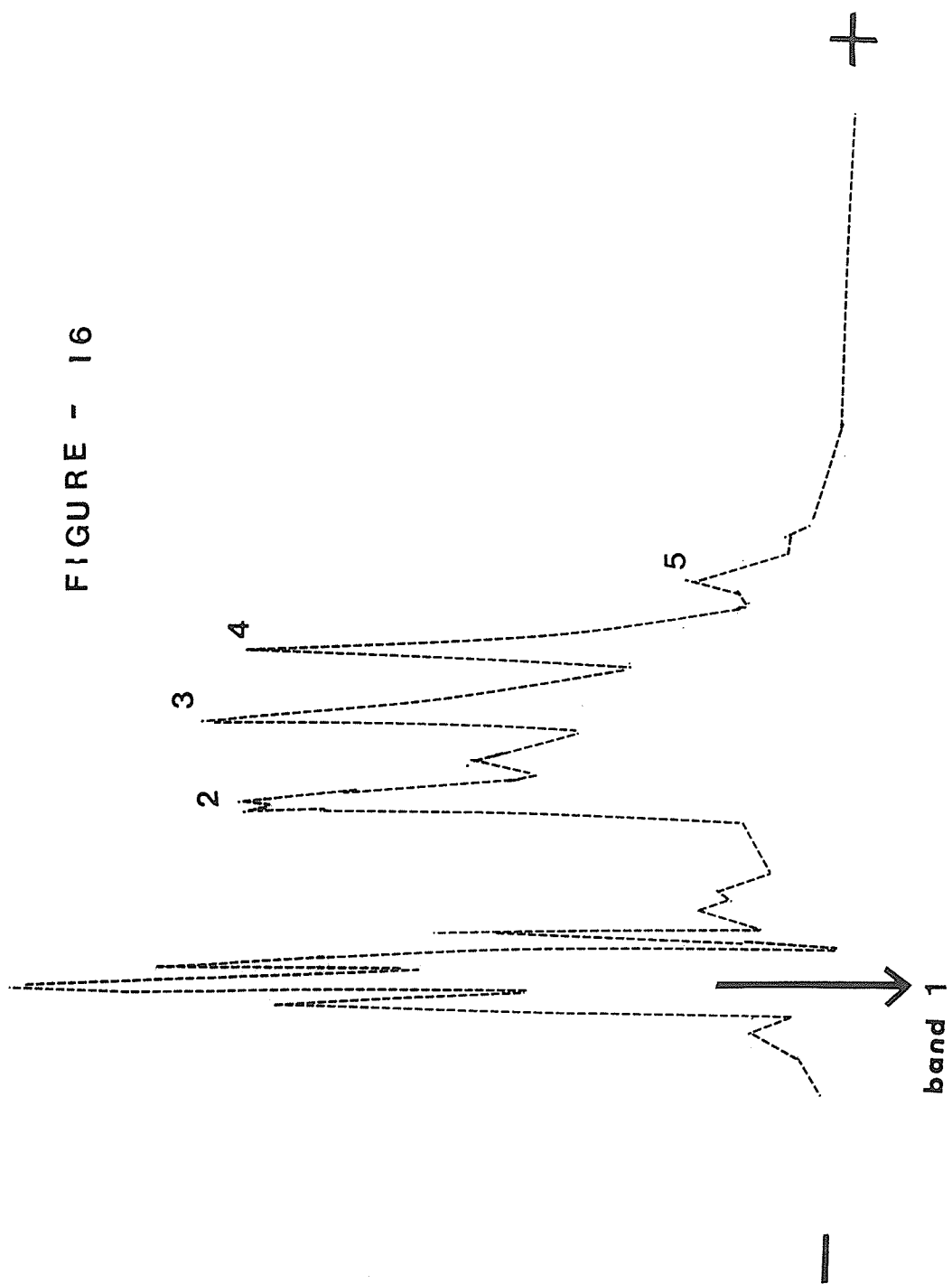


Figure - 17

Blot of M-subunit bands resolved on Sephadex G-75-urea gels

Eight to ten mg of Boehringer CK-MM were dissociated into subunits in an 8 M urea, 1 mM EDTA, 100 mM mercaptoethanol, 1% ampholyte buffer and applied about three inches from the anode onto a Sephadex G-75, S.F.-8 M urea gel. The catholyte (-) was 0.5 M ethanolamine; the anolyte (+) was 0.2 M citric acid. The gel was isoelectrically focused for 16 hours, 8 W., 13.5⁰ C.

After isoelectric focusing, the gel was blotted with Whatman #3 filter paper as described in Methods. The M-subunits were visualized with a Coomassie stain on the filter paper.

FIGURE-17

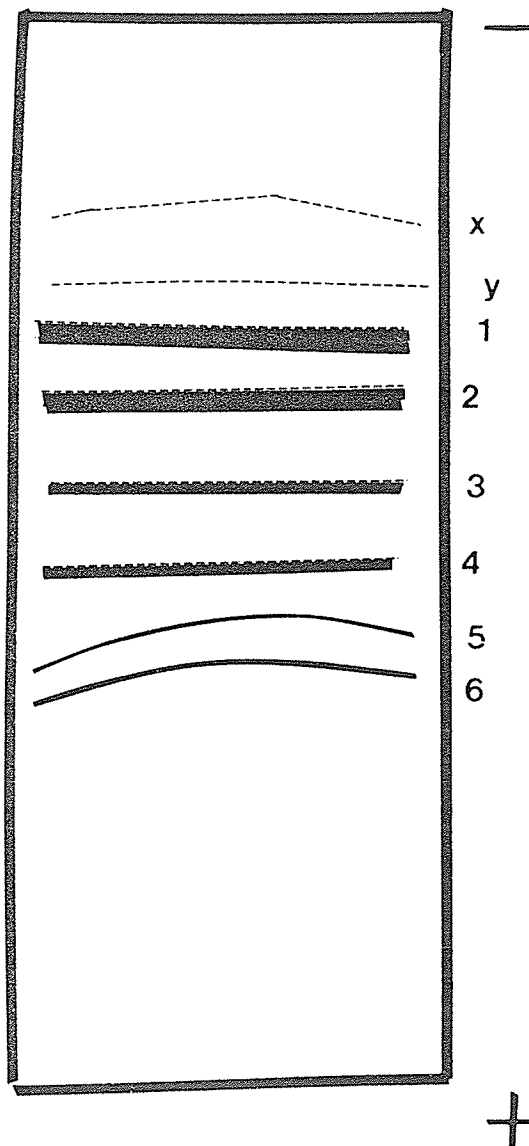


Figure - 18

Assay for activity for renatured M-subunit Bands 1-6 isolated from
a Sephadex-urea gel

Samples of Bands 1-6 were isolated from a primary Sephadex G-75, S.F.-8 M urea gel after isoelectric focusing and tested for activity as described in Methods. The degree of activity was determined after the addition of creatine phosphate (CP) from the difference in slope from the initial line. The activity was expressed as the rise of mV/min. 1 mV/min. equals 1 Unit.

FIGURE -18

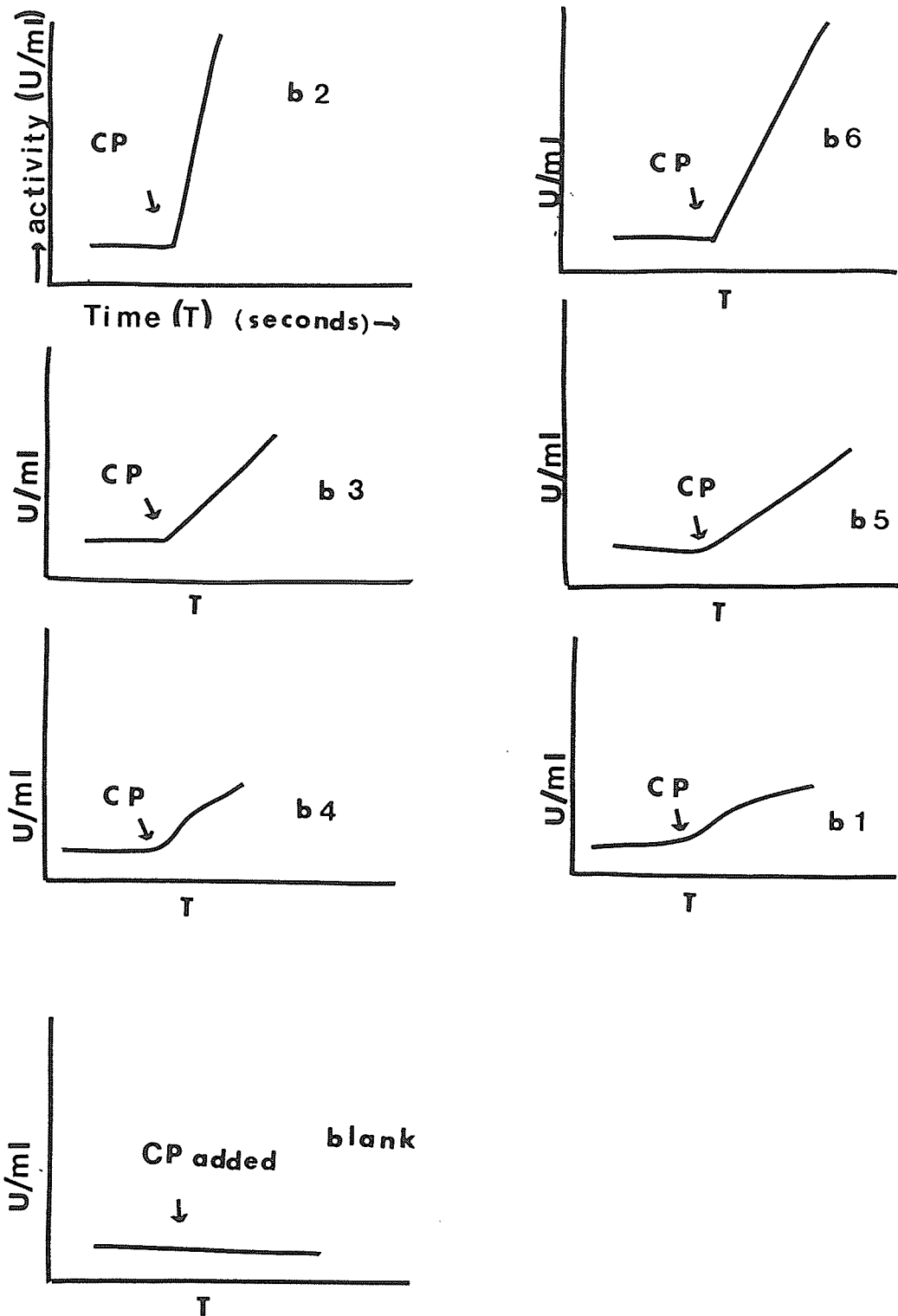


Figure - 19

Blot of isolated M-subunit bands 2 and 3 refocused on a second

Sephadex-urea gel

M-subunit bands 2 and 3 were isolated from a primary Sephadex G-75, S.F.-8 M urea gel and applied to a second Sephadex G-75, S.F.-8 M urea gel. The gel was isoelectrically focused for 16 hours, 13.5⁰ C., at 8 W. as described in methods.

A blot using Whatman #3 filter paper was made of the gel and fixed and stained as described in Methods.

a) M-subunit band 2 refocused

b) M-subunit band 3 refocused

FIGURE - 19

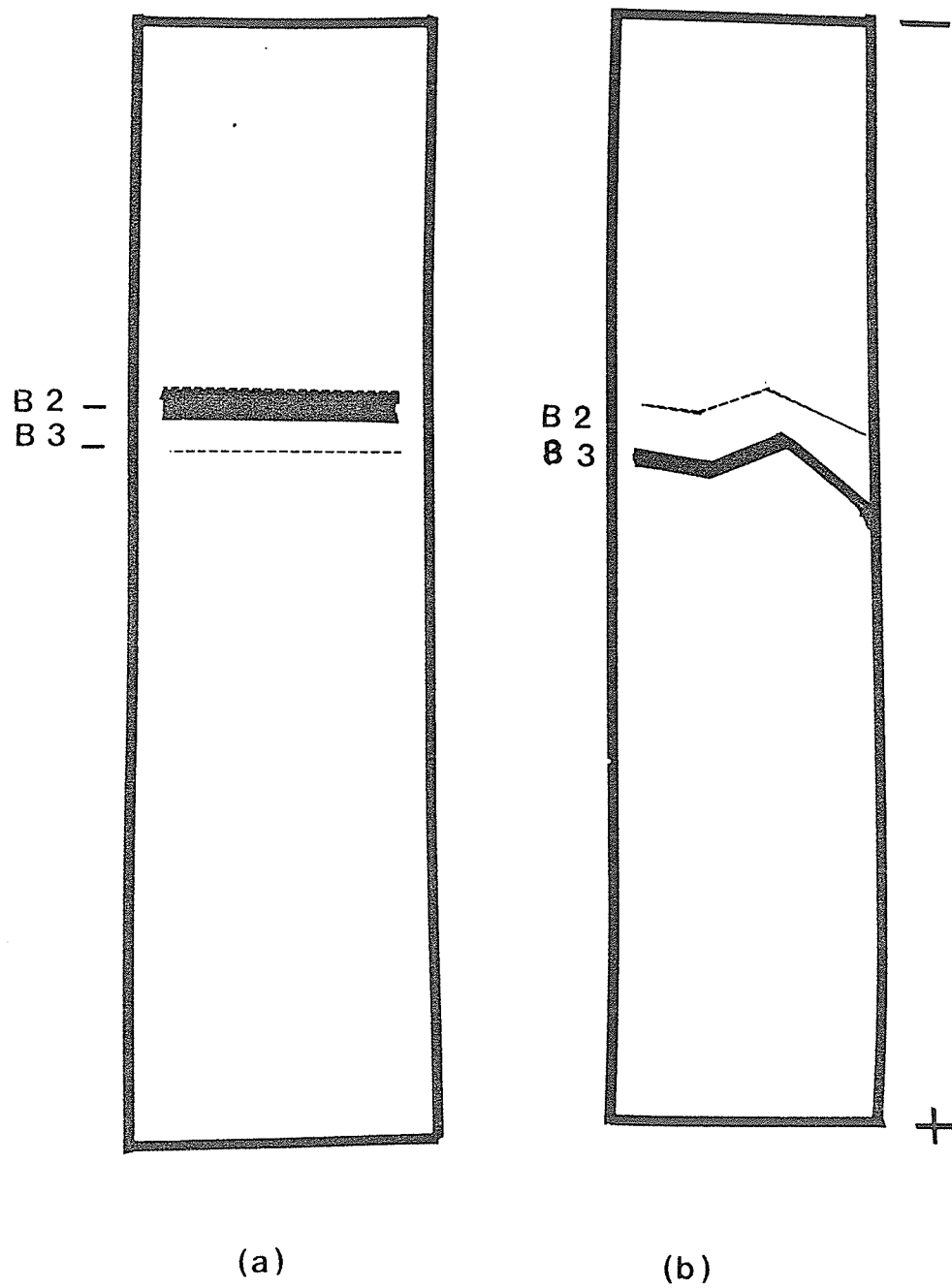


Figure - 20

Elution Profiles of M-subunits after gel filtration

Bands were isolated from primary or secondary Sephadex G-75, S.F.-8 M urea preparative gels. Individual bands were added to the surface of a Sephadex G-50 gel filtration column (1.5x50 cm) and eluted with 0.1 M ammonium bicarbonate, pH 7.0, 100 mM mercaptoethanol buffer. Two ml fractions were collected in separate test-tubes (t.t.).

The elution profile of the renatured band material was recorded on a Isco UA-5 Absorbance Monitor.

a.1) elution profile for M-subunit band 3 material

a.2) t.t. 16 - middle of protein peak of renatured CK-MM

b.1) elution profile for M-subunit band 2 material

b.2) t.t.16 - middle of protein peak of renatured CK-MM

FIGURE - 20

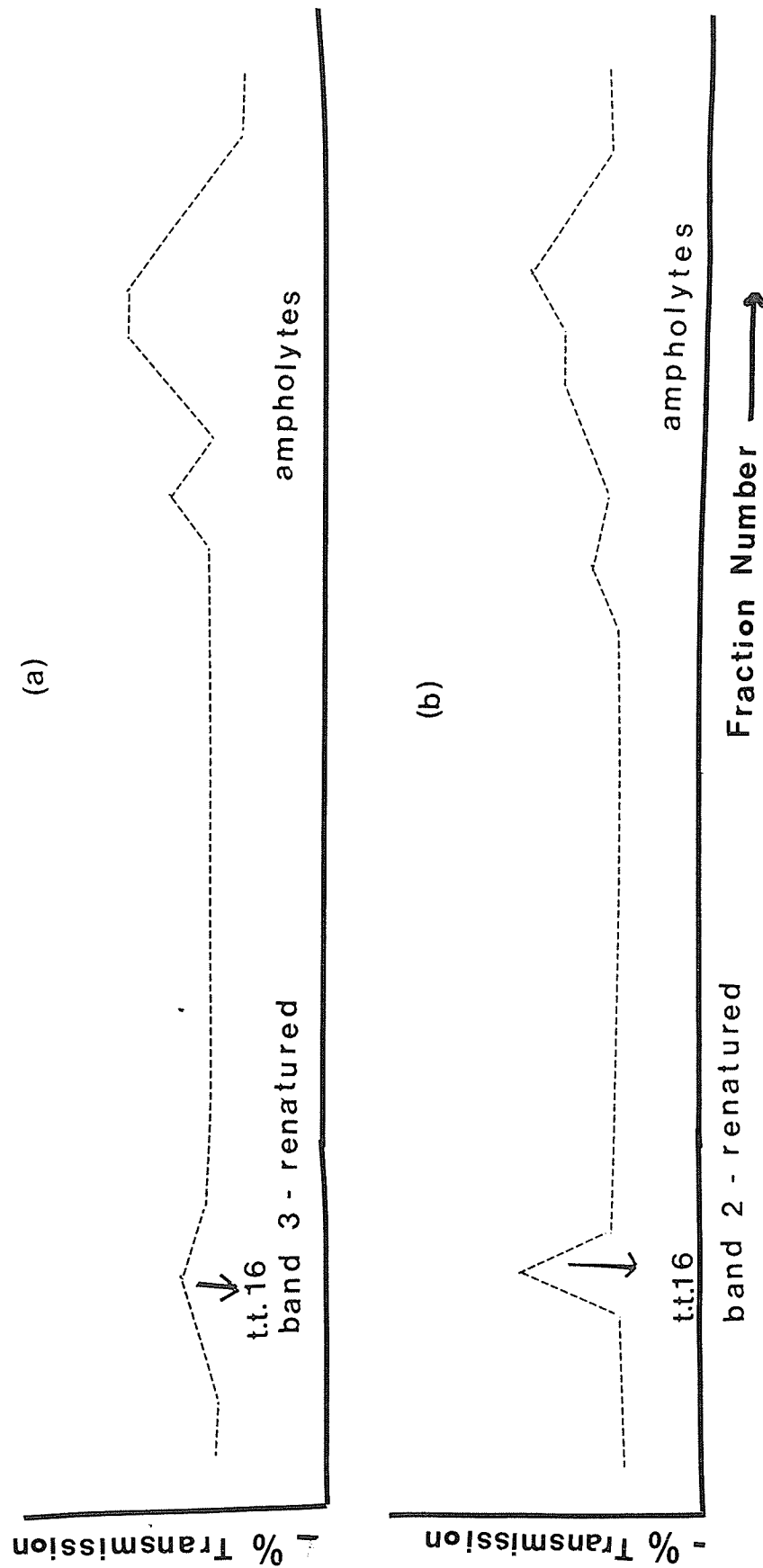


Figure - 21

Detection of M-subunit bands from Band 1 and Band 2 material isolated
from a primary Sephadex-urea gel

Band 1 and 2 material were isolated from a primary preparative Sephadex-urea gel and isoelectrically focused on an agarose-5 M urea gel for 5 hours, 13.5⁰ C., 4 W., constant power. The gel was then fixed and stained with a Coomassie stain as described in Methods.

CK = CK-MM (80 µg/lane) dissociated into subunits in an 8 M urea buffer before isoelectric focusing on the agarose-urea gel.

1 = Band 1 material isolated from a primary Sephadex-urea gel

2 = Band 2 material isolated from a primary Sephadex-urea gel

Figure - 22

Detection of M-subunit bands from Bands 3, 4, 5, and 6 material
isolated from a primary Sephadex-urea gel

M-subunit bands 3, 4, 5, and 6 were each isolated from a primary preparative Sephadex-urea gel and applied to an agarose, IEF-5 M urea gel. The gel was focused as described in Figure 21.

The gel was fixed and stained with a Coomassie stain as described in methods.

mp - Horse heart myoglobin marker proteins

3 - Band 3 material isolated from a primary Sephadex-urea gel

4 - Band 4 material isolated from a primary Sephadex-urea gel

5 - Band 5 material isolated from a primary Sephadex-urea gel

6 - Band 6 material isolated from a primary Sephadex-urea gel

FIGURE-21

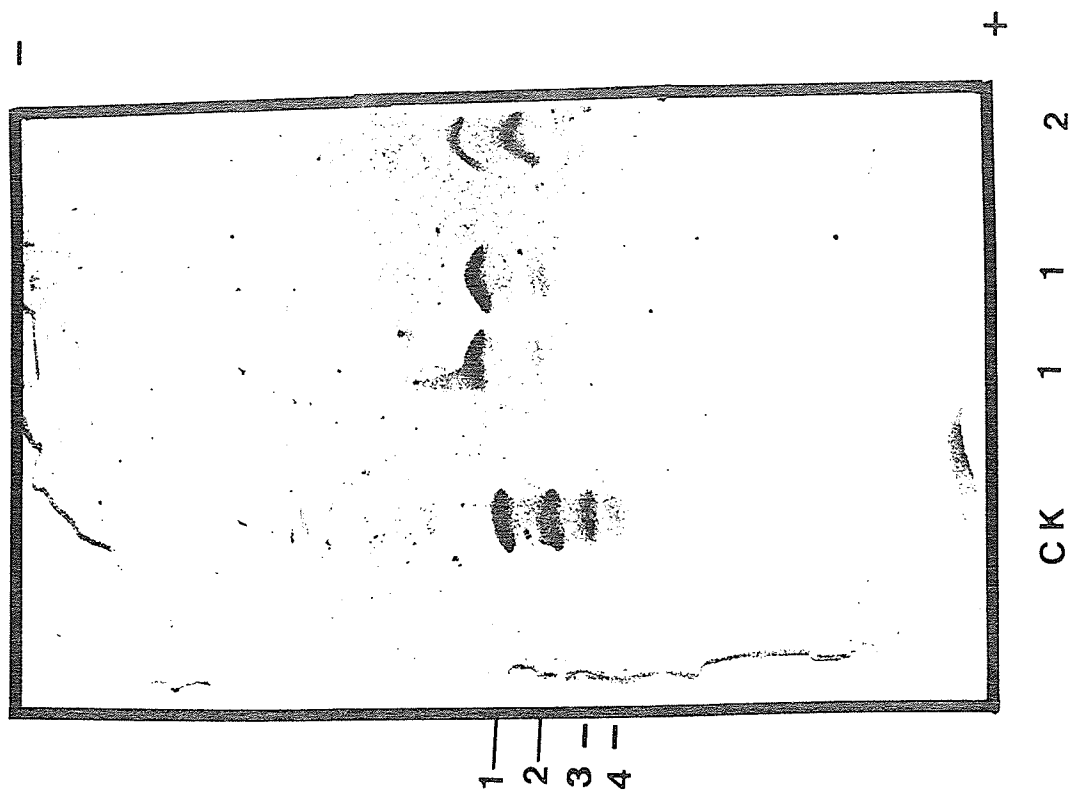


FIGURE-22

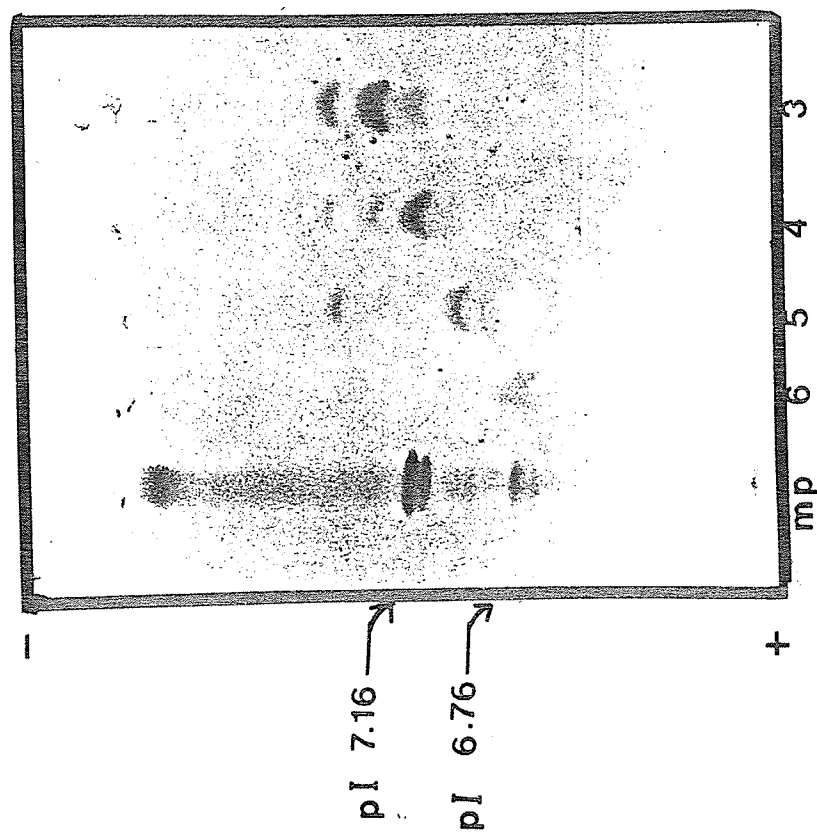


Figure - 23

Detection of active forms of Creatine kinase for Bands 1 and 2
isolated from Sephadex-urea gels

Aliquots of Band 1 (isolated from a primary Sephadex-urea gel) and Band 2 (isolated from a secondary Sephadex G-75, S.F.-8 M urea gel) were pipetted onto LKB pads on a native agarose(IEF) gel and isoelectrically focused for 4 hours at 4⁰ C., 4 W., constant power. An activity stain was applied to half the gel as described in Methods.

a) Photocopy of gel after activity stain.

1 - Aliquot of Band 1 isolated from a primary Sephadex-urea gel.

CK - CK-MM solubilized in DDW (100 ug/lane).

2 - Aliquot of Band 2 isolated from a secondary Sephadex-urea gel.

b) Polaroid photograph of gel after activity stain.

1, CK, 2 - same as above

FIGURE-23

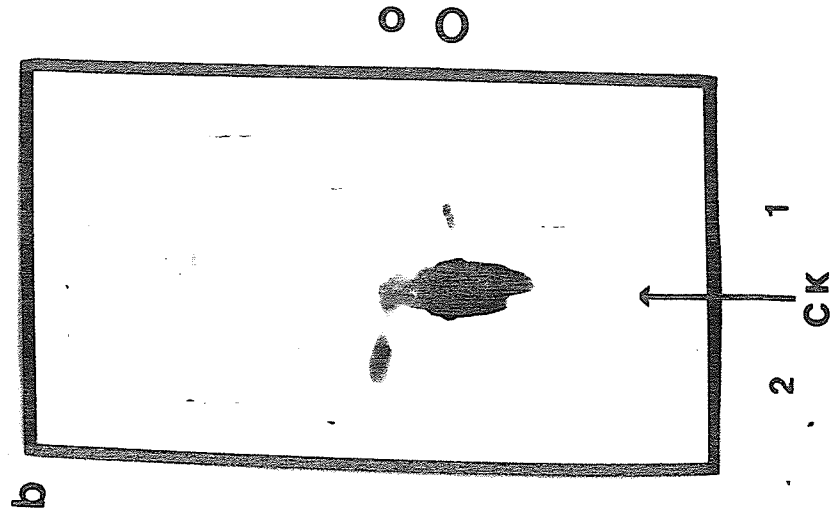
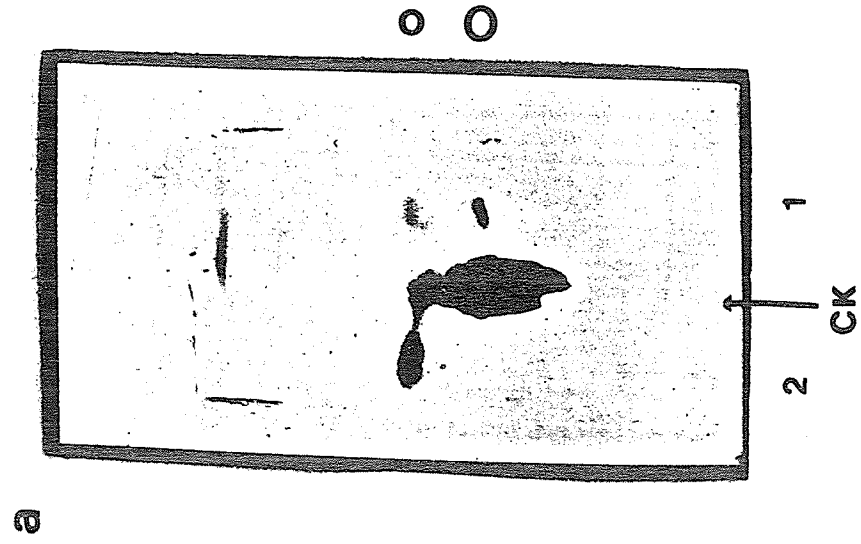


Figure - 24

Detection of unknown protein peak eluting before Boehringer Band 1
material from a Sephadex G-50 gel filtration column

Boehringer or Sigma band 1 material (isolated from a primary (preparative) Sephadex G-75, S.F.-8 M urea gel) was eluted through a Sephadex G50, S.F. gel filtration column using 0.1 M ammonium bicarbonate, pH 7.0, 100 mM mercaptoethanol.

The protein peaks were recorded on a Isco UA-5 Absorbance Monitor.

- a) Elution profile of Boehringer Band 1 material.
- b) Elution profile of Sigma Band 1 material.

FIGURE- 24

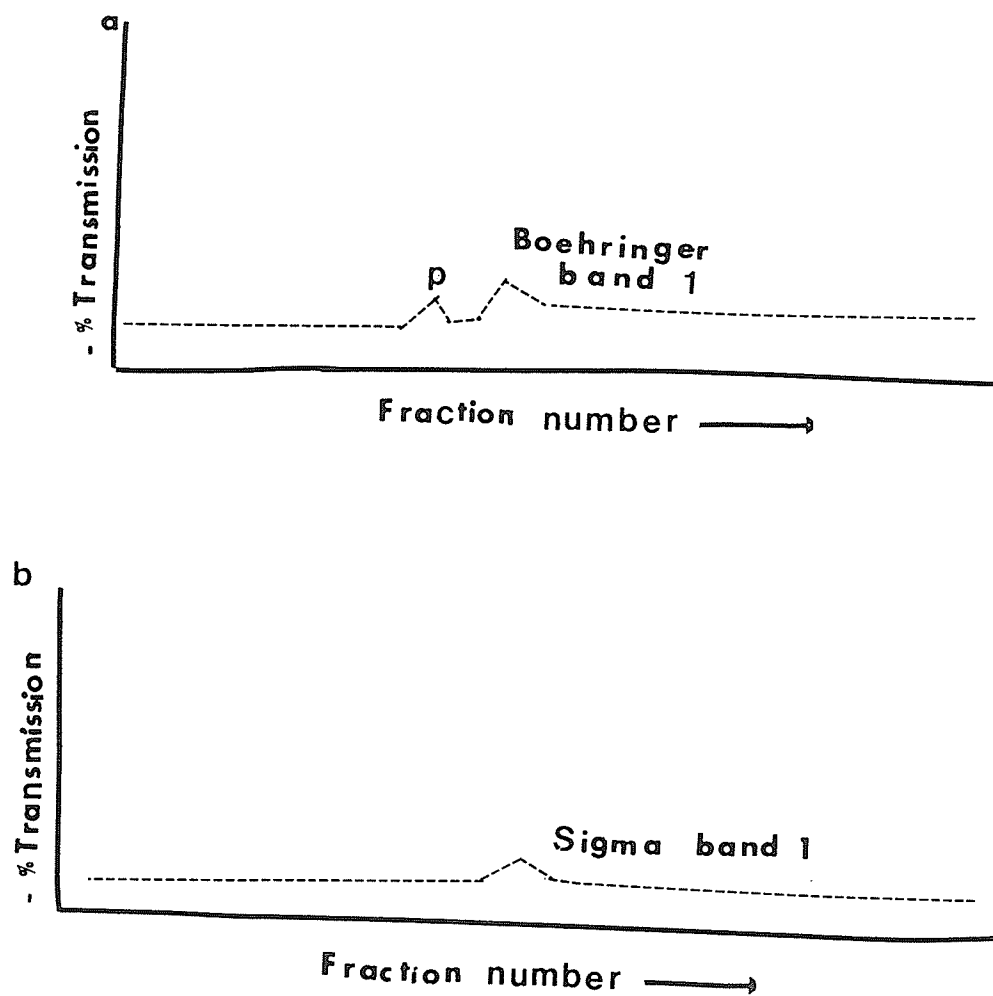


Figure - 25

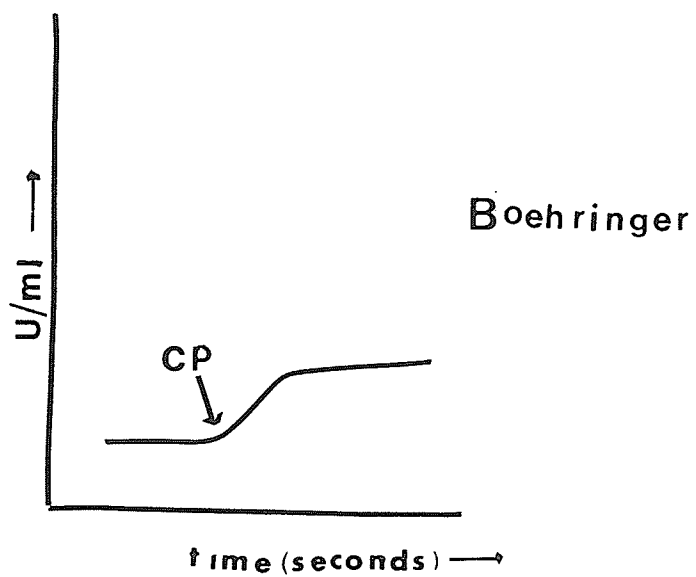
Assay for activity for renatured Boehringer Band 1 material
and renatured Sigma Band 1 material

Band 1 material from Sigma and Boehringer CK-MM was isolated from primary Sephadex G-75, S.F.-8 M urea gels after isoelectric focusing and eluted through a Sephadex G-50, S.F. gel filtration column. After concentration, the renatured bands were tested for activity as described in Methods. The amount of activity was expressed as the rise of mV/min. after the addition of creatine phosphate (CP). 1 mV/min. equals 1 Unit.

- a) Activity profile for renatured Boehringer Band 1 material.
- b) Activity profile for renatured Sigma Band 1 material.

FIGURE - 25

A



B

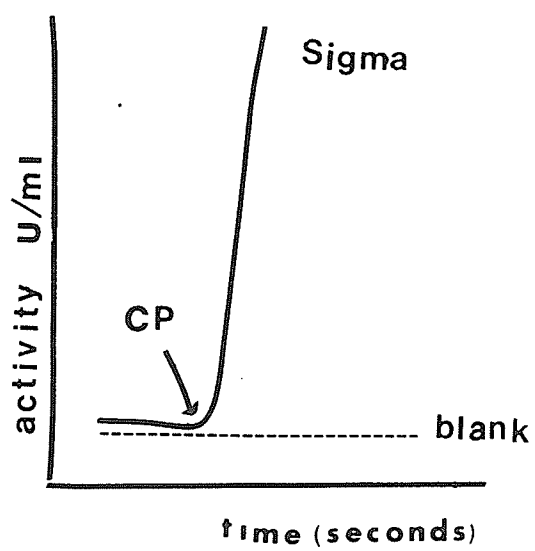


Figure - 26

Molecular weight estimation for Band 1 electrophoresed on SDS-Page

M-subunit band material was isolated from primary Sephadex G-75, S.F.-8 M urea gels, eluted through a Sephadex G-50, S.F. gel filtration column to remove urea, and concentrated. Aliquots of the bands were treated as described in Methods and applied to the top of an SDS-PAGE stacking gel. Gels were electrophoresed for 6 hours, 40 C., 30 m.a., constant current. Gels were fixed and stained as described in Methods.

Lane 1 - Creatine kinase standard

Lane 4 - Boehringer Band 2 Material

Lane 6 - Boehringer Band 3 Material

Lane 7 - Boehringer Band 3 plus Trypsin

Lane 9 - Sigma Band 1 Material

FIGURE - 26

