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A study of the differences in the kinetic properties of human creatine kinase isoenzymes.

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A STUDY OF THE DIFFERENCES IN THE KINETIC PROPERTIES
OF HUMAN CREATINE KINASE ISOENZYMES

by
Georges Lindsay Chong

A Thesis
Submitted to the Faculty of Graduate Studies through
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University of Windsor

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1983

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ABSTRACT

A STUDY OF THE DIFFERENCES IN THE KINETIC PROPERTIES OF HUMAN CREATINE KINASE ISOENZYMES

by

Georges Lindsay Chong

Creatine kinase isoenzymes from human skeletal muscle, heart and brains were purified to homogeneity by using a combination of isolation techniques that included $(\text{NH}_4)_2\text{SO}_4$ and ethanol fractionations, Sephadex G-100 chromatography, DEAE-Sephadex A-50 chromatography and Affi-Gel Blue affinity chromatography.

The substrate affinity of the individual CK isoenzyme for creatine phosphate was re-examined using the optimized coupled assay recommended by the Scandinavian Committee on Enzymes. The K_m values calculated revealed small but reproducible differences in substrate affinity for the different isoenzymes.

Measurement of the CK-B subunit in a preparation of purified CK-MB, by an immunoinhibition method which made use of anti-CK-MM antibodies, indicate that the activity of the CK-M subunit is about one and a half times greater than the CK-B subunit, under the specified conditions. This suggests that the CK-B subunit is greatly inhibited by this assay system.

DEDICATION

To
Mom and Dad

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LIST OF ABBREVIATIONS

ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
CK	creatine kinase
CK-BB	creatine kinase-BB isoenzyme
CK-MB	creatine kinase-MB isoenzyme
CK-MM	creatine kinase-MM isoenzyme
CPK	creatine phosphokinase
CSF	cerebrospinal fluid
C.V.	coefficient of variation
DAPP	diadenosine pentaphosphate
DEAE	diethylaminoethyl
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
EGTA	ethylene-glycol-(bis- β -aminoethylether)- N,N,N',N'-tetraacetic acid
G6PDH	glucose-6-phosphate dehydrogenase
h	hour
HK	hexokinase
K_m	Michaelis constant
L	litre
m	milli
mA	milliampere
min	minute
mmol	millimoles

NADP ⁺	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
nm	nanometre
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
RIA	radioimmunoassay
S.D.	standard deviation
S.E.	standard error
Tris	tris-(hydroxymethyl)-aminomethane
U	unit
uv	ultraviolet
V _{max}	maximum velocity

CHAPTER I

INTRODUCTION

A. GENERAL

A knowledge of the multiple molecular forms of enzymes - currently named isoenzymes - has opened new perspectives in molecular pathology. Isoenzymes catalyse the same main reaction, but differ in certain biochemical and physical aspects. These include pH-optimum, thermostability, amino-acid composition, different affinities for substrates or coenzymes (expressed as differences in Michaelis constants), different reactions towards inhibitors - including excess of either substrate or product, different catalytic efficiency expressed as a turnover number of substrate per molecule enzyme per minute, and possibly allosteric influences as well (1). It is now well known that these molecular forms may vary according to tissue, age, and also in some pathological conditions.

Isoenzymology is now finding its way into many areas, for instance, into the field of inherited metabolic diseases. An enzymatic defect, being rarely complete, seems to indicate

that either the aberrant gene is not entirely silent but directs the synthesis of a functionally related protein, or that the function can be taken over by other similar proteins.

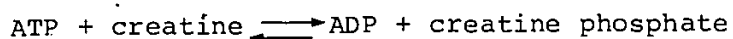
Of direct interest to diagnostic biochemistry is that part of isoenzymology concerned with the specific detection of tissue changes. Necrosis, membrane permeability changes or just an increase in tissue mass can often be revealed by isoenzyme analysis of fluids in more or less direct contact with the affected organs (2). Determination of the serum isoenzyme pattern is often a valuable aid in directing the physician's attention to one particular tissue; moreover, the isoenzyme pattern may show abnormalities while the total serum enzyme is normal (3).

"Creatine kinase isoenzymes" or "multiple forms of creatine kinases" in a number of different tissues have been described by several authors (4-8) who made use of a wide range of separation techniques. The creatine kinases represent a heterogeneous group of enzymes containing at least four well-defined isoenzymes.

B. CREATINE KINASE

In 1934, Lohmann (9) was the first to show that skeletal muscle extracts catalyzed the phosphorylation of creatine by ATP. Soon after Lehmann (10) established the nature of the

reaction and it was found that the following reaction occurred:



The enzyme catalyzing this reaction has been known under different names: the Lohmann enzyme, creatine kinase, creatine phosphokinase, creatine phosphoperase, ATP-creatine phosphoperase, creatine phosphoryltransferase, and ATP-creatine transphosphorylase. In this work, it will be referred to as creatine kinase (ATP: creatine phosphotransferase, EC 2.7.3.2).

C. CREATINE KINASE ISOENZYMES

The heterogeneity of creatine kinase was first established by Burger (11). It was later shown that creatine kinase exhibited three electrophoretically distinguishable isoenzymes (12). These multiple molecular forms of creatine kinase are hybrids of parent subunits which combine to form active dimers (13). The mechanism controlling the formation of these systems remains unknown. There are two primary parent subunits of creatine kinase which combine together in groups of two to give the enzymic dimer. These two primary parent proteins have been designated M and B as they are individually prominent in muscle and brain, respectively. Varying the combination of these subunits results in the occurrence of three isoenzymes

designated the MM (CK-3) muscle, MB (CK-2) heart, and BB (CK-1) brain forms. These isoenzymes are found in differing proportions in various tissues (14,15). The MM isoenzyme occurs in large quantities in skeletal muscle, whereas the BB isoenzyme occurs largely in the brain. The CK-MB form is found predominantly in heart muscle.

The isoenzyme composition of the serum was first shown to be of considerable diagnostic value, especially in the diagnosis of myocardial infarction (16). This discovery prompted the further study of variations in the isoenzyme content of the blood serum.

Further studies have shown that creatine kinase isoenzymes differed from each other in a variety of respects other than in their electrophoretic and chromatographic properties. Differences in chemical composition (17), substrate specificities (18), thermal stabilities (19), stabilizing effect of thiols (20), and susceptibilities to inhibitors (21) have been reported. Moreover, a high degree of immunochemical specificity has been observed: antisera have been prepared which inhibit the action of particular isoenzymes without affecting that of others (22).

D. THE MITOCHONDRIAL CREATINE KINASE ISOENZYME

A distinct form of creatine kinase has been found in the mitochondria of human heart (23), brain (24), and liver (25).

It is known as mitochondrial creatine kinase isoenzyme (CK-Mt). CK-Mt is a dimeric molecule made up of two identical subunits. Its molecular mass is identical to that of the cytoplasmic isoenzymes, but the amino acid composition and the amino-terminal amino acid differ from those of the other creatine kinase isoenzymes (25). Electrophoretically, it moves cathodally compared to CK-MM. CK-Mt does not react with either anti-CK-M or anti-CK-B antibodies, and no hybrid formation occurs with CK-MM or CK-BB. CK-Mt not only occurs in the dimeric state but also in the oligomeric state with a higher molecular mass (26).

E. CREATINE KINASE VARIANTS

Electrophoretic creatine kinase isoenzyme separations revealed an "atypical CK" band moving between CK-MM and CK-MB. This band has been reported in about 1 of 1000 cases, and is often confused with the CK-MB isoenzyme (27). Immunoinhibition studies have shown it to be CK-BB activity; and it was termed "idiopathic serum CK-BB". Both atypical CK and idiopathic CK-BB turned out to be in many cases a macromolecular form of CK-BB, produced by linking CK-BB to IgG immunoglobulins, and termed "macro CK" (28,29). In addition, Yuu et al. (30) reported on the formation of a macro CK complex between IgA and CK-MM in three cancer patients.

During further investigation, another form of macro-

molecular CK was found, which is not an immunoglobulin complex but is closely related to mitochondrial CK. For differentiation, these two forms have been named macro CK type 1 and type 2, respectively (31). The macro CK type 2 form migrates cathodally or very close to CK-MM (25). The macro CK type 2 is very probably oligomeric mitochondrial CK. Both macro CK type 2 and freshly prepared CK-Mt show a greater molecular mass than the usual dimeric creatine kinase isoenzymes (25). The activation energy of macro CK type 2 and CK-Mt are similar and much greater than for all other creatine kinase isoenzymes (32).

Although the macro CK types reveal themselves by their abnormal migration pattern on electrophoresis, two relatively simple tests can also be utilized to differentiate between them and the other soluble isoenzymes. Both the macro CK types 1 and 2 are much more heat stable than CK-MB and CK-BB, and so by heating samples for 20 min at 45°C the presence of thermostable macrotypes can be demonstrated (29). Macro CK type 2 can be differentiated from macro CK type 1 and the "free" CK isoenzymes by its much higher activation energy (32).

Creatine kinase isoenzymes can apparently form complexes with lipids or lipoproteins in the free state (33) as well as in the macro state (34).

Genetic variants of creatine kinase have been found in some families whose blood cells (which do not normally show

CK activity) contain CK isoenzyme activity, mostly of the BB type (35). Another type of genetic variant may be the rise of lymphocyte clones producing CK-BB in patients with lymphoblastic lymphosarcoma (36).

F. ISOENZYME DISTRIBUTION

Creatine kinase isoenzymes are found in differing proportions in various tissues. The results of some quantitative studies show considerable discrepancies between the values obtained for the same tissue by different investigators (14,15,37). These may be partly due to variations in technique and in the treatment of the specimen before electrophoresis, and also possibly to disproportionate loss of the more labile, fast-moving BB isoenzyme. Other likely causes of variation, especially in the case of human specimens, include post-mortem autolysis and contamination of certain tissues with blood. Despite these anomalies, there is broad agreement that in the heart, the hybrid form CK-MB predominates.

G. TECHNIQUES FOR THE SEPARATION OF CREATINE KINASE ISOENZYMES

The separation of creatine kinase isoenzymes can be achieved by several different procedures: (1) electrophoresis; (2) isoelectric focusing; (3) ion-exchange chromatography; (4) affinity-chromatography.

1. Electrophoretic Techniques

The development of simplified procedures for the zone-electrophoretic separation of serum proteins during the early fifties provided a convenient means for the separation of isoenzymes. In fact, the existence of creatine kinase isoenzymes was first demonstrated by electrophoretic separation on agar gel (11). In recent years, however, a number of alternative methods employing a variety of supporting media, namely, cellulose acetate (38), agarose (39), starch gel (40), and polyacrylamide (41) has been introduced.

Cellulose acetate and agarose are most commonly used in clinical laboratories since complete kits are available commercially. Both methods use the Rosalki's system for the visualization of the separated isoenzymes under uv light.

2. Isoelectric Focusing

Isoelectric focusing in sucrose density gradient has been used for the isolation of CK-MM isoenzyme from human serum (42). It was found that the CK-MM isoenzyme has pI values between 6 and 7. The MB isoenzyme appears to have its isoelectric point near pH 5.2.

Agarose gel isoelectric focusing of creatine kinase isoenzymes has also been reported (43). This technique

is an analytical procedure with a high resolution, often exceeding that obtainable by means of electrophoresis. Using this technique, sub-bands of the CK-MM isoenzyme have been demonstrated in various human tissue extracts (43).

3. Ion-Exchange Chromatography

The application of ion-exchange column chromatographic procedures has enabled the various fractions of creatine kinase isoenzymes to be separated on a preparative scale. DEAE-Sephadex and DEAE-Cellulose were the first ion-exchange resins used in the purification of tissue isoenzymes of creatine kinase (44,45). Since then, other more efficient resins have been developed, namely, DEAE-Sepharose CL-6B (46).

A simplified 'batch' procedure for the differential determination of serum creatine kinase isoenzymes has been reported (47). This technique utilizes BioRad macroporous, strongly basic anion exchange resin AGMP-1; CK-MB and CK-BB are bound to the resin while CK-MM is not.

4. Affinity Chromatography

Creatine kinase from human skeletal muscle has been purified by an affinity chromatography technique (48). The enzyme was retained on a column of p-mercuribenzoate-2-aminoethyl-Sepharose and was selectively eluted with a

gradient of 2-mercaptoethanol.

Other affinity ligands used in the purification of creatine kinase include 8-azo-ADP-, 8-azo-ATP-, 8-(6-aminohexylamino)-ATP-, and 3-(aminopyridine)-AD⁺-Sepharoses. Using these ligands, the enzyme can be eluted with an appropriate ATP gradient (49).

H. STABILITY OF CREATINE KINASE ISOENZYMES

The measurement of creatine kinase activity and consequently the clinical interpretation of such measurements are often affected by the rapid inactivation of the enzyme in serum (50,51). Creatine kinase undergoes two types of inactivation (52). One is irreversible and highly temperature dependent (53), the other is reversible and may be affected by thiol compounds, via the deblocking of sulphhydryl groups at the active site of the enzyme molecule (54).

A considerable fraction of enzyme activity is lost when blood specimens are drawn (55). Szasz et al. (55) observed 30 to 80% reversible inactivation within 20 min of blood sampling, especially in sera from patients with myocardial infarction - which contain the MB isoenzyme. It is believed that the creatine kinase-containing cells release the enzyme which is already partly in the inactive form or it is inactivated in the circulation. In any case, cell damage is only reflected by how much enzyme is spilled

into the circulation and not by how much is active. Maximum reactivation and protection against inactivation between blood sampling and activity measurement is, therefore, very important not only to reproducible results, but also for intelligent clinical interpretation of the creatine kinase activity in serum.

All three isoenzymes of creatine kinase are affected by temperature. There is an increasing stability in the sequence: BB, MB, and MM isoenzyme. Above 30°C irreversible inactivation is very rapid, even in the presence of thiols (55). The endogenous MM isoenzyme in serum is the most stable and needs no thiol protection below 30°C. It is, therefore, necessary to cool the specimens below 30°C as soon as possible after blood sampling. It is, in fact, common practice to freeze the specimens for CK-MB measurement.

Thiols greatly increase the stability of the BB and MB isoenzymes at 30, 25 or 4°C, if the thiol concentration is adequate (55). N-Acetylcysteine is the thiol compound which has been most widely used for the protection and reactivation of the CK isoenzymes. Other thiol compounds which have been used include glutathione (56), 2-mercaptoethanol (57), 1-thioglycerol (58), dithiothreitol (59), and, β -D-thioglucose (58).

Serum pH is also critically important to the stability of human creatine kinase activity. The pH of sera left in plastic sample cups overnight can increase by as much as 1 pH

unit because of loss of CO_2 (60). As the pH of serum increases above 7.0, the creatine kinase activity becomes less stable. A pH of 6.5 is recommended as the best storage pH for the creatine kinase isoenzymes (61).

Several studies have recommended the addition of metal chelators such as ethylenediamine tetraacetate (EDTA) or ethylene-glycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetate (EGTA) to either the creatine kinase assay mixture (62-64), or to the sample prior to storage (65,66). The need for such chelators has been proposed because of the inhibitory effect of Ca^{2+} on the CK (62,64).

The effect of Ca^{2+} is greatest on human CK-BB ($k_i = 0.95$ mmol/L) and least on human CK-MM ($k_i = 4.35$ mmol/L), the effect on CK-MB being intermediate ($k_i = 1.85$ mmol/L) (67). Ca^{2+} acts as a competitive inhibitor of Mg^{2+} for the activity of creatine kinase (68). EDTA has a greater affinity for Ca^{2+} than for Mg^{2+} , and therefore addition of EDTA minimizes the amount of "free" Ca^{2+} competing with the available Mg^{2+} . EGTA, in contrast to EDTA, binds Ca^{2+} ions more specifically and hence does not chelate the Mg^{2+} ions necessary for creatine kinase activity.

Based on previous studies and on his own, Nealon et al. (61) concluded that for the protection of the stability of CK, monothioglycerol was the best thiol agent, EGTA the best chelator, and 6.5 the best storage pH. The effect of these

parameters is more marked on the CK-BB isoenzyme.

I. DIFFERENCES IN AMINO ACID COMPOSITION

The occurrence of isoenzymes very often involves variation in the primary structure of the enzyme protein. Such a mechanism has been established to account for the existence of the isoenzymes of creatine kinase, three electrophoretically and chromatographically distinct forms of which occur in human tissues (19,69).

Studies on the amino acid composition of the various isoenzymes reveal the occurrence of a larger number of basic amino acids in BB than in MM, thus explaining its faster electrophoretic mobility. Also, the absence of a tyrosine residue in human BB is in contrast to that of the MM form. The amino acid composition of human CK-MM and CK-BB are shown in Table I.

J. PHYSIOLOGICAL ROLE OF THE CK ISOENZYMES

The cellular functions of the creatine kinases are now better understood after the studies of Saks *et al.* (70). It has been shown that creatine kinase MM is bound to the myofibrils, sarcoplasmic reticulum, and plasma membranes of cardiac and skeletal muscle cells (70). In cardiac muscle, creatine kinase MB is also located on the myofibrils.

It has been suggested that ATP produced within the mitochondria by oxidative phosphorylation is transported

TABLE I
 AMINO ACID COMPOSITION OF
 HUMAN CK ISOENZYME

Amino acid/ mole enzyme	MM	BB
Asp	76.7	85.9 ^a
Thr	39.0	30.0
Ser	48.9	42.2
Glu	76.5	74.1 ^b
Pro	36.9	36.0
Gly	64.4	70.4
Ala	30.5	40.8
Val	58.3	42.4
Met	16.6	19.5
Ileu	22.8	21.4
Leu	63.2	72.1
Tyr	15.7	none
Phe	25.8	29.3
His	39.2	19.2
Try	6.2	- ^c
Lys	69.7	38.5
Arg	35.0	29.7
Cys	6.4	- ^c
Amide	- ^c	- ^c
Total	732	- ^c
Molecular weight	81,800	

^aAsp + Asn.

^bGlu + Gln.

^cData not available

Sources: A. Warren, W. A. (1973) Prep. Biochem. 3, 199-208

B. Zweig, M. H., van Steirteghem, A. C. & Schechter, A. N. (1978) Clin. Chem. 24, 422-428

across the inner mitochondrial membrane by an ATP-ADP translocase. Mitochondrial creatine kinase (CK-Mt) bound to the exterior surface of the inner mitochondrial membrane, catalyzes the phosphorylation of creatine to form ADP and creatine phosphate. The ADP is recycled into the mitochondria for oxidative phosphorylation via the translocase. The creatine phosphate diffuses into the cytoplasm, where it serves as a substrate for the cytoplasmic creatine kinase MM and MB.

K. DIAGNOSTIC SIGNIFICANCE OF CK ISOENZYMES

1. Myocardial Infarction

Human myocardium contains creatine kinase activity of about 1600 U/g, about 20 percent of the activity being contributed by CK-MB (65). Elevated serum CK activity as an index of myocardial infarction was first described in 1960 (71) and was found soon after to be a sensitive test for the detection of acute myocardial injury with positive results in 95 to 100 percent of patients (72).

Total serum CK activity generally increases 4 to 8 h after the onset of chest pain, peaks within 12 to 24 h, and returns to the normal range within 72 to 96 h (73). However, despite its sensitivity, elevation of total serum CK activity lacks specificity for the diagnosis of acute myocardial infarction since activity also increases in many noncardiac disorders (74).

Analysis of serum CK isoenzyme profiles provide more specific diagnostic information regarding myocardial infarction (75). The results of analysis of CK isoenzyme profiles in human tissues and in serum after injury to skeletal muscle suggest that elevated serum CK-MB is a virtually specific index of injury to myocardium (76). The level of CK-MB in a sample is dependent upon the time at which the sample is taken after myocardial infarction. Samples taken too early or too late may give normal CK-MB values in spite of the incidence of myocardial infarction (77).

2. Other Cardiac Disorders

Besides myocardial infarction, a number of other cardiac disorders can affect the levels of creatine kinase and the MB-isoenzyme. Angina, coronary insufficiency and congestive cardiac failure can increase CK activity (78,79). Pulmonary embolism and myocardial infarction are often concurrent and one may serve as a complicating feature of the other (80). Acute inflammatory myocarditis exhibits an isoenzyme pattern which can often resemble that of myocardial infarction (81). Cardioversion, which is used to correct cardiac arrhythmias by subjecting patients to electric countershock, is known to increase serum creatine kinase activity (82,83). However, the increase in the MB isoenzyme is much less than that observed in myocardial infarction.

3. Muscle Disorders

The measurement of serum creatine kinase activity was first used to detect patients with progressive muscular dystrophies (84). Raised serum creatine kinase activities are often observed in the sex-linked forms of muscular dystrophy, Duchenne Muscular Dystrophy and Becker Muscular Dystrophy (85). It has been reported that the prenatal diagnosis of Duchenne Muscular Dystrophy by intrauterine fetal blood sampling may be possible (86), and successful applications of such techniques utilizing creatine kinase measurements have been reported (87).

Other muscle disorders such as polymyositis (88), rhabdomyolysis (89), acromegalic myopathy (90), hypokalemic periodic paralysis myopathy (91), viral myositis (92), and alcohol myopathy (93) have been described as causing increased creatine kinase activities and in some cases elevated creatine kinase-MB has been detected.

4. Effect of Surgery

Creatine kinase isoenzyme MB is important in the assessment of myocardial infarction, but this isoenzyme is also present in skeletal muscle and can be released into the circulation when severe muscle trauma occurs as, for instance, during surgery (94,95). The level of creatine kinase MB in serum after surgery varies considerably, but it is found increased mostly in coronary artery surgery

(96), aortic valve surgery (97), and coronary-artery bypass surgery (98).

5. Malignant Hyperthermia

Malignant hyperthermia or hyperpyrexia, induced by anaesthesia, is characterized by a rapid rise of body temperature, acidosis, hyperkalaemia, and muscle rigidity (99). The condition is inherited as an autosomal dominant characteristic (100). In susceptible patients, serum creatine kinase activities can be elevated. Creatine kinase can reach very high levels and peaks 24 to 48 h following an acute crisis (101).

The isoenzyme pattern in the serum of malignant hyperthermia patients has not been clearly resolved, and a number of studies made mention of the presence of all three isoenzymes (102). Other workers have only been able to demonstrate an increase in creatine kinase MM (103). The use of different separation techniques may be the reason for the anomalies.

6. Thyroid Disorders

The association between creatinuria and thyrotoxicosis (104) led to attempts to establish a correlation between myopathy and hyperthyroidism (105). As urinary creatine is primarily of muscle origin it was proposed that serum creatine kinase levels might be increased (106). Hypothyroid

patients have been found to have elevated serum CK levels (107). Subsequent studies of serum CK activity in euthyroid, hyperthyroid and hypothyroid people have shown an inverse relationship between serum enzyme activity level and thyroid status (108). Aquaron et al. (109) did not confirm this general inverse correlation but did show a marked association between clinical hypothyroidism, or reduced plasma protein bound iodine (PBI) concentration, and elevated serum CK activity. Only the CK-MM is present in these disorders.

Thyroid disorders produce an increase in serum CK level because of a toxic effect upon muscle. Myopathy of chronic form is commonly seen in thyrotoxicosis (110). Patients with hypothyroidism may present with muscle pain and weakness, acroparaesthesiae or less commonly, definite myopathy (111). Since skeletal muscle is involved, this explains why the CK isoenzyme present in these diseases is predominantly MM.

7. Effect of Exercise

Increases in creatine kinase activity due to exercise have been reported (112,113), and seem to vary considerably. Comparison of a group of Olympic athletes with untrained academics has led to the suggestion that changes in creatine kinase activity could be used as an index of physical fitness attained by an individual (114). It is believed that conditioned skeletal muscle has an increased availability of

ATP which is able to maintain the integrity of the cell membrane during work and thus reduce enzyme efflux (115).

8. Brain Injury

High CK-BB isoenzyme activity in cerebrospinal fluid (CSF) has been demonstrated in patients with various neurological diseases and is considered to reflect brain damage (116-118).

Due to the extreme instability of the CK-BB isoenzyme, enzymatic activity tends to decrease very rapidly in specimens drawn. A very acute fall in activity to 3 percent of the initial value 24 h later, has been reported (118). Therefore, the time passed between the insult and the lumbar puncture has to be taken into consideration if CSF-CK activity is to be used as an indicator of brain damage.

Increased total CK activity in the CSF has also been reported in bacterial meningitis (119). A large percentage of the activity was accounted for by the increased level of CK-BB isoenzyme.

Using a radioimmunoassay technique, serum CK-BB has also been shown to increase rapidly after serious head injury reaching 30 to 40 times the mean control value (120). Radioimmunoassay is proving to be a useful technique in detecting the presence of the CK-BB isoenzyme since it measures the protein mass and not the enzyme activity, hence overcoming the difficulty of assaying an unstable enzyme.

9. Tumour-Associated Creatine Kinase

In recent years, several reports have accumulated on the elevated levels of creatine kinase in the plasma of patients with carcinomas (121-124). Most of the data available are from studies on prostatic carcinoma. Prostatic tumour homogenates have significant creatine kinase BB and it has been suggested that a correlation could exist between the amount of creatine kinase BB isoenzyme and tumour activity (125).

The CK-BB isoenzyme has been classified as a foeto-placental protein because it is the fetal form of creatine kinase (126). In tumour growth, as de-differentiation takes place, the fetal enzyme form is produced. One of the reasons why elevated creatine kinase BB levels have not been found in all cases of carcinomas is associated with its instability in serum (55).

Although creatine kinase BB is synthesized by tumours, it is quite widespread in its distribution (37). This has been the cause of problems with interpretation of elevated plasma levels of creatine kinase BB. Measurement of this isoenzyme is probably more useful as a tumour marker when it is used in combination with other biochemical tests.

10. Miscellaneous Conditions

High serum levels of creatine kinase in patients with Reye's syndrome have been reported (127). The isoenzyme

pattern obtained shows the presence of all 3 isoenzymes (127), but there is a predominance of the CK-MM isoenzyme in all the reported cases.

It has been suggested that measuring the creatine kinase isoenzymes may aid in determining the severity of certain liver diseases (128).

During normal childbirth, increases in serum creatine kinase of up to 6 times normal have been observed and the activity returns to normal after 6 weeks (129).

Serum creatine kinase can be frequently elevated in cases of wasp/bee stings with the increased activity being due to damage to muscle fibers (130).

Due to the abundance of creatine kinase in many tissues, elevated levels of this enzyme have been described in many conditions. However, the data are not conclusive enough to be of any diagnostic value.

L. METHODOLOGIES FOR THE QUANTITATION OF CK ISOENZYMES

The studies of isoenzymes can be divided into two classes of methods. The first class of methods rests on classical procedures for protein separation, which are often followed by a conventional assay of the isolated fractions. This class includes electrophoresis, ion-exchange, etc.

The second class of methods relies on differential enzymic or immunologic behaviour to reveal the composition of a mixture of isoenzymes. It makes use of the fact that

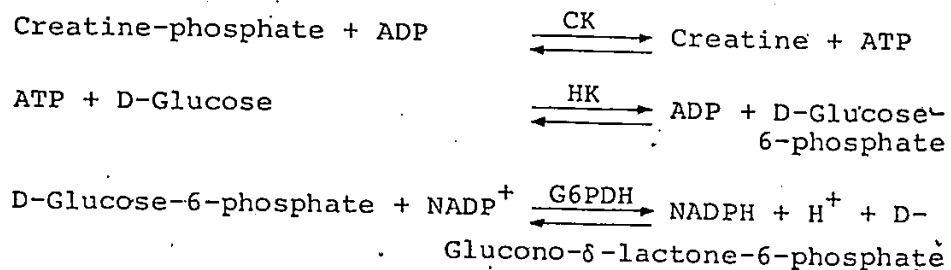
activity and specificity sites, as well as immunogenic sites are modulated by protein structure. This class includes differential activation, immunoinhibition, radioimmunoassay, etc.

The methods used in the study of creatine kinase isoenzymes fall into both classes. The value of separating the isoenzymes for diagnostic purposes is well established (76, 131). The quantitation of the CK-MB isoenzyme for the monitoring of myocardial damage is of special clinical importance (81).

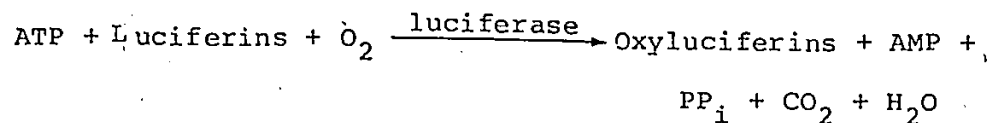
Creatine kinase activity can be determined by end-point or by kinetic procedures. Early methods for determining CK activity involved:

1. Coupling ADP production to NADPH oxidation (132) which results in decreasing absorbance at 340 nm.
2. Reacting the phosphate formed with ammonium molybdate to form a blue complex read at 690 nm. (133).
3. A colorimetric assay for creatine which forms a red complex with α -naphthol and diacetyl having maximum absorption at 520 nm (134).
4. A fluorimetric method based on a reaction between creatine and ninhydrin in strongly basic medium (135).

However, the most commonly utilized method is the one designed by Oliver (136), modified by Rosalki (137), and later optimized by Szasz (138). In this assay, the ATP released in the reverse reaction of creatine kinase is coupled to auxiliary reactions catalyzed by hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6PDH) where the NADPH produced is a sensitive and direct measure of creatine kinase activity:



More recently, a bioluminescence assay in which the ATP formed in the reverse reaction is measured by means of firefly luciferase (139) has been proposed. The light emission is proportional to the amount of ATP present:



The presence of the CK-MB isoenzyme in the serum is a sensitive and specific marker of myocardial infarction (74,75). Various methods have been introduced for the isolation and quantitation of this isoenzyme. These are briefly reviewed.

M. DETERMINATION OF CK-MB ISOENZYME IN SERUM

The methods currently utilized to separate and to quantitate the CK-MB isoenzyme include:

1. Electrophoretic separation
2. Ion-exchange procedures
3. Immunological methods
4. Differential isoenzyme activity assays
5. Radioimmunoassay

Electrophoresis is one of the earliest methods used to separate the isoenzymes of creatine kinase. It is usually carried out on cellulose acetate strips or agarose-gel. The separated isoenzymes are visualized under uv light by first reacting them with the coupled-assay system of Rosalki (137). Although technically simple, electrophoresis is less sensitive than chromatography (140). It is at best semi-quantitative. However, its main advantage is that the separated isoenzyme bands can be visualized, and any extraneous or abnormal band is readily detected. This is of importance, especially in cases where IgG-CK-BB complexes are present in serum and may be mistaken for CK-MB by the other assay methods (27,141).

Separation of CK-MB by chromatography on anion-exchange mini-columns (44), resins (47), or Glycophase glass beads (142) has been reported. The main disadvantage of anion-exchange chromatography is the merging of CK-MM into CK-MB

on an unsatisfactory column or in a sample with a very high CK-MM activity (81). In some chromatographic methods CK-MB and CK-BB are eluted together, which is undesirable. Chromatography is more time consuming than electrophoresis, but with good technique it can be more precise than electrophoresis (81).

Immuno-inhibition provides a method for the specific measurement of CK-MB in serum in the presence of other CK isoenzymes. It makes use of anti-CK-MM antibodies to inhibit the M-subunit of creatine kinase, thus allowing the B-subunit of the MB isoenzyme to be measured. Again, the coupled assay of Rosalki is used to measure the residual B-subunit activity. Recently, an optimized bioluminescence assay has been used to measure the B-subunit activity after the immuno-inhibition procedure (143). It claims increased sensitivity. It is common practice to multiply the activity for the B-subunit by two to obtain a measurement of CK-MB, assuming similar activities for both M- and B-subunits (144).

The immuno-inhibition procedure may suffer from serious interference due to the presence of the CK-BB isoenzyme and in some cases atypical CK's as well as adenylate kinase. Wicks et al. have devised a procedure to remove such interferences (145).

The selective activation procedure, which makes use of two different sulphhydryl activators to differentially

activate individual CK isoenzymes as a means to quantitate them (146), has failed to gain recognition as a reliable assay.

Radioimmunoassay is several-fold more sensitive than enzyme assays based on detection of activity, because abnormally high concentrations of MB in serum can be detected within 2 to 4 h of the onset of symptoms (147), as opposed to 6 to 8 h by conventional methods. Several immunoassays have been developed for quantitating CK isoenzymes by use of antibodies specific for the enzyme polypeptide subunits M or B (148-150). To overcome the problem of measuring CK-MB specifically in the presence of CK-MM and CK-BB, a new two-site immunoradiometric assay was recently developed (151). Despite their high specificity and sensitivity, radioimmunoassays have the disadvantage of being time consuming compared to more conventional techniques.

Several papers have been published comparing the various techniques for the measurement of CK-MB and comparing clinical observations with analytical results (152-155). Boone et al. (156) have provided data on interlaboratory variability of methods commonly used to measure CK-MB.

N. THE STUDY

Only few data have been published concerning kinetic differences between the isoenzymes of creatine kinase (8,18). Moreover, owing to variations in the purity of the enzyme preparations and in isolation procedures it is difficult to compare the values obtained.

The object of this work is to characterize creatine kinase preparations of different tissue origins using their kinetic constants. The optimized assay procedure, recommended by the Scandinavian Committee on Enzymes (157, 158), was used to investigate the differences in Michaelis constants of the creatine kinase isoenzymes of human skeletal and heart muscles and brain.

It is commonly assumed that the 3 main creatine kinase isoenzymes, namely CK-MM, CK-MB and CK-BB, have the same catalytic efficiency, *i.e.*, they turn over the same amount of substrate per molecule of enzyme per minute. Owing to the fact that these isoenzymes are very unstable *in vitro*, especially CK-BB (155), it is virtually impracticable to verify this assumption by measuring a turnover number for each isoenzyme. However, using an immunoinhibition method (145), the activity of the M-subunit was compared to that of the B-subunit in purified preparations of CK-MB.

To ensure that the results obtained with enzyme preparations are comparable to enzyme directly released into the serum, additional experiments were carried out using pooled human inactivated sera.

CHAPTER II :

MATERIALS AND METHODS

A. EQUIPMENT

Spectrophotometers: For kinetic assays of creatine kinase activity, the GEMINI Miniature Centrifugal Analyzer (Electro-Nucleonics, Inc., Fairfield, NJ 07006) was used. Determination of the kinetic constants of the isoenzymes was carried out on a GILFORD- Stasar III spectrophotometer (Gilford Instrument Laboratories Inc.). An LKB 2138 Unicord S spectrophotometer (LKB-Produkter AB, Bromma 1, Sweden) was used to monitor protein concentration in the column eluates.

pH meter: Measurements of pH during the preparation of the enzyme reagent were made on a Corning Model 12 Research pH meter manufactured by Corning Scientific Instruments, Medfield, MA 02052, U.S.A.

Balance: For weight determinations below 1 g a Mettler AC 100 (Mettler Instrumente AG, CH-8606, Greifensee-Zurich) was used.

B. MATERIALS

Tissues: Autopsy material was the source of human tissues, which were stored frozen at -20°C until required for use. Permission to use human tissues was approved by the University of Windsor Human Research Ethics Committee.

Reagents: Sephadex DEAE-A-50 and Sephadex G-100 gels were from Pharmacia Fine Chemicals, Uppsala 1, Sweden. The GEMINI CPK Reagent Kit for the determination of creatine kinase activity was obtained from Electro-Nucleonics, Inc., Fairfield, NJ 07006. Cellulose acetate strips (Sepraphore III) and barbital buffer for isoenzyme analysis by electrophoresis were from Gelman Instrument Co., Ann Arbor, MI 64106.

Affi-Gel Blue and the reagents for polyacrylamide gel electrophoresis were from Bio-Rad Laboratories, Mississauga, Ontario L4X 2C8. The following chemicals were obtained from Sigma Chemical Co., St. Louis, MO 63178: Imidazole (Grade III, crystalline); ADP (Grade I, sodium salt); AMP (Type III, sodium salt); creatine phosphate (disodium salt); dithiotreitol (DTT); D(+)-glucose (anhydrous, grade III); glucose-6-phosphate dehydrogenase (crystallized from yeast); hexokinase (crystallized from yeast); NADP+ (grade V, sodium salt); diadenosine pentaphosphate (lithium salt).

The reagents for the immunochemical separation and selective inhibition of the isoenzymes of CK (the Isomune-CK kit) were obtained from Roche Diagnostics, Division of Hoffmann-La Roche Inc., NJ 07110. The immunochemical reagents consist of goat antiserum against CK-MM and a second antibody suspension consisting of polymerbound antigoat immunoglobulin.

The CK/LD Isoenzyme Control used in the identification

of the creatine kinase isoenzymes was from Helena Laboratories, P.O. Box 752, Beaumont, TX. 77704. All the other reagents used were of the highest grade and purity available. All solutions were prepared in doubly distilled de-ionized water. Type I water, as specified by the National Committee for Clinical Laboratory Standards (NCCLS), was used to prepare the enzyme reagents (Hotel Dieu Hospital).

C. METHODS

1. Purification of Human CK-MM Isoenzyme

The CK-MM- isoenzyme was purified from human skeletal muscle and heart tissue obtained within about 12 h post-mortem. The tissues were either processed immediately or stored at -20°C . The purification scheme was based on the procedure of Miller *et al.* (159), with some modifications. All preparative procedures were carried out in a cold-room ($4-8^{\circ}\text{C}$) or in an ice bath. Protein concentrations and creatine kinase activity were determined at each stage of purification.

(a) Homogenization

After being rinsed with distilled, de-ionized water, the tissues were trimmed of fat. About 100 g of tissue were cut into small pieces with scissors and homogenized in a Willems Polytron using 10-15 s bursts, in a 2-fold volume of Tris-HCl buffer (0.05 M, pH7.4), containing 2 mmol of 2-mercaptoethanol per litre. The homogenate was gently stirred for about 4 h. It was then centrifuged at $31,000\times g$

for 15 min, and the supernatant was filtered through eight layers of cheesecloth.

(b) Ammonium sulphate fractionation

The filtered supernatant was brought to an ammonium sulphate concentration of 50% saturation by slow addition of 31.3 g of solid ammonium sulphate per 100 mL., with continuous stirring. After stirring for 2 h, the mixture was centrifuged at 22,500xg for 45 min, and the pellet was discarded. The supernatant solution was brought to 80% ammonium sulphate concentration by the further addition of 21.5 g of solid ammonium sulphate. The 50-80% ammonium sulphate precipitate was recovered after 2-h stirring, by centrifugation at 31,000xg for 40 min and redissolved in a minimum volume of 0.05 M Tris-HCl buffer, pH 8.5, containing 2 mmol/L 2-mercaptoethanol. The solution was then dialyzed vs the same Tris-HCl buffer for 24 h with at least 2 buffer changes.

(c) Gel filtration

The dialysate was applied to a 2.5 x 90-cm column of Sephadex G-100, which had previously been equilibrated with 0.05 M Tris-HCl buffer, pH 8.0, containing 2 mmol/L of 2-mercaptoethanol. The column was developed with the same buffer at a flow rate of about 10 mL/hr. The active fractions were pooled and concentrated under nitrogen pressure in an Amicon chamber with the use of a UM-10 filter.

(d) Anion-exchange chromatography

The preparation was further purified on a 40 x 2.6-cm column of Sephadex DEAE-A-50 equilibrated with 0.05 M Tris-HCl buffer, pH 8.0, containing 2 mmol/L of 2-mercaptoethanol. After washing the column with one bed volume of the same buffer, the enzyme was eluted with a 0.05 M Tris-HCl buffer, pH 8.0, containing 50 mmol/L NaCl and 2 mmol/L of 2-mercaptoethanol, at a flow rate of 20-25 mL/h. The eluate was collected in 5-mL fractions, which were examined for protein spectrophotometrically at A_{280} and measured for creatine kinase activity, followed by electrophoresis on cellulose acetate strips for creatine kinase isoenzymes. Fractions containing creatine kinase activity were pooled and concentrated by ultrafiltration. The concentrated fractions were rechromatographed under the same conditions. The active fractions were pooled and dialyzed for 24 h against a 0.05 M Tris-HCl buffer, pH 7.5, containing 5 mmol/L 2-mercaptoethanol. After dialysis, the isoenzyme was concentrated by ultrafiltration and stored at -20°C until required.

2. Purification of Human CK-MB Isoenzyme

MB creatine kinase was isolated from human heart in the same procedure used for the MM isoenzyme.

(a) Anion-exchange chromatography

After elution of the CK-MM isoenzyme from the previous Sephadex DEAE-A-50 column, a salt gradient, 50 to 500 mmol/L of NaCl was used to elute a second peak of creatine kinase activity representing isoenzyme MB. The active fractions were pooled, concentrated by ultrafiltration, and then dialyzed for 24 h vs a 0.05 M buffer, pH 7.5, containing 5 mmol/L of 2-mercaptoethanol.

(b) Affinity chromatography

The MB preparation was further purified on a 1.0 x 20-cm column packed with "Affi-Gel Blue" (100-200 mesh), previously equilibrated with a 0.05 M Tris-HCl buffer, pH 8.0, containing 2 mmol/L of 2-mercaptoethanol. After the MB preparation was applied, the column was washed with a 0.05 M Tris-HCl buffer, pH 8.0, containing 250 mmol/L of NaCl and 2 mmol/L of 2-mercaptoethanol. The fractions containing MB activity were pooled and dialyzed vs the starting buffer, to remove the salt. The pooled fractions were concentrated by ultrafiltration and stored in liquid nitrogen until used.

3. Purification of Human CK-BB Isoenzyme

BB creatine kinase was isolated from human brain obtained at autopsy. The homogenization procedure was similar to that used for the muscle isoenzyme.

(a) Ethanol fractionation

To the supernatant, 95% ethanol (pre-cooled to -20°C) was added dropwise to give a final concentration of 50%. The mixture was stirred for 30 min and then centrifuged at 1,000xg for 15 min, and the pellet discarded. A second ethanol extraction was performed on the supernatant by adding 95% ethanol to give a final concentration of 70%. After stirring the mixture for another 30 min, it was centrifuged at 1,000xg for 15 min. The pellet was resuspended in a minimum volume of Tris-HCl buffer, pH 8.0, containing per litre, 50 mmol Tris-(hydroxymethyl)-amino-methane and 1 mmol DTT, and centrifuged at 31,000xg for 15 min. The supernatant was collected and dialyzed for two hours vs the same buffer to remove the ethanol.

(b) Anion-exchange chromatography

The dialyzed fraction of the BB creatine kinase was further purified by anion-exchange chromatography on a 40 x 2.6-cm column of Sephadex DEAE-A-50, previously equilibrated with a 0.05 M Tris-HCl buffer, pH 8.0, containing 1 mmol/L DTT. After application of the sample, the column was washed with one bed volume of the same buffer. The BB isoenzyme was eluted by using a salt gradient containing 100 to 450 mmol/L of NaCl. The fractions containing creatine kinase activity were pooled and concentrated by ultrafiltration. The concentrated fraction was

then dialyzed for 24 h vs a 0.05 M Tris-HCl buffer, pH 8.5, containing 2 mmol/L DTT. The dialyzed sample was further purified by a second Sephadex DEAE-A-50 fractionation on a column equilibrated with the same pH 8.5 buffer. The isoenzyme was eluted by using a salt gradient containing 140-300 mmol/L of NaCl. Fractions containing creatine kinase activity were pooled, concentrated and dialyzed for 24 h vs a 0.01 M Tris-HCl buffer, pH 7.5, containing 2 mmol/L of DTT. The dialysate was then recovered and stored in liquid nitrogen until required.

4. Protein Determinations

The concentration of protein in fractions eluted from the chromatographic columns was determined spectrophotometrically at A_{280} on an LKB 2148 Uvicord S.

The biuret procedure described by Gornall *et al.* (160) was used with bovine serum albumin as standard during the early stages of purification. To determine the specific activity of the isoenzymes, a modified Lowry method was used (161) for measuring protein concentrations.

5. Polyacrylamide Gel Electrophoresis of the CK Isoenzymes

The purified isoenzymes were examined for homogeneity by disc-gel electrophoresis as described by Davis (162) using a 7.7% acrylamide running gel overlaid with a 2.6% acrylamide

stacking gel. Bromophenol blue was used as the tracking dye and the enzymes samples were applied in 20% glycerol solutions. The buffer used was 0.05 mol/L Tris/0.38 mol/L glycine buffer, pH 8.3. A current of 1.5 mA per tube was applied for the first 30 min and then increased to 3 mA per tube for the next two and a half hours. Protein bands were stained with Coomassie Brilliant Blue G-250 in 5% TCA solution. The gels were subsequently destained and stored in a 7% solution of acetic acid.

6. Electrophoresis on Cellulose Acetate

Identification of the CK isoenzymes was done by electrophoresis at room temperature on cellulose acetate strips using the Gelman system. A 2- μ L sample of isoenzyme preparation was applied to a Sepraphore III cellulose acetate strip and electrophoresed for 20 min at 250 volts, using barbital buffer, pH 8.8. Samples with a CK activity greater than 1000 U/L were diluted in 0.9% saline before sample application. The CK/LD Isoenzyme Control was run simultaneously on each strip. Following electrophoresis, the strips were layered with the CK reagent, blotted gently, incubated in the dark at 37°C for 30 min. The strip was then dried for 10 min under a stream of cool air and the isoenzyme bands identified using a uv lamp.

7. Enzyme Assay

Creatine kinase activity, in fractions eluted from the chromatographic columns, was determined spectrophotometrically by following the formation of NADPH at 340 nm at 37°C using the coupled enzyme system of Rosalki (137). The reaction was followed kinetically on the GEMINI Miniature Centrifugal Analyzer. Assays were performed by reacting 15 μ L of the sample with 700 μ L of the enzyme reagent (163).

For the determination of the kinetic constants, the optimized method recommended by the Scandinavian Committee on Enzymes was used, (157,158). The reagent consisted of, per litre, 100 mmol imidazole acetate, 2 mmol ADP, 20 mmol glucose, 2 mmol NADP⁺, hexokinase 3500 U, glucose-6-phosphate dehydrogenase 2000 U, 20 mmol NAC, 5 mmol AMP, 10 μ mol DAPP, 10 mmol magnesium acetate and 2.0 mmol EDTA.

Three types of specimens were used: pure creatine kinase isoenzymes MM, MB and BB diluted to the appropriate activity with a 0.05 M Tris-HCl buffer, pH 8.0, containing 2 mmol/L 2-mercaptoethanol; heat inactivated pooled human serum containing appropriate quantities of purified creatine kinase isoenzymes MM, MB and BB; and routine clinical specimens containing endogenous CK-MM or both CK-MM and CK-MB.

Absorbances were measured with a Gilford Stasar III spectrophotometer at 340 nm, in a thermostated cuvette at

37°C. Enzyme activity was calculated with a Syva Model CP-5000 EMIT Clinical Processor (Syva, a Syntex Company, Palo Alto, CA 94303, U.S.A.).

The procedure was as follows: 1 mL of buffer and 0.05 mL of the sample were pipetted into a 5-mL disposable polypropylene tube, and the tube was incubated for 4 min in a water-bath set at 37°C. Substrate (100 μ L) was then added and mixed. The mixture was then aspirated into the cuvette. The initial absorbance was measured 60 s after sampling, and $\Delta A/\text{min}$ recorded every 15 s for 10 min. The initial rate was taken as the first linear portion of the progress curve, *i.e.*, when $\Delta A/\text{min}$ is constant. The 'procedure for kinetic mode' (see APPENDIX A) was used to obtain the rate of reaction of the isoenzymes.

A unit (U) of enzyme is defined as that amount of enzyme that is required to liberate 1 μ mol of NADPH per minute under the conditions described above. Thus,

$$U/L = \frac{\Delta A/\text{min} \times 10^3 \times V_t}{6.22 \times 10^3 \times b \times V_s}$$

where

$\Delta A/\text{min}$ = absorbance change per minute

10^3 = factor to convert moles to micromoles

V_t = total assay volume in mL

6.22×10^3 = molar absorptivity of NADPH at 340 nm

b = light path in cm

V_s = sample volume in mL

8. Immunochemical Assay of CK-MB

The procedure for the immunoinhibition of the M-subunit of CK-MB isoenzyme was that described by Wicks et al. (145). 200 μ L of CK-MB preparation in pooled inactivated human sera were added to each of two tubes. Then, 250 μ L of goat anti-CK-MM was added to the first tube and the mixture was incubated for 20 min at room temperature to allow immunoinhibition of the CK-M subunits; the residual CK activity in this tube represented the CK-B subunit activity present in CK-MB. To the second or "blank" tube, 50 μ L of goat anti-CK-MM was added and the mixture was incubated at room temperature for 5 min. The second antibody (200 μ L), was then added and the tube was again incubated at room temperature for another 5 min. Following incubation, the tube was centrifuged at 1,000 \times g for 5 min. The supernatant in this tube served as a "blank" and accounted for any extraneous activity. The CK activities in both tubes were measured at 37°C on a centrifugal analyzer (GEMINI) using 25 μ L of sample from each tube, 500 μ L of enzyme reagent and 50 μ L of creatine phosphate solution. The activity in tube 2 was subtracted from tube 1. The difference reflected the B-subunit activity of CK-MB. The activity due to the CK-M subunits was obtained by subtraction from the total CK-MB activity.

CHAPTER III

RESULTS AND DISCUSSION

A. PURIFICATION OF HUMAN CREATINE KINASE ISOENZYMES

Results of the isolation of CK-MM and CK-MB from human psoas muscle and myocardium are summarized in Table II.

The procedure was based upon that of Miller and co-workers (159) with some modifications. The purification scheme produced two major fractions - one containing the CK-MM isoenzyme and the other the CK-MB isoenzyme. The CK-MM fraction was purified 21 times after chromatography on DEAE-Sephadex A-50 and had a specific activity of 361 U/mg protein. The CK-MB fraction was purified 18 times after the Affi-Gel Blue chromatography, with a specific activity of 317 U/mg protein.

Purification of the isoenzymes involved 3 major steps after homogenization. The enzyme homogenates were first purified by ammonium sulphate fractionation. The fraction collected between 50-80% ammonium sulphate saturation was dissolved in Tris-HCl buffer and further purified by Sephadex G-100 chromatography (Fig. 1). Further chromatography by ion-exchange, using DEAE-Sephadex A-50, yielded

TABLE II

PURIFICATION OF CREATINE KINASE-MM AND CREATINE
KINASE-MB FROM HUMAN PSOAS AND HEART MUSCLE

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purity (fold)
MM fraction:					
Supernatant	5425	94395	17.4	-	-
(NH ₄) ₂ SO ₄ fractionation					
(50-80% saturation)	833.8	51946	62.3	55.0	3.6
Sephadex G-100	201.6	22478	111.5	23.8	6.4
1st Sephadex DEAE-A-50	38.0	7908	208.1	8.4	13.0
2nd Sephadex DEAE-A-50	12.6	4546	360.8	4.8	20.7
MB fraction:					
Sephadex DEAE-A-50	20.3	3252	160.2	3.5	-
Affi-Gel Blue	3.1	982	316.6	1.0	18.2

Note: The human skeletal and heart muscles were pooled during the purification procedure.

FIGURE 1

SEPHADEX G-100 CHROMATOGRAPHY OF THE PROTEIN
SAMPLE OBTAINED AFTER AMMONIUM SULPHATE
FRACTIONATION (50-80% SATURATION)Legend

Column size: 90 x 2.5 cm. The column was pre-equilibrated in 0.05 M Tris-HCl buffer, pH 8.0, containing 2.0 mmol/L 2-mercaptoethanol. Protein samples (ca. 0.7 g) were applied to the column. Elution was carried out with the same buffer at a flow rate of about 10 mL/h. Fractions (5 mL) containing the highest enzyme activities were pooled and concentrated.

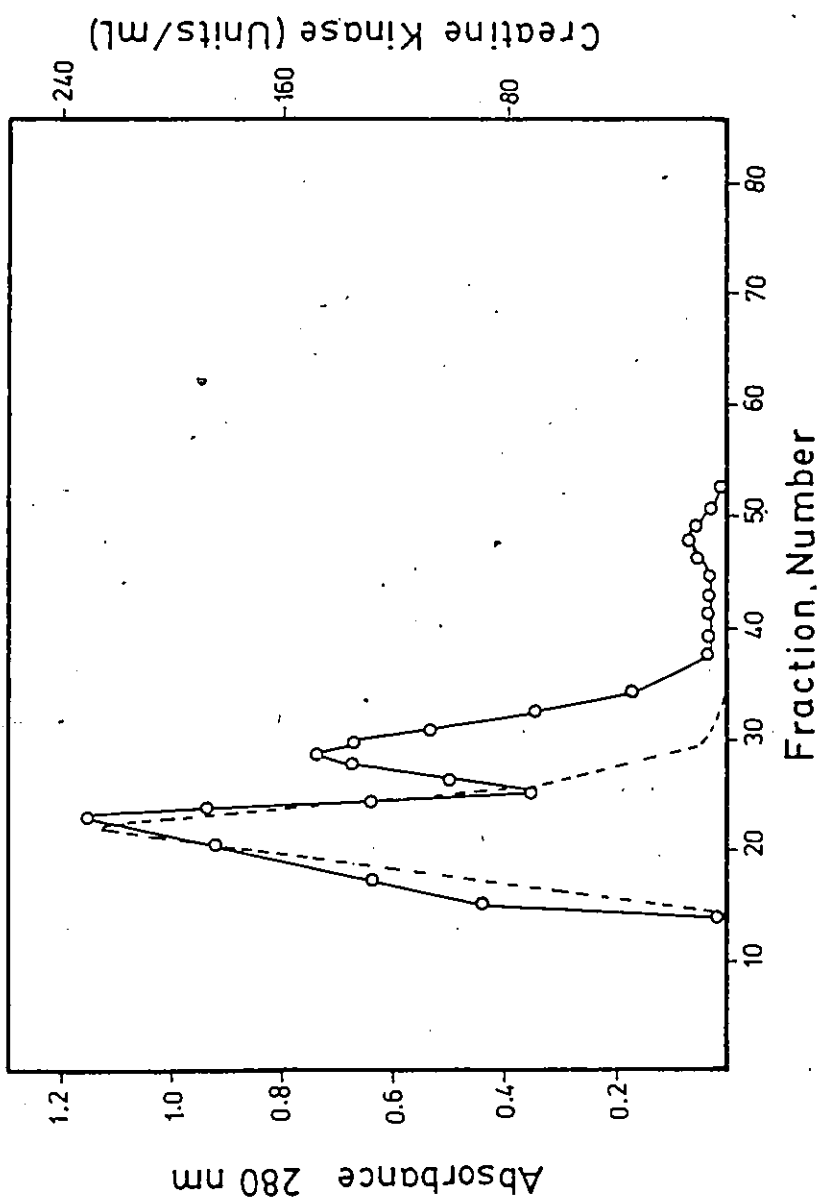
Creatine kinase activity was determined in the fractions eluted from the column as described in METHODS, CHAPTER II, C. 7, p. 39. The isoenzymes were identified by electrophoresis on cellulose acetate. A CK control (murine source, from Helena Laboratories), containing all the 3 isoenzymes, was run simultaneously on each cellulose acetate strip.*

(----) = creatine kinase activity

(—○—) = A_{280}

* See APPENDIX D.

FIGURE 1



two fractions (Fig. 2). The first fraction containing the CK-MM isoenzyme, was further purified on a second DEAE-Sephadex A-50 column (Fig. 3). The second fraction, containing the CK-MB isoenzyme, was subjected to Affi-Gel Blue chromatography to remove contaminating albumin (Fig. 4).

A representative purification procedure for the CK-BB isoenzyme is presented in Table III. The homogenization of the human brain tissue was carried out in the same fashion as for the muscle tissues. The isoenzyme was then extracted with 95% pre-chilled ethanol. The pellet obtained between 50-70% ethanol concentration was dissolved in Tris-HCl buffer and further purified by successive ion-exchange chromatography on DEAE-Sephadex A-50 (Figs. 5 and 6).

The specific enzyme activity was 285 U/mg protein, with a 84-fold purification. The CK-BB isoenzyme is extremely labile and the amount present in human brain is much less than the MM or MB creatine kinase in their respective tissue. Brains obtained 4 h after death contain very low enzyme activity and the yield may be extremely small.

The isoenzyme preparations were used in the kinetic characterization studies. They were stored at -20 °C or in liquid nitrogen and were used within 2 weeks of isolation.

FIGURE 2

DEAE-SEPHADEX A-50 CHROMATOGRAPHY OF THE PROTEIN
SAMPLE CONTAINING CK-MM AND CK-MB ISOENZYMESLegend

Column size: 40 x 2.6 cm. The column of Sephadex DEAE-A-50 was equilibrated with 0.05 M Tris-HCl buffer, pH 8.0, containing 2 mmol/L 2-mercaptoethanol. About 250 mg of protein were applied to the column. After washing the column with one bed volume of the same buffer, the CK-MM isoenzyme was eluted with 0.05 M Tris-HCl buffer, pH 8.0, containing 2 mmol/L 2-mercaptoethanol and 50 mM NaCl. Fractions (5 mL) were collected at a flow rate of 20 mL/h. After elution of the CK-MM isoenzyme, a salt gradient (indicated by the arrow) 50 to 500 mmol/L of NaCl was used to elute a second peak containing enzyme activity representing isoenzyme MB. Fractions containing enzyme activity were pooled and concentrated.

Creatine kinase activity was determined in the fractions eluted from the column as described in METHODS, CHAPTER II, C. 7, p. 39. The isoenzymes MM and MB were identified by electrophoresis on cellulose acetate. A CK control (murine source; from Helena Laboratories), containing all the 3 isoenzymes, was run simultaneously on each cellulose acetate strip.

(---) = creatine kinase activity

(—) = A_{280}

* See APPENDIX D.

FIGURE 2

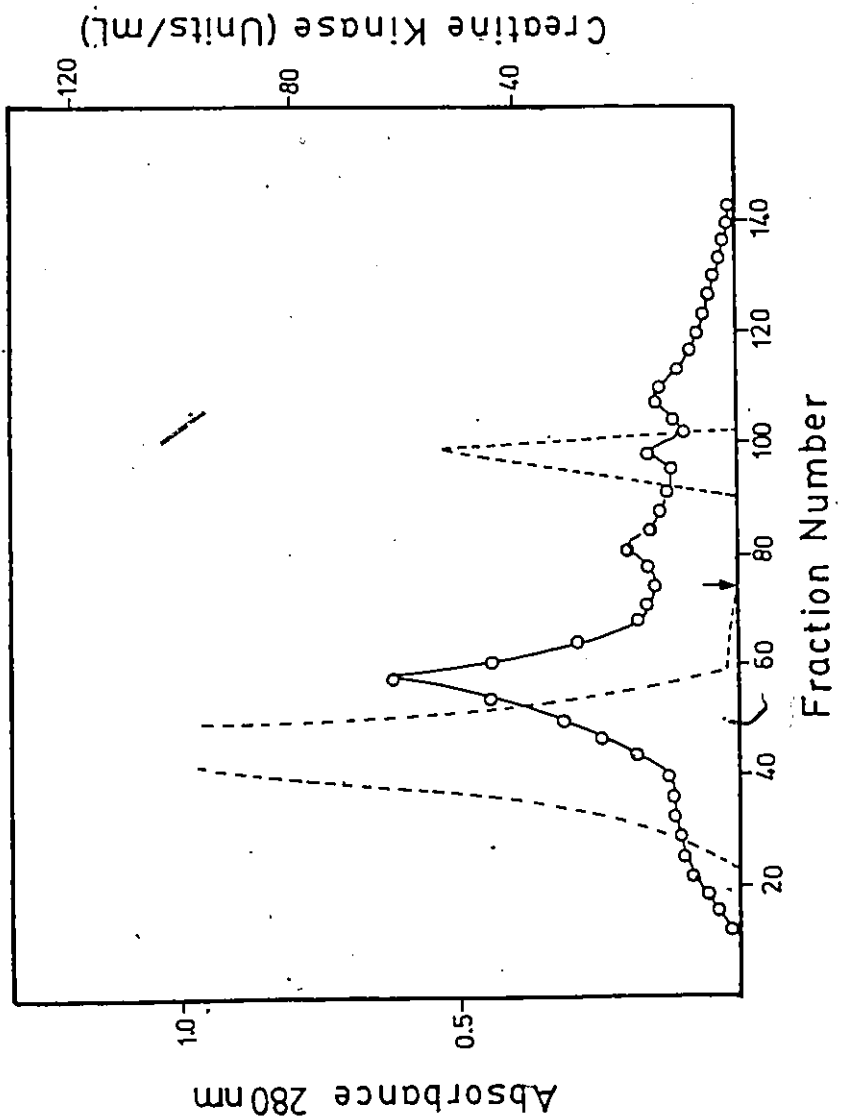


FIGURE 3

SECOND DEAE-SEPHADEX A-50 CHROMATOGRAPHY
OF CK-MM ISOENZYMELegend

Column size: 40 x 2.6 cm. The concentrated protein fraction obtained after the first Sephadex DEAE A-50 chromatograph was rechromatographed under the same conditions. After washing the column with starting buffer, the CK-MM isoenzyme was eluted with a 0.05 M Tris-HCl buffer, pH 8.0, containing 100 mmol/L NaCl and 2 mmol/L 2-mercaptoethanol.

Creatine kinase activity was determined in the fractions eluted from the column as described in METHODS, CHAPTER II, C.7, p. 39. The CK-MM isoenzyme was identified by electrophoresis on cellulose acetate. A CK control (murine source, from Helena Laboratories), containing all 3 isoenzymes, was run simultaneously on each cellulose acetate strip.

(---) = creatine kinase activity

(—○—) = A_{280}

See APPENDIX D.

FIGURE 3

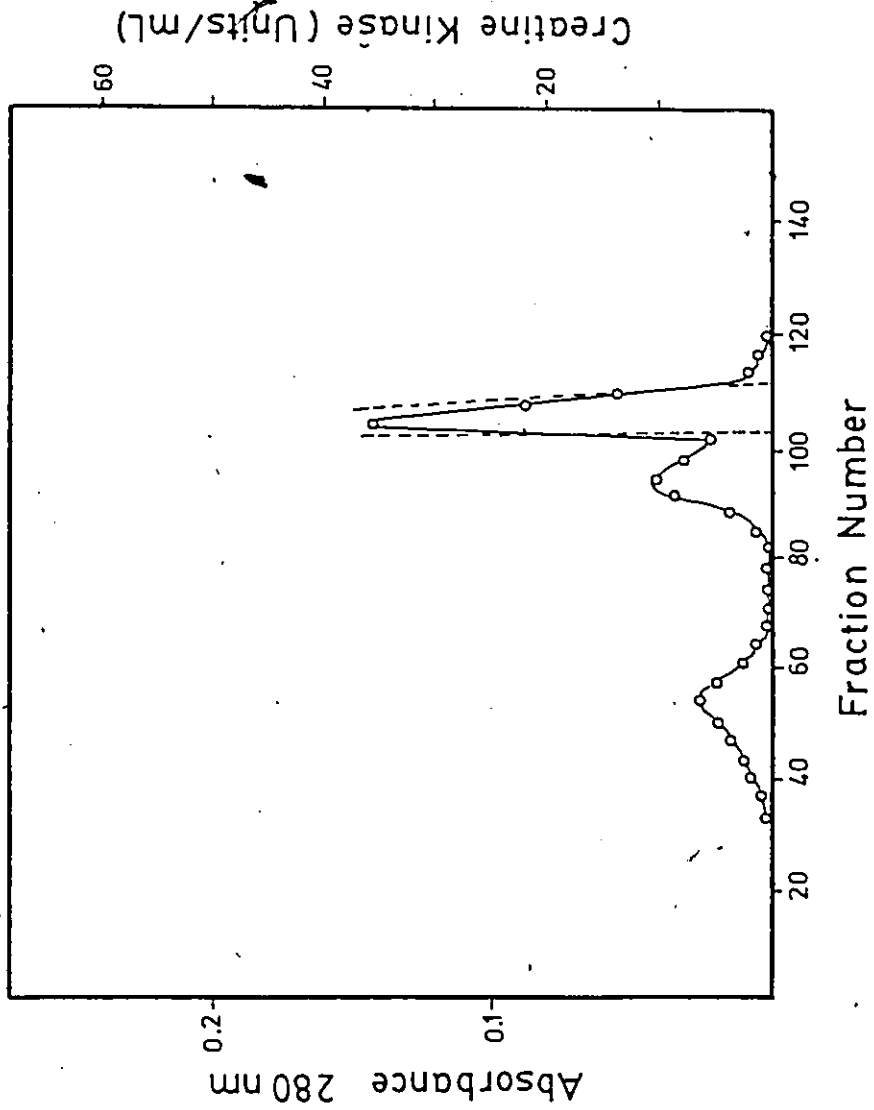


FIGURE 4

AFFI-GEL BLUE CHROMATOGRAPHY OF THE PROTEIN
SAMPLE CONTAINING CK-MB ISOENZYMELegend

Column size: 20 x 1.0 cm. The column was pre-equilibrated in 0.05 M Tris-HCl buffer, pH 8.0, containing 2 mmol/L 2-mercaptoethanol. After application of the protein sample, the column was washed with 2 bed volumes of the same buffer. The MB isoenzyme was eluted with a 0.05 M Tris-HCl buffer, pH 8.0 containing 250 mmol/L NaCl and 2 mmol/L 2-mercaptoethanol.

Creatine kinase activity was determined in the fractions eluted from the column as described in METHODS, CHAPTER II, C. 7, p. 39. The CK-MB isoenzyme was identified by electrophoresis on cellulose acetate. A CK control (murine source, from Helena Laboratories), containing all 3 isoenzymes, was run simultaneously on each cellulose acetate strip.*

(---) = creatine kinase activity

(—○—) = A_{280}

*See APPENDIX D.

FIGURE 4

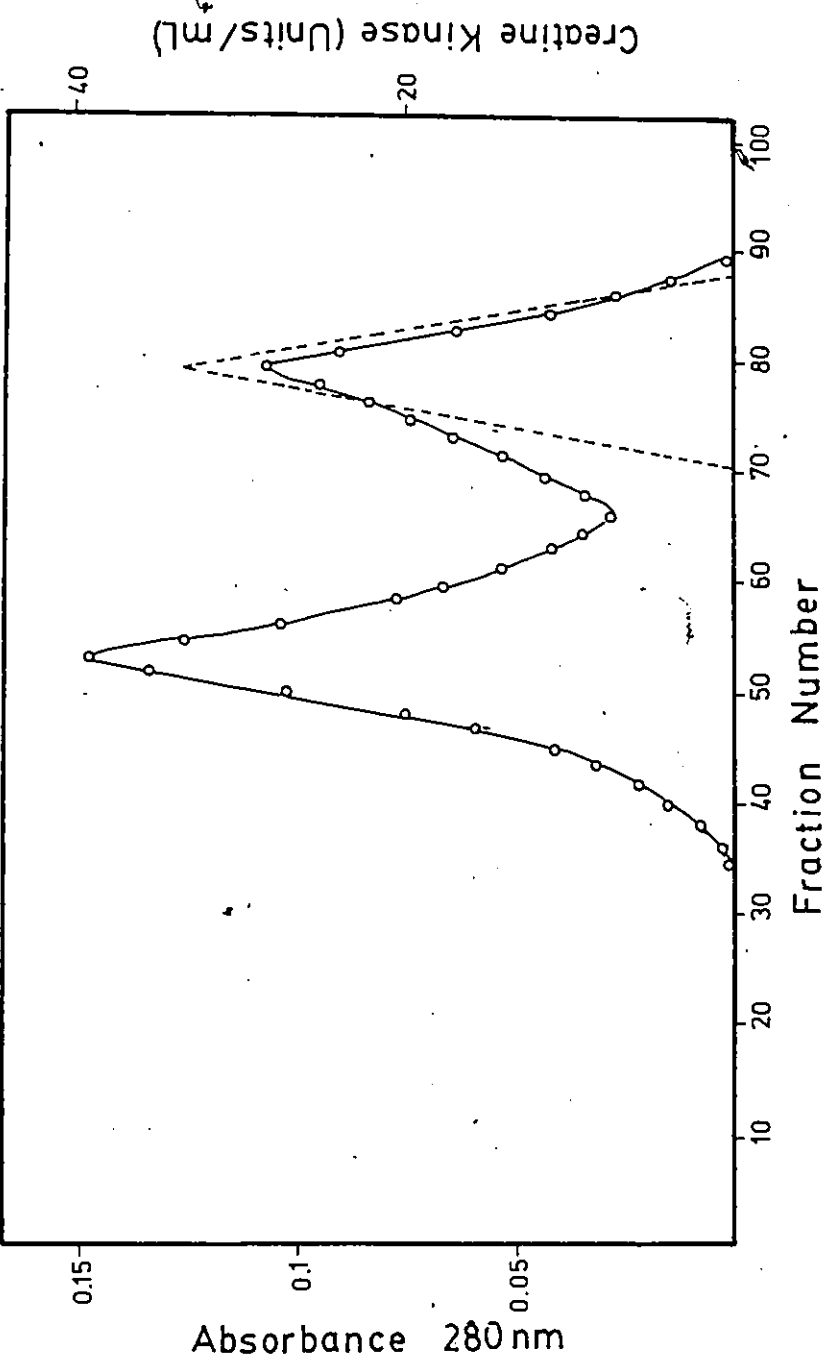


TABLE III
 PURIFICATION OF CREATINE KINASE-BB ISOENZYME
 FROM HUMAN BRAIN

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purity (fold)
Supernatant	1596	5426	3.4	-	-
50% ethanol	252	4347	17.3	80.1	5.1
70% ethanol	75.9	3952	52.1	72.8	15.3
1st Sephadex DEAE-A-50	30.3	3068	101.3	56.5	29.8
2nd Sephadex DEAE-A-50	4.2	1197	285.0	22.1	83.8

Note: The CK-BB isoenzyme was purified by the procedure of Ritter *et al.* (Ritter, C.S., Munim, S. R., and Roberts, R. (1981) Clin. Chem. 27, 1878-1887).

FIGURE 5

FIRST DEAE-SEPHADEX A-50 CHROMATOGRAPHY
OF THE PROTEIN SAMPLE OBTAINED AFTER
70% ETHANOL FRACTIONATIONLegend

Column size 40 x 2.6 cm. The column was equilibrated with a 0.05 M Tris-HCl buffer, pH 8.0, containing 1 mmol/L DTT. After application of the protein sample, the isoenzyme was eluted with a salt gradient (indicated by the arrow) containing 100 to 450 mmol/L of NaCl. The fractions (5 mL) containing creatine kinase activity were pooled and concentrated.

Creatine kinase activity was determined in the fractions eluted from the column as described in METHODS, CHAPTER II, C. 7, p. 39. The CK-BB isoenzyme was identified by electrophoresis on cellulose acetate. A CK control (murine source, from Helena Laboratories), containing all 3 isoenzymes, was run simultaneously on each cellulose acetate strip.

(---) = creatine kinase activity

(—○—) = A_{280}

*See APPENDIX D.

FIGURE 5

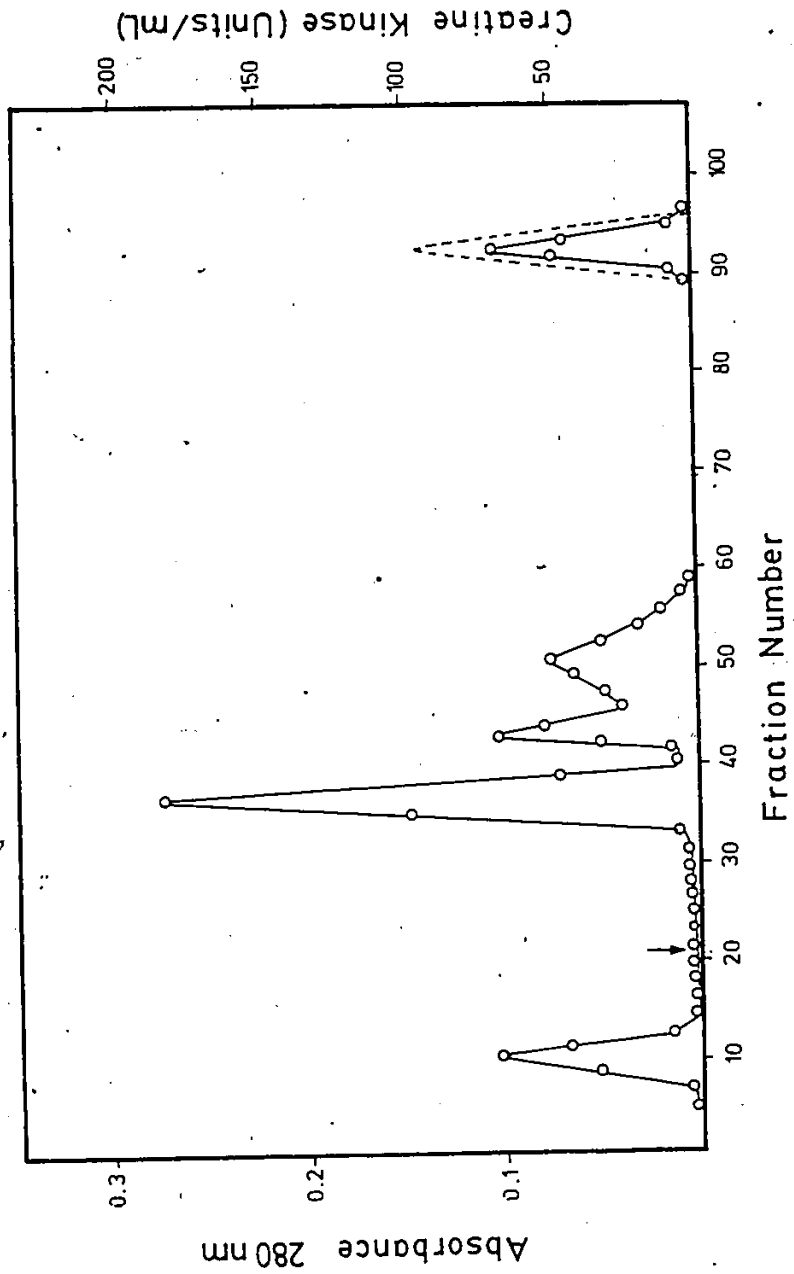


FIGURE 6

SECOND DEAE-SEPHADEX A-50 CHROMATOGRAPHY
OF THE PROTEIN SAMPLE CONTAINING
CREATINE KINASE-BBLegend

Column size: 40 x 2.6 cm. The column was equilibrated with a 0.05 M Tris-HCl buffer, pH 8.5, containing 2 mmol/L DTT. The isoenzyme was eluted with a salt gradient (indicated by the arrow) containing a 140-300 mmol/L of NaCl. Fractions (2 mL) containing creatine kinase activity were pooled and concentrated.

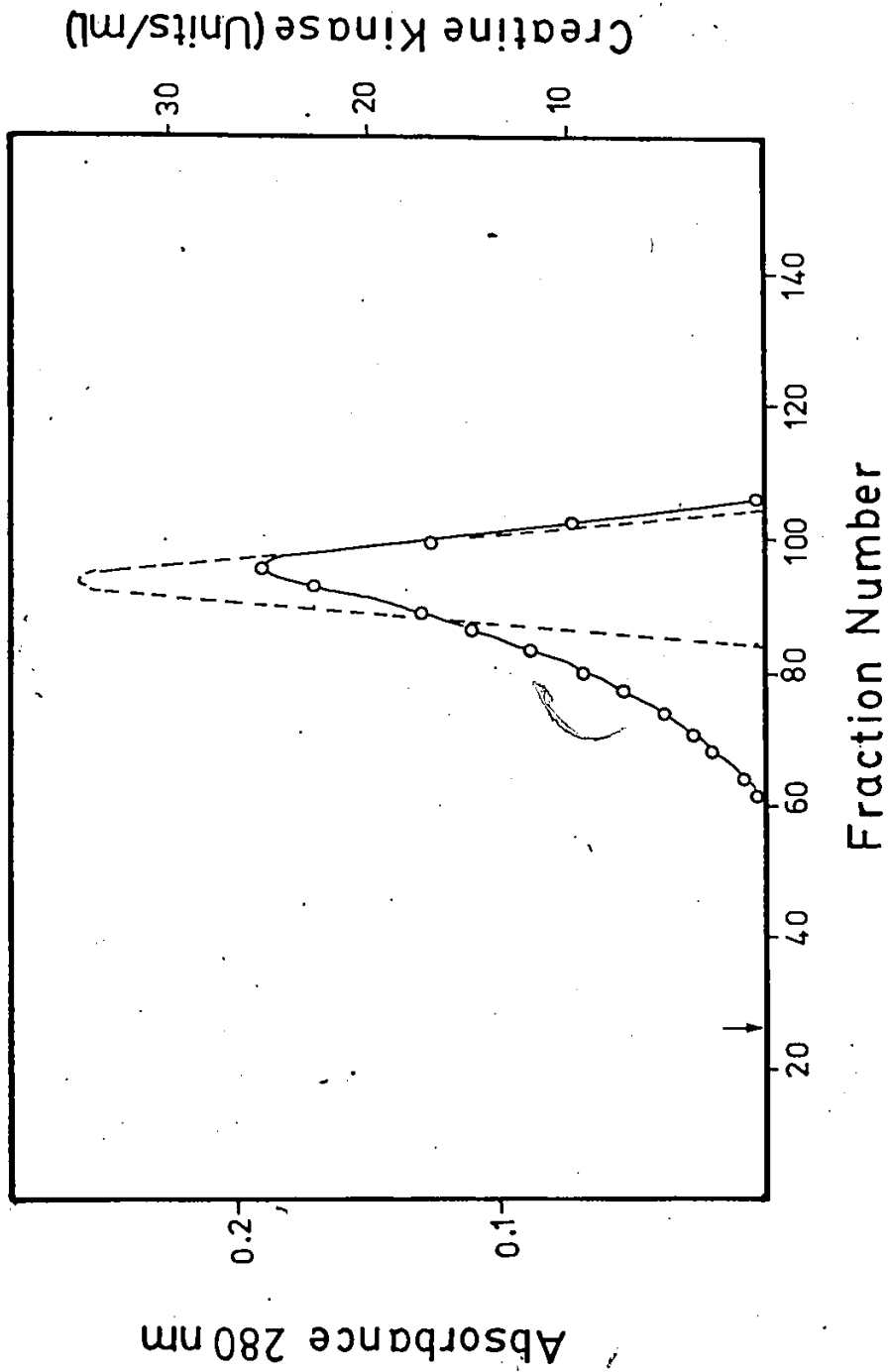
Creatine kinase activity was determined in the fractions eluted from the column as described in METHODS, CHAPTER II, C. 7, p. 39. The CK-BB isoenzyme was identified by electrophoresis on cellulose acetate. A CK control (murine source, from Helena Laboratories), containing all 3 isoenzymes, was run simultaneously on each cellulose acetate strip.*

(----) = creatine kinase activity

(—○—) = A₂₈₀

*See APPENDIX D.

FIGURE 6



B. . . HOMOGENEITY OF THE PURIFIED CREATINE KINASE ISOENZYMES

The final preparations of the CK-MM, -MB and -BB isoenzymes exhibited single isoenzyme bands after electrophoresis on cellulose acetate. A CK control, containing all 3 isoenzymes, was run together with each preparation in order to identify the isoenzyme isolated.

Disc-gel electrophoresis on 7.7% polyacrylamide gels of the samples, obtained during the final steps of the purification scheme, confirmed a homogeneous protein preparation in each case, producing only one band on the electrophoretic gels (Figs. 7 and 8).

C. . . DETERMINATION OF MICHAELIS CONSTANTS

The affinity of the individual creatine kinase isoenzymes for creatine phosphate was characterized by their apparent Michaelis constants. Initially, the K_m was determined graphically from the double-reciprocal plots of Lineweaver and Burk (164) by means of regression analysis (Figs. 9, 10, and 11). Cleland (165) suggested that an ideal spread of points might be from $0.2 K_m$ to $5 K_m$. Therefore, only the creatine phosphate concentration varied between 0.5 and 8.0 mmol/L, whereas all the other conditions remained constant. The concentrations were chosen to give evenly spaced reciprocals and the plots were based on seven or eight different pairs of data, each being determined in triplicate. Eight velocity measurements are usually necessary; using more, however, will

FIGURE 7

DISC-GEL ELECTROPHORESIS ON POLYACRYLAMIDE OF THE
PREPARATIONS OF CREATINE KINASE-MMLegend

- A : Crude muscle extract
- B : 50-80% $(\text{NH}_4)_2\text{SO}_4$ fractionation
- C : Active protein fraction obtained after Sephadex G-100 chromatography.
- D : Active protein fraction obtained after the 1st DEAE-A50 chromatography.
- E : Active protein fraction obtained after the 2nd DEAE-A-50 chromatography.

FIGURE 7

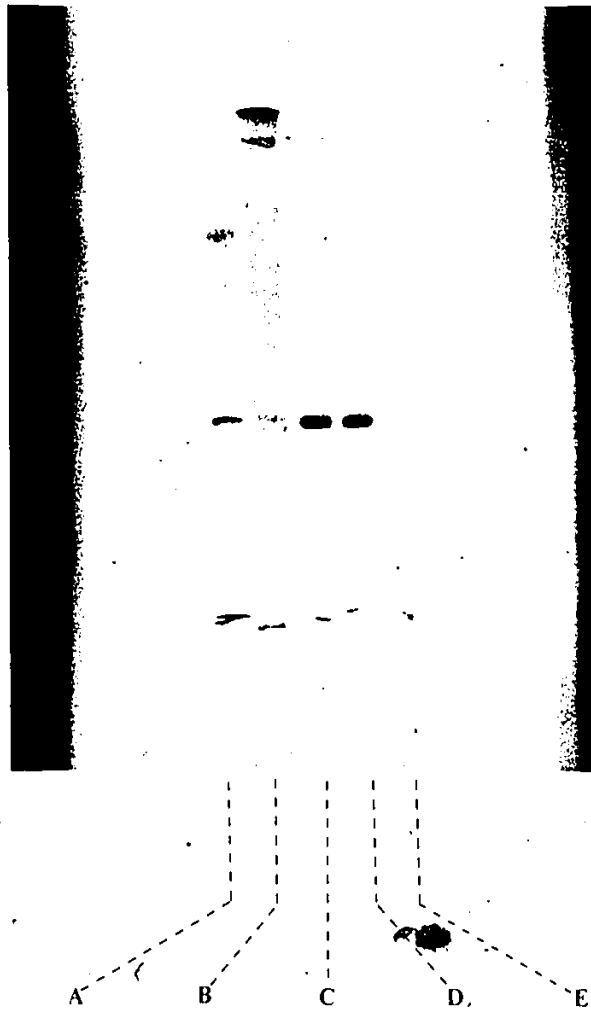
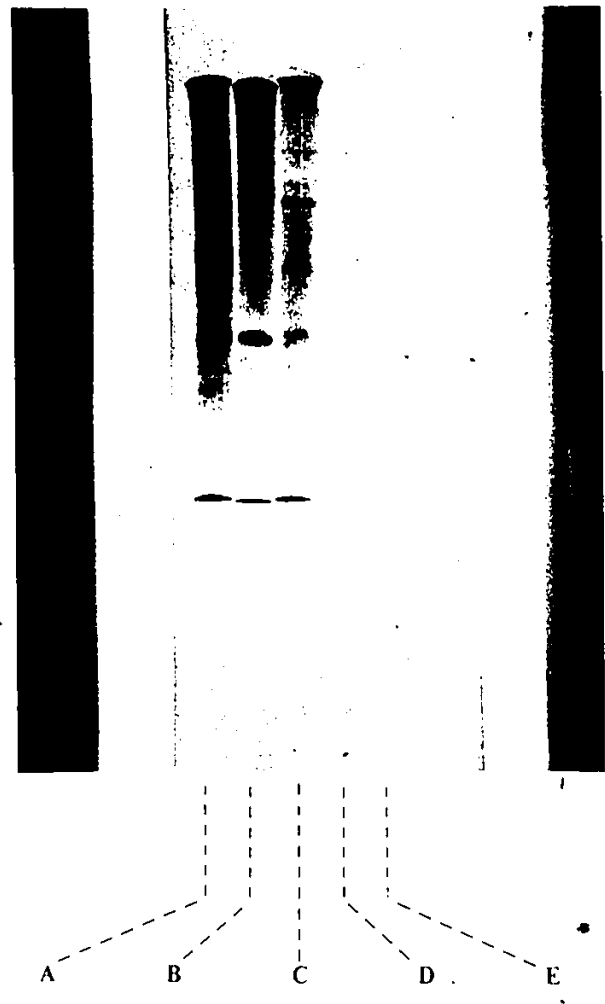


FIGURE 8

DISC-GEL ELECTROPHORESIS ON POLYACRYLAMIDE OF THE
PREPARATIONS CONTAINING CREATINE KINASE-BBLegend

- A : Crude extract of brain tissues
- B : Protein fraction obtained after 50% ethanol precipitation
- C : Protein fraction obtained after 70% ethanol precipitation
- D : Active protein fraction obtained after 1st DEAE A-50 chromatography
- E : Active protein fraction obtained after 2nd DEAE A-50 chromatography

FIGURE 8



often offset the perturbation produced by a single outlier (166).

Although a knowledge of K_m and V_{max} is often useful in comparing isoenzymes from different tissues or organisms, there is still a lack of conformity in the methods used for calculating the valuable parameters, and no lack of discussion about which of the methods is best (166). Of the three linear transformations of the Michaelis-Menten equation that can be used for estimating K_m and V_{max} , the Lineweaver-Burk double-reciprocal plot is the most commonly used. It gives estimates of K_m and V_{max} based on unweighted fits to the Michaelis-Menten equation, and has been described as the worst one to use (167).

In order to verify the K_m values, especially the differences among the individual isoenzyme, obtained from the Lineweaver-Burk plots, another procedure for the determination of these parameters was sought. Wilkinson (168) has described a method using least-squares-fitting techniques, which gives the best estimate of both K_m and V_{max} , and the standard errors of these values when the correct weighting factors are applied. A computer programme of Wilkinson's method was written in FORTRAN IV to calculate the required parameters (see APPENDIX B). The statistical calculations used to obtain an estimate of the K_m of the individual creatine kinase isoenzymes are shown in APPENDIX C. According to Cleland (165), reasonably precise estimates of K_m and V_{max} would have standard errors of less

than 10% of the mean values.

Figures 9 and 10 show the Lineweaver-Burk plots for human CK-MB and CK-BB isoenzyme preparations diluted with Tris-HCl buffer and dissolved in inactivated sera. A similar plot was obtained for endogenous CK-MM isoenzyme in serum and for CK-MM diluted with Tris-HCl buffer (Fig. 11). The correlation coefficient, r , was 0.997 or greater for all the plots.

The Lineweaver-Burk plots exhibit a linear relationship -- an indication that the Michaelis-Menten relationship is obeyed. Porter and Traeger (169) have suggested that, once the Michaelis-Menten model was satisfied, more precise estimates of K_m and V_{max} could be obtained by collecting data at only two substrate concentrations: one well above the K_m and the other well below the K_m . At least four velocity determinations would be required at each point. However, in this study, perfectly adequate K_m and V_{max} values were provided by a spread of different substrate concentrations, and it would have seemed somewhat redundant to repeat the experiment at only two substrate concentrations.

The results are summarized in Table IV. The K_m values obtained by the two methods are in good agreement. The CK-BB isoenzyme preparations in buffer and in inactivated sera showed the highest affinity to creatine phosphate, i.e., they had the lowest K_m value. The CK-MM isoenzyme preparation in Tris-HCl buffer had a 2.5-fold higher K_m value than the endogenous

FIGURE 9

LINEWEAVER-BURK PLOTS OF THE KINETIC BEHAVIOUR OF
PURIFIED CK-MB ISOENZYME DILUTED IN TRIS-HCl
BUFFER AND IN INACTIVATED HUMAN SERA FOR
VARIOUS CONCENTRATIONS OF CREATINE
PHOSPHATE

Legend

The concentration of creatine phosphate ranged between 0.5 to 8.0 mmol/L. The reaction medium was as described in METHODS, CHAPTER II, C. 7, p. 39. The plots were based on eight different pairs of data, each being the mean of triplicate assays, and were obtained by means of regression analysis.

(▲) = purified MB preparation

(Δ) = pure MB diluted in inactivated sera

FIGURE 9

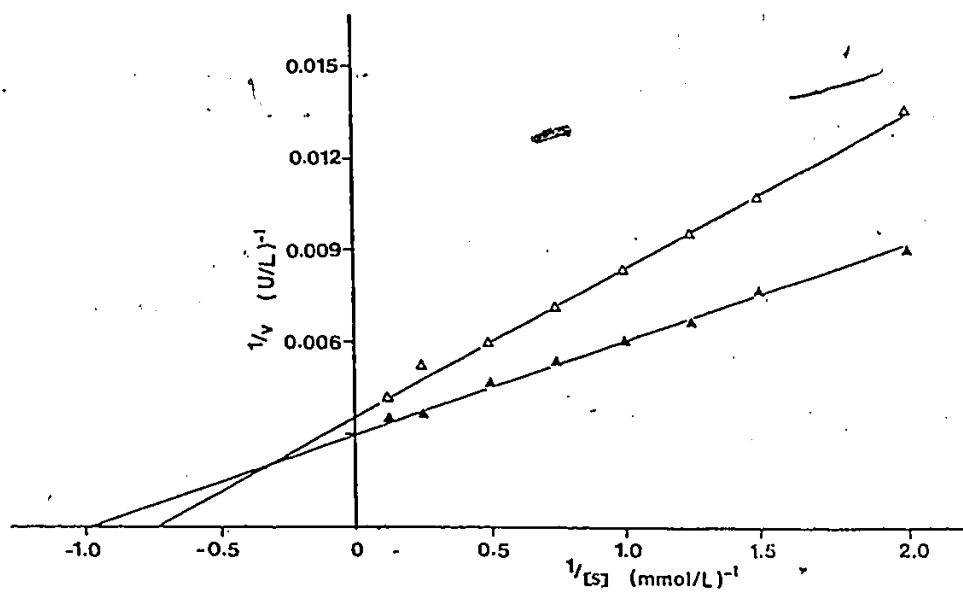


FIGURE 10

LINWEAVER-BURK PLOTS OF THE KINETIC BEHAVIOUR OF
PURIFIED CK-BB ISOENZYME DILUTED IN TRIS-HCl
BUFFER AND IN INACTIVATED HUMAN SERA FOR
VARIOUS CONCENTRATIONS OF CREATINE
PHOSPHATE

Legend

The range of creatine phosphate concentration used were between about 0.5 and 8.0 mmol/L. The reaction medium was as described in METHODS, CHAPTER II, C. 7, p. 39. The plots were based on eight different pairs of data, each being the mean of triplicate assays, and were obtained by means of regression analysis.

(Δ) = purified BB preparation

(\blacktriangle) = pure BB diluted in inactivated sera

FIGURE 10

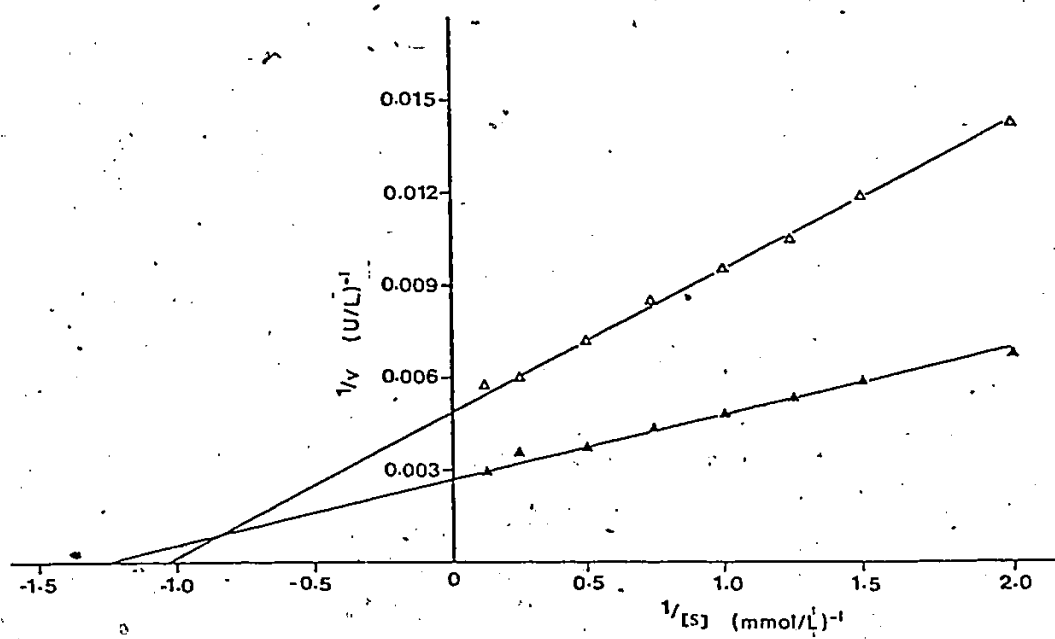


FIGURE 11

LINEWEAVER-BURK PLOTS OF THE KINETIC BEHAVIOUR OF
PURIFIED CK-MM ISOENZYME AND OF THE CK-MM
ISOENZYME ENDOGENOUS IN HUMAN SERUM FOR
VARIOUS CONCENTRATIONS OF CREATINE
PHOSPHATE

Legend

The range of creatine phosphate concentration used were between 0.5 and 8.0 mmol/L. The reaction medium was as described in METHODS, CHAPTER II, C. 7, p. 39. The plots were based on eight different pairs of data, each being the mean of triplicate assays, and were obtained by means of regression analysis.

(Δ) = purified MM preparation

(\blacktriangle) = endogenous MM in serum

FIGURE 11

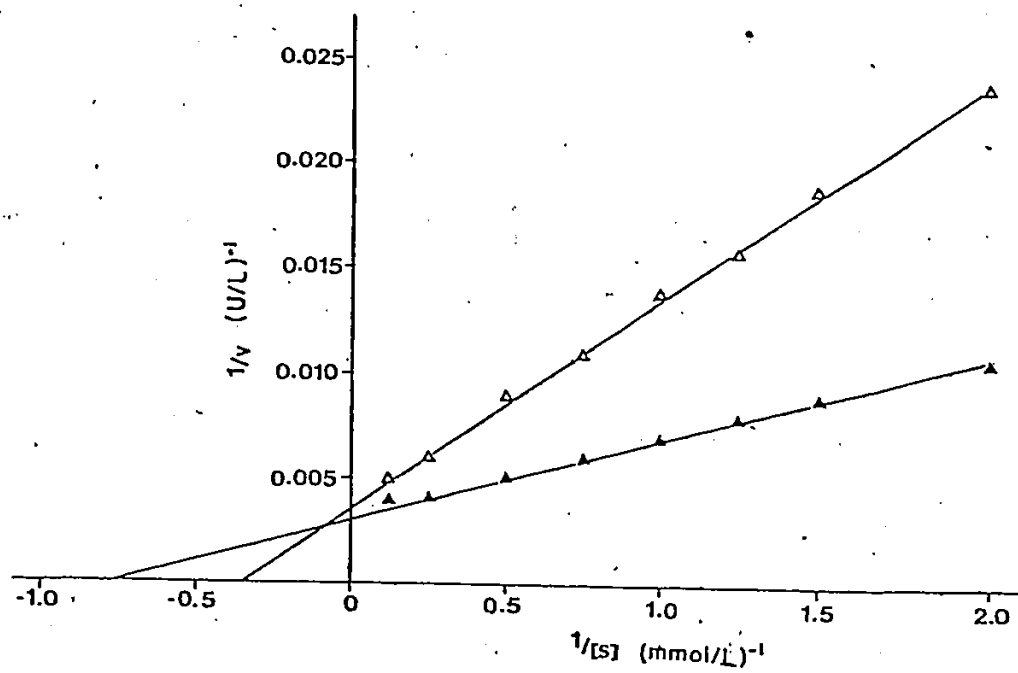


TABLE IV

MICHAELIS CONSTANTS OF THE PURIFIED PREPARATIONS OF HUMAN CREATINE KINASE ISOENZYMES FOR CREATINE PHOSPHATE

Method	CK-isoenzymes: K_m (mmol/L) ^c		
	BB	MB	MM
Lineweaver-Burk ^a	0.98±0.06 (3)	1.12±0.06 (3)	3.08±0.07 (3)
Wilkinson ^b	0.98±0.04 (3)	1.05±0.03 (3)	3.12±0.05 (3)
Lineweaver-Burk ^a	0.75±0.04 (6)	1.40±0.05 (5)	1.46±0.07 (6)
Wilkinson ^b	0.76±0.01 (6)	1.39±0.02 (5)	1.45±0.02 (6)

Note: The first set of experiments were with isoenzyme preparations in Tris-HCl buffer. The second set of experiments were with isoenzyme preparations diluted in inactivated sera.

^aMean ± S.D.

^bValues are the means ± S.D. See APPENDIX C.

^cNumbers in parenthesis indicate the number of individual determinations.

serum CK-MM isoenzyme. The CK-MB isoenzyme preparation in inactivated sera exhibited a K_m value close to that obtained for the endogenous serum CK-MM isoenzyme. However, one unexpected finding was that the pure CK-MB preparation in Tris-HCl buffer had a lower K_m value compared to the preparation in inactivated sera.

D. DETERMINATION OF THE K_m FOR CK-B SUBUNIT

Using the reagents of Wicks et al. (145), the M-subunit of a preparation of CK-MB isoenzyme in inactivated sera was inhibited and the K_m of the B-subunit determined as described previously. The Lineweaver-Burk plot is shown in Fig. 12. The K_m was found to be similar to the value obtained for the CK-BB isoenzyme dissolved in inactivated sera. The results indicate that the B-subunit of CK-MB and that of CK-BB are kinetically identical (Table V). This provides evidence that there is no "steric hindrance" or "allosteric effect" on the CK-B subunit after inhibition of the CK-M subunit of CK-MB. Wicks et al. (145) also gave evidence that there was no cross-reactivity between the anti-CK-MM and the B-subunit of creatine kinase or any component of human serum.

E. DIFFERENCES IN ACTIVITY BETWEEN THE M AND B SUBUNITS OF CK-MB

The difference in activity between the M- and the B-subunits of the CK-MB isoenzyme was investigated using goat anti-CK-MM antibody. After measuring the activity of the CK-MB

FIGURE 12

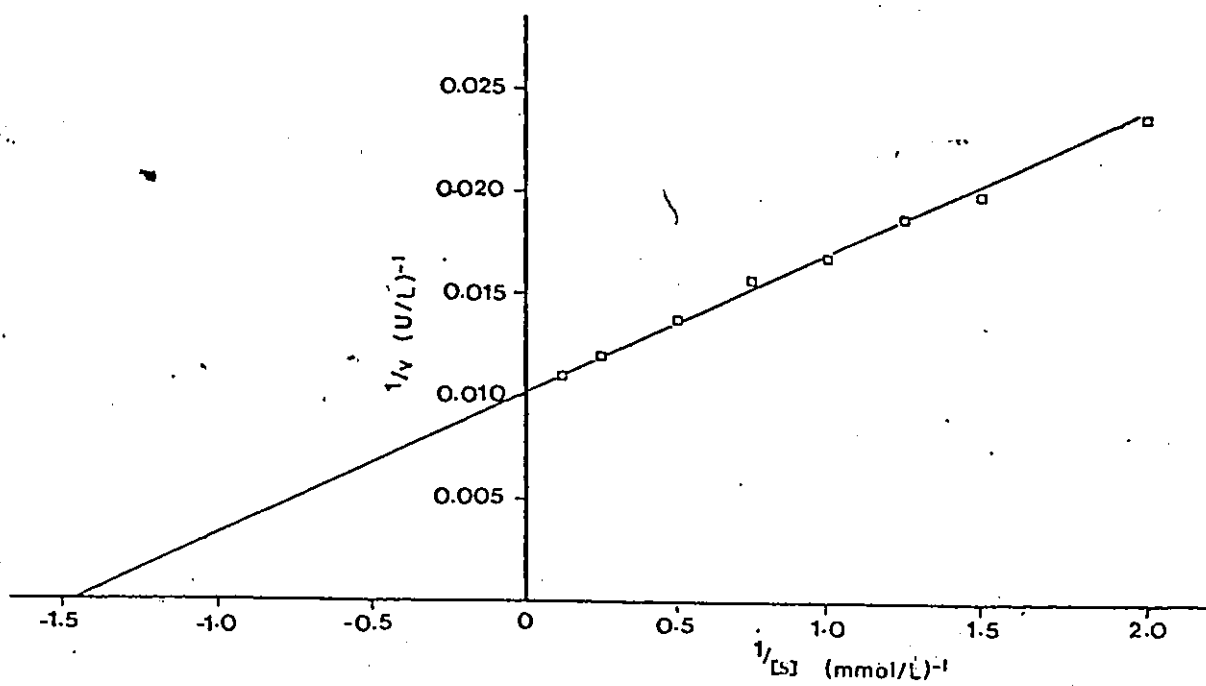
LINEWEAVER-BURK PLOT OF THE KINETIC BEHAVIOUR
OF THE CK-B SUBUNIT AFTER IMMUNOINHIBITION
OF THE CK-M SUBUNIT OF CK-MB FOR VARIOUS
CONCENTRATIONS OF CREATINE PHOSPHATE

Legend

Samples were diluted in inactivated human sera. The range of creatine phosphate concentration varied between 0.5 to 8.0 mmol/L. The reaction medium was as described in METHODS, CHAPTER II, C. 7, p. 39. The plots were based on eight different pairs of data, each being the mean of triplicate assays, and were obtained by means of regression analysis.

(□) = B-subunit of CK-MB

FIGURE 12



isoenzyme, the M-subunit was inactivated with the anti-CK-MM antibody. The residual activity due to the CK-B subunit was measured. The activity of the CK-M subunit was obtained by difference. A ratio of CK-M subunit activity to CK-subunit activity was calculated and found to be 1.52 ± 0.094 (Table VI).

The results showed that the activity of the M-subunit was approximately one and a half times greater than that of the B-subunit under the particular conditions specified. This was probably due to the fact that the B-subunit was inhibited by the high creatine phosphate concentration used (30 mM) in the assay. This seems to suggest that the common assumption that the M-subunit of creatine kinase has the same activity as that of the B-subunit - hence multiplication by 2 of the B-subunit activity obtained in immunoinhibition experiments (144) - may not be analytically correct. In this particular study, the activity of the MB isoenzyme would be calculated as $(1.52 \times \text{B-subunit activity}) + \text{B-subunit activity}$ instead of $2 \times \text{B-subunit activity}$. However, it should be noted that the calculated ratio was obtained using the Roche kit and the coupled assay of the Scandinavian Committee on Enzymes. Further investigation using different immunoinhibition systems and coupled assays for determining the residual activity of the B-subunit may reveal a different ratio.

The spectrophotometric assay of B-subunit activity after immunoinhibition of the M-subunit activity is a rapid and convenient method of assaying the CK-MB isoenzyme. However,

TABLE V

MICHAELIS CONSTANTS OF HUMAN CREATINE KINASE-BB
ISOENZYME AND CREATINE KINASE -B SUBUNIT

	Isoenzyme preparations: K_m (mmol/L) ^a	
	BB	B-subunit
Lineweaver-Burk ^b	0.75 ± 0.04	0.70 ± 0.05
Wilkinson ^c	0.76 ± 0.01	0.72 ± 0.02

^aBoth preparations were diluted with inactivated sera before the assay. The values are from the data of 6 experiments.

^bMean ± S.D.

^cValues are the means ± S.D. (See APPENDIX C).

TABLE VI

M-SUBUNIT: B-SUBUNIT RATIO OBTAINED IN THE
IMMUNOINHIBITION STUDIES ON PURIFIED HUMAN
CREATINE KINASE-MB ISOENZYME

	Estimated M:B Ratio		
	Mean	S.D.	C.V. (%)
Samples (N=72)	1.52	0.094	0.062

Note: The activities of the CK-MB isoenzyme used varied between 100 to 700 U/L.

the limited sensitivity of the spectrophotometric assay of the CK-B necessitates that the CK-B be considerably above the normal range of expected activity (143).

An optimized bioluminescence assay of the CK-B-subunit has recently been described (143). It claims more sensitivity than the spectrophotometric assay. Bioluminescence assays, however, have not found wide acceptance into many clinical laboratories and are still mainly utilized as a research tool. Optimization of the spectrophotometric assay for the CK-B subunit may prove to be more profitable.

The Scandinavian assay reagent used in this study is optimized for the determination of CK-MM endogenous in serum (157). Preliminary studies carried out in this laboratory indicates that lowering the creatine phosphate concentration decreases the M:B ratio - thus indicating that inhibition of the CK-B subunit is decreased. Optimization of the other components of the assay system may increase the sensitivity of the assay, thus allowing early detection of the CK-MB isoenzyme in serum.

CHAPTER IV

SUMMARY AND CONCLUSIONS

The creatine kinase -MM, -MB and -BB isoenzymes were purified to homogeneity from human skeletal muscle, heart and brain, respectively. After isolation and purification, the kinetic properties of the individual isoenzyme were studied.

The affinity of human skeletal muscle, heart and brain creatine kinase for creatine phosphate as substrate was re-examined. The determination of the K_m values for the different creatine kinases has shown them to be distinct molecules having different affinities for creatine phosphate as substrate. The results showed small but reproducible differences in K_m values for creatine kinases from the different tissues.

Work should continue to show if examination of isoenzyme patterns in crude tissue homogenates may be achieved without application of solubilization and separation techniques, which would possibly modify essential characteristics of the isoenzyme of interest.

Using the immunoinhibition assay of Wicks et al. (145) (145) and the optimized creatine kinase coupled assay

recommended by the Scandinavian Committee on Enzymes, the CK-M subunit of the CK-MB isoenzyme was shown to have approximately one and a half times more activity than the CK-B subunit. The CK-B subunit is known to be inhibited under such reaction conditions.

Optimization of the assay system for the measurement of CK-B subunit activity may increase its sensitivity and thus prove rewarding and useful, especially in detecting the early presence of CK-MB in serum.

APPENDIX A

OPERATING PROCEDURE FOR THE KINETIC MODE ON THE
EMIT CP-5000 MICROPROCESSOR

In the Kinetic Mode, the CP-5000 has three methods of monitoring reactions in the spectrophotometer (Gilford STASAR III):

1. By pressing the KIN button, the operator chooses to take a preset number of readings and to observe $\Delta A/\text{min}$ (that is, the change in absorbance per minute) multiplied by a specified constant.
2. By pressing the ΔA button, the operator chooses to take a preset number of readings and to observe ΔA multiplied by a specified constant.
3. By pressing the ABS button, the operator chooses to take a preset number of readings and to observe the absorbance multiplied by a specified constant.

To operate in the Kinetic Mode, Option 1 was used. For example, to monitor $\Delta A/\text{min}$, press KIN.

```
*****  
KIN MODE  
*****  
DELAY TIME? 15  
READ INT? 10
```

In the above example, the instrument was set to have a delay time of 15 seconds and to monitor the reaction every 10 seconds. In this study, the following specifications were entered into the minicomputer:

```
*****  
KIN MODE  
*****  
DELAY TIME? 60  
READ INT? 15  
# OF RDGS? 0  
ENTER K= 1
```

If 0 is entered for # OF RDGS?, the CP-5000 will take readings continuously until the cell is purged or CANCEL is pressed. The program will ask the operator to enter a constant (K). Each subsequent reading taken by the spectrophotometer will then be multiplied by the constant, and the result printed. If the constant = 1, then the result will equal one times the spectrophotometer reading.

A sample printout:

```
*****  
RUN #      1  
ID #      12  
A0 =    103  
  
ΔA/MIN =  4459.83  
ΔA/MIN =  4459.84  
ΔA/MIN =  4459.83  
ΔA/MIN =  4384.47  
ΔA/MIN =  4329.11  
*****
```

The CP-5000 takes readings in milliabsorbance units. To convert to absorbance units, divide by 1000.

APPENDIX B

COMPUTER PROGRAM FOR ESTIMATING K_m
AND ITS STANDARD ERROR

```

//GEORGE JOB (R121.....), 'G.L.CHONG', CLASS=W
// EXEC WATFIV
//BO.SYSIN DD *
$JOB WATFIV R121..... CHONG
C
C   MICHAELIS-MENTEN PROGRAM
C
C   THIS PROGRAM FITS EXPERIMENTAL DATA TO THE
C   MICHAELIS-MENTEN EQUATION ACCORDING TO THE
C   LEAST-SQUARE METHOD OF WILKINSON (BIOCHEM. J.,
C   1961, 80, 324-332)
C
C   THE METHOD MAKES INITIAL ESTIMATES OF  $K_M$  AND
C    $V_{MAX}$  BY ONE OF THE LINEAR TRANSFORMATIONS.
C   THESE ESTIMATES ARE THEN REFINED BY AN ITERATIVE
C   TECHNIQUE UNTIL THEY CONVERGE ON THE BEST FIT
C   VALUES BY THE LEAST-SQUARES CRITERION.
C
C   PROVISIONAL ESTIMATES OF  $K_M$  AND  $V$ 
C
REAL KM
DIMENSION S(50),V(50),X(50),Y(50),Z(50),F(50),FD(50)
SALPHA=0
SBETA=0
SGAMMA=0
SDelta=0
SEPSI=0
SUMA=0
SUMB=0
SUMC=0
SUMD=0
SUME=0
SUMVZ=0
READ, N
PRINT, 'N=',N
DO 10 I=1,N
READ ,S(I),V(I)
X(I)=V(I)**2
Y(I)=X(I)/S(I)
SALPHA=V(I)*X(I) + SALPHA
SBETA=X(I)**2 + SBETA
SGAMMA=V(I)*Y(I) + SGAMMA
SDelta=X(I)*Y(I) + SDelta
SEPSI=Y(I)**2 + SEPSI
10 CONTINUE
PRINT, 'S', 'V', 'X=VE2', 'Y=VE2/S'
DO 20 I=1,N
20

```

APPENDIX B - Cont'd)

```

PRINT, S(I),V(I),X(I),Y(I)
20 CONTINUE
DELTA=(SALPHA*SEPSI)-(SGAMMA*SDELTA)
  KM=((SBETA*SGAMMA)-(SALPHA*SDELTA))/DELTA
  VMAX=((SBETA*SEPSI)-(SDELTA**2))/DELTA
PRINT, 'DELTA=',DELTA,'KM=',KM,'VMAX=',VMAX
C
C REFINEMENT OF KM AND VMAX
C
DO 50 J=1,N
  Z(J)=S(J) + KM
  F(J)=VMAX*S(J)/Z(J)
  FD(J)=(-1*VMAX*S(J))/(Z(J)**2)
  SUMA=SUMA + F(J)**2
  SUMB=SUMB + FD(J)**2
  SUMC=SUMC + F(J)*FD(J)
  SUMD=SUMD + V(J)*F(J)
  SUME=SUME + V(J)*FD(J)
  SUMV2=SUMV2 + V(J)**2
50 CONTINUE
PRINT, 'S', 'V', 'S+KM', 'F=VS/(S+KM)', 'FD=-VS/(S+KM)E2'
DO 40 J=1,N
PRINT, S(J),V(J),Z(J),F(J),FD(J)
60 CONTINUE
DELTA2=(SUMA*SUMB)-(SUMC**2)
  B1=((SUMB*SUMD)-(SUMC*SUME))/DELTA2
  B2=((SUMA*SUME)-(SUMC*SUMD))/DELTA2
  VM=B1*VMAX
  ADDKM=KM + (B2/B1)
PRINT, 'V=',VM,'KM2=',ADDKM
C
C ESTIMATION OF STANDARD ERRORS
C
SSQUAR=((SUMV2-(B1*SUMD)-(B2*SUME))/(N-2)
  RS=SQRT(SSQUAR)
  SEKM=(RS/B1)*SQRT(SUMA/DELTA2)
  SEV=VMAX*RS*SQRT(SUMB/DELTA2)
PRINT, 'S.E.(KM)=' ,SEKM,'S.E.(V)=' ,SEV
STOP
END
$ENTRY
C
C DATA ARE ENTERED HERE
C
$IBSYS
$STOP
//

```

APPENDIX C

AVERAGING PARAMETER ESTIMATES AND
THEIR STANDARD ERRORS

If a measurement x has been made many times so that its standard error σ has been precisely calculated, then the correct weight, w , for that point in fitting to an equation is

$$w = 1/\sigma^2$$

If a kinetic analysis is repeated several times, to test the effect of day-to-day variation, for example, several estimates of K_m or V_{max} are obtained, each with its calculated standard error. Since the standard errors are likely to be different, the weighted mean must be calculated (170, 171), and is obtained as:

$$\bar{x} = \sum w_i x_i / \sum w_i$$

So, for several K_m values

$$\bar{K}_m = \left(\frac{K_1}{\sigma_1^2} + \frac{K_2}{\sigma_2^2} + \dots + \frac{K_n}{\sigma_n^2} \right) / \left(\frac{1}{\sigma_1^2} + \frac{1}{\sigma_2^2} + \dots + \frac{1}{\sigma_n^2} \right)$$

Provided that the standard error on one day is uncorrelated with that on another, the standard error of the mean K_m may be calculated from

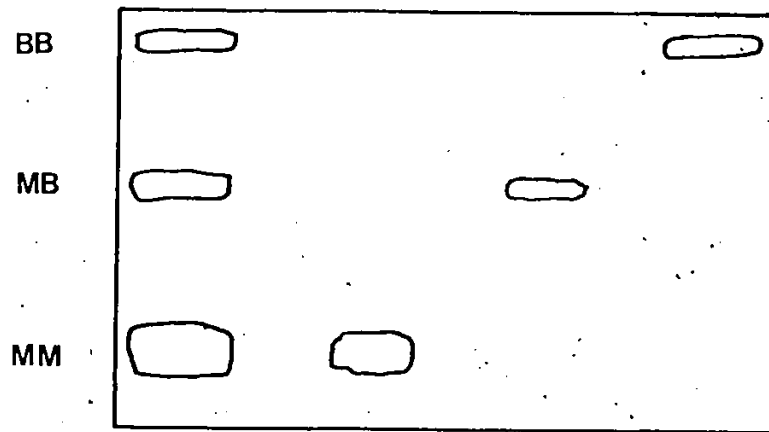
$$\sigma_{\text{mean}} = \sqrt{1/\sum (1/\sigma_i^2)}$$

i.e.,

$$\sigma_{\bar{K}_m} = 1 / \sqrt{(1/\sigma_1^2 + 1/\sigma_2^2 + \dots + 1/\sigma_n^2)}$$

APPENDIX D

MIGRATION PATTERNS OF CK ISOENZYMES AFTER
ELECTROPHORESIS ON CELLULOSE ACETATE



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