CATALYTIC AND KINETIC PROPERTIES
OF MITOCHONDRIAL CREATINE KINASE
FROM BOVINE HEART

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In Partial Fulfillment of the

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Klaus H. Philipp

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BY

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A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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to Ingrid

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ABSTRACT

Kinetic properties of homogeneous creatine kinase from bovine heart have been investigated. pH activity profiles have been determined at 30°C and yield apparent pH optima of 8.1 for the forward reaction, the phosphorylation of creatine, and of 6.3 for the reverse reaction, the phosphorylation of MgADP. Temperature activity profiles have been established for pH levels which are at or near the respective optima. Apparent optimal temperatures are 34° for the forward and 44° for the reverse reaction. Sodium chloride and inorganic phosphate have been found to inhibit enzyme activity strongly.

Initial velocity studies have been conducted at three different pH values at 30° on both the forward and reverse reactions utilizing a pH-stat assay. The double reciprocal plots are linear and intersecting at all pH levels and are thus indicative of a sequential kinetic mechanism. With increasing pH the affinity of the enzyme for its substrates decreases to some extent. The inhibition constants for adenine nucleotides are the smallest observed for any of the creatine kinase isoenzymes.

To identify the kinetic mechanism more accurately product inhibition experiments have been performed at pH 7.4 and 30°. The results are consistent with an Ordered Bi Bi mechanism in which no dead end complexes are formed by the enzyme with any of its substrates or products. Inhibition patterns obtained with the fixed substrates present at a saturating concentration are in accord with such a mechanism. Experiments involving the use of the competitive inhibitor guanidinoacetate, a structural analog of creatine, lend further support to the Ordered mechanism.

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ABBREVIATIONS

adN, MgadN - adenosine 5'-di- or triphosphate and their Mg^{2+} complexes

AMP, ADP, ATP - adenosine 5'-mono-, di- and triphosphate

BSA - bovine serum albumin

CDP - cytidine 5'-diphosphate

CK - creatine kinase

Cr - creatine

Cr∿P - creatine phosphate

dCDP - 2'-deoxycytidine 5'-diphosphate

DTE - 1,4-dithioerythritol

E - enzyme

EDTA - ethylenediaminetetraacetic acid

GAA - guanidinoacetic acid

IMP - inosine 5'-monophosphate

K_i - inhibition constant

K_m - Michaelis constant

MgADP, MgATP, MgATP-P - Mg^{2+} complexes of adenosine 5'-di-, tri-, and tetraphosphate

MgdATP, MgITP - Mg^{2+} complexes of the 5'-triphosphates of 2'-deoxyadenosine and inosine

NTP - any nucleoside 5'-triphosphate

TDP - thymidine 5'-diphosphate

Tris - tris(hydroxymethyl)aminomethane

UDP - uridine 5'-diphosphate

Unit of enzyme activity - micromole per minute

V_{max} - maximal velocity

V_1, V_2 - maximal velocity of forward and of reverse reaction

I. INTRODUCTION

Creatine kinase (ATP-creatine phosphotransferase E.C.2.7.3.2) belongs to a group of phosphotransferases which transfer in a reversible reaction the terminal phosphoryl group of ATP to some guanidino compound. The resulting N-phosphorylated compound, called phosphagen, serves as storage compound for the high bond energy. Various phosphagens occur in invertebrates, whereas only creatine phosphate is found in vertebrates. Creatine kinase exists in the form of at least five different isoenzymes, which exhibit considerable tissue specificity and are particularly abundant in nervous and muscle tissue.

The reaction catalyzed by creatine kinase forms the basis for intracellular energy transport. ATP generated in the mitochondrial matrix during oxidative phosphorylation is carried across the inner membrane by an adN translocator. Mitochondrial creatine kinase located on the outer surface of the inner membrane utilizes the magnesium complex of ATP to produce creatine phosphate. While the ADP formed is immediately shuttled back to the matrix via the translocase system, the creatine phosphate is able to diffuse through the outer membrane into the cytoplasm and on to the contractile apparatus of the muscle cell. A second particulate isoenzyme of creatine kinase associated with the myofibrils reconverts the energy stored in creatine phosphate to energy stored in ATP which is then utilized in the process of contraction. As many as three soluble forms of the enzyme, denoted MM, BB, and MB, may be found in the cytoplasm depending on the type of cell. Their function is the formation of creatine phosphate by means of the ATP formed in glycolysis. 50% of the total creatine kinase activity in heart cells is due to the cytoplasmic

isoenzymes MM and MB, 30% are accounted for by mitochondrial creatine kinase, and 20% by the myofibrillar enzyme.

The cytoplasmic forms from many sources have been extensively studied. The MM isoenzyme from rabbit muscle, in particular, has received much attention which has resulted in a thorough knowledge of its physical, chemical, catalytic and kinetic properties and also of the details of the reaction catalyzed. The kinetic behavior of the enzyme is consistent with a rapid-equilibrium Random mechanism which includes an abortive ternary complex formed by the enzyme with MgADP and creatine.

Whereas little is known as yet about myofibrillar creatine kinase, several groups of workers have reported studies concerned with mitochondrial creatine kinase. These investigations have involved the use of whole mitochondria as enzyme and their general thrust has been in the direction of the role played by the enzyme in oxidative phosphorylation.

The present study is part of the continuing investigation in this laboratory into the physical, chemical, and kinetic properties of the mitochondrial isoenzyme isolated from bovine heart and purified to homogeneity. Its purpose is to determine the catalytic and kinetic properties of the enzyme in vitro and to assign a kinetic mechanism on the basis of initial velocity measurements.

II. LITERATURE REVIEW

A. The Beginnings

In 1927 Eggleton and Eggleton (1) and Fiske and Subbarow (2) recognized that the supposed inorganic phosphate of muscle was in fact a labile organic compound. Fiske and Subbarow (3) subsequently isolated and characterized this compound and named it phosphocreatine. Further studies were carried out by Meyerhof and Lohmann who categorized it as a high energy compound (4) and linked its resynthesis after muscle contraction to ATP hydrolysis (5). Lundsgaard (6) had previously shown that muscle poisoned with iodoacetate may contract with only a breakdown in phosphagen.

Lohmann (7) proposed that the hydrolysis of creatine phosphate was mediated by the enzyme creatine kinase. He believed that both ADP and AMP could serve as acceptors of the phosphoryl group. This view was shared by Lehmann (8), who established that the reaction catalyzed was reversible and, in addition, found that Mg^{2+} ion was probably required and that the reaction velocity was pH dependent. In contrast to the crude extracts used by these workers, a partially purified enzyme was employed by Banga (9) a few years later. She was able to confirm the pH dependence observed by Lehmann, but she noticed that her enzyme preparation was able to transfer only the terminal phosphoryl group of ATP to creatine. She proposed the existence of another enzyme fraction capable of phosphorylating creatine by means of ADP. After another attempt at purifying the enzyme by Sorenyi and Degtyar (10), it was the work by Askonas in 1951 (11) which constituted the important breakthrough in the technique of purifying the enzyme: she discovered its remarkable resistence to denaturation by solvents which was to become the basis of all subsequent purification schemes.

The early period of research into creatine kinase and the reaction it catalyzes concluded with Narayanaswami (12) describing properties of a crude creatine kinase preparation obtained from cerebral cortical tissue, whereas all previous studies had been conducted on extracts of skeletal muscle.

B. The Middle Period

In 1954 a period of rapid advancement of research began to which two groups of workers were the main contributors: the Wisconsin group around Kuby and the Canberra group initially represented by Ennor and Rosenberg and later by Morrison and coworkers. In concurrent publications these groups showed that Banga's claim of another creatine kinase fraction being responsible for the phosphorylation of AMP was due to contamination by adenylate kinase and that the reaction catalyzed was restricted to the reversible phosphorylation of ADP by phosphocreatine (13,14).

With both groups employing adaptations of Askonas' propanol fractionation technique, it was Kuby et al. who were able to crystallize the enzyme from rabbit muscle (14) and to demonstrate its homogeneity (15); they also described its physical properties. The major emphasis in these investigations lay on the kinetics of the reaction, however. The effects of pH (13,16) and temperature (16) were determined; enzyme stability, inhibition by products (16) and other inhibitors (13) were studied.

The focal point of these kinetic studies, however, was the role played by Mg^{2+} and also by Ca^{2+} and Mn^{2+} in the catalytic mechanism. The workers were fully aware of the absolute requirement of the reaction for one of these metal ions as well as of the inhibitory effect produced by an excess of the ions (13,16,17,18). The major point of contention

arose from the mechanism by which the metal ion exerts its influence on the reaction velocity. Kuby et al. (16) proposed very early that the magnesium complexes of the nucleotides were the true substrates of the enzyme, a view that was shared by Noda et al. (19), but not by Morrison et al. (20) who claimed that Mg^{2+}, creatine phosphate and ADP could bind independently to the enzyme. This means that the ion was seen as forming a bridge between the protein and the nucleotide, which was the role previously assigned to it in the case of arginine kinase (21). The issue was complicated by a lack of reliable values for the stability constants of the metal nucleotides which made it impossible to calculate accurately the concentrations of the various species. (See Kuby and Noltmann (22) for a detailed review of this problem.)

From NMR studies involving the use of the paramagnetic Mn^{2+} ion in place of Mg^{2+} ion, Cohn (23) concluded that the metal ion does not form a bridge between the enzyme and the nucleotide but rather that it is complexed to the nucleotide which is then bound to the enzyme. In 1965 Morrison (24) conceded that the formation of an enzyme -Mg^{2+} complex still had to be considered uncertain and tacitly adopted the Mg^{2+}-nucleotide concept in the next paper (25).

Ennor and Rosenberg (13) found that cysteine will increase the reaction rate while sulfhydryl and oxidizing reagents will inhibit enzyme activity strongly. They concluded from these observations that the enzyme possesses a free SH group or groups which is necessary for enzyme activity. Using arsenicals they characterized creatine kinase as a monothiol enzyme (26). Noltmann et al. (27) carried out an amino acid analysis and found a total of six sulfhydryl groups per mole, two of which were classified as reactive residues on the basis of inhibition titration experiments with iodoacetate (28). O'Sullivan and Morrison (29) observed that trace

contamination by heavy metal ions leads to oxidation of SH groups and concomitant loss of activity. Watts et al. (30) determined that alkylation of two reactive thiols by iodoacetate is accompanied by complete loss of activity and concluded that creatine kinase contains a maximum of two catalytic sites per molecule. Watts (31) linked the inactivation that results from modifying the SH groups to the catalytic mechanism rather than to a conformational change.

Inhibition by competitive inhibitors was first observed by Kuby et al. (16) who used MgADP to inhibit the forward reaction. From the type of inhibition which was competitive relative to MgATP and noncompetitive relative to creatine, it was inferred that separate binding sites exist for the Mg^{2+}-nucleotide and the guanidino substrates. The results of equilibrium binding measurements made by the same workers indicate two nucleotide binding sites per molecule (32). Noda et al. (19) identified $HATP^{3-}$ as a strong competitive inhibitor relative to MgATP and ATP^{4-} as a weak one. They also showed that small inorganic ions such as nitrate, phosphate and chloride inhibit the forward reaction competitively relative to MgATP (19) and the reverse reaction competitively relative to creatine phosphate (33). This suggested to the authors that the anions interact with the enzyme at the same locus at which the γ-phosphate of MgATP and the phosphoryl group of creatine phosphate interact with the enzyme. Millner-White and Watts (34) and Watts (35) attributed the inhibition to the ability of these small anions to form a stable and inactive enzyme-creatine-MgADP-anion complex. The existence of this complex has been substantiated by the application of EPR and NMR techniques by Cohn's group (36,37,38).

The quaternary complex mimics the transition state in the rapid-equilibrium Random kinetic mechanism that has been established for creatine

kinase by Morrison and James (25) on the basis of initial velocity experiments performed with products present and absent. The mechanism was confirmed in isotope exchange studies which are, moreover, in accord with the formation of an enzyme-MgADP-creatine dead end complex but not with the formation of an enzyme-MgATP-creatine phosphate complex (39).

The rapid-equilibrium Random mechanism persists when Ca^{2+} or Mn^{2+} are substituted for Mg^{2+} (40). However, using chromium nucleotides as inhibitors, Schimerlik and Cleland (41) were able to show that at pH 7 the rapid-equilibrium Random mechanism changes into an equilibrium Ordered mechanism in the forward reaction with MgATP adding before creatine.

In 1975 Engelborghs et al. (42) reported the results of quenched-flow studies which are consistent with the rapid-equilibrium Random mechanism, and lately there has been renewed interest in the kinetic mechanism on the part of NMR spectroscopists (38,43,44,45,46). Several of these studies have presented evidence for the relay of conformational changes from one subunit to the other in an enzyme which has been modified with thiol reagents (43,45,46).

The specificity of the enzyme for guanidino substrates is very narrow. Of the naturally occurring substrates of guanidino phosphotransferases only creatine and glycocyamine, or GAA, and their phosphates are substrates of creatine kinase (47,48). Glycocyamine is a poor substrate at that with respect to both V_{max} and K_m values (49) and so is phosphoglycocyamine which produces only a low maximum velocity (48). A few synthetic compounds produce relatively high activities, the most notable being cyclocreatine which is even metabolized in the living organism (50). The activity of this substrate analog shows that creatine is phosphorylated on the guanidino nitrogen atom that is trans to the N-methyl group. The low activity of glycocyamine which lacks the N-methyl group and of

other creatine analogs which have bulkier N-alkyl groups supports the view that the N-methyl group plays an important role in the catalytic mechanism (48).

Specificity for the metal-nucleotide substrate is broad except with regard to the length of the phosphate chain. The forward reaction requires the triphosphate and the reverse reaction the diphosphate (16). AMP and adenosine cannot function as substrates, but do bind to the enzyme (51). The magnesium complexes of the diphosphates of guanosine, inosine, cytidine and uridine have been found to be active though weak substrates as well as MgITP and MgdATP (16,51). Morrison and Uhr (17) have observed that the affinity of the enzyme-creatine phosphate complex for the metal-ADP complex varies with the identity of the metal ion.

C. The Present

The third period of discovery began in 1964 with the observation by Burger et al. (52,53) and also by Deul and Van Breemen (54) of three electrophoretically different bands obtained from homogenates of brain, skeletal and smooth muscle and the recognition of these bands as isoenzymes of creatine kinase. The isoenzyme from brain was observed to move toward the anode, that from skeletal muscle toward the cathode. The third isoenzyme found in smooth muscle and particularly in heart (54,55) possessed intermediate mobility. Eppenberger et al. (55) suggested that the isoenzymes might consist of combinations of two different monomers yielding the dimers MM, BB, and MB. Dawson et al. (56) and later Keutel et al. (57) were able to show this to be correct by producing the MB hybrid from dissociated MM and BB type subunits. While these isoenzymes could be extracted from the cytoplasm, a number of researchers have found creatine kinase activity associated with myofibrils (58,59,60).

Wood (61,62) had isolated and characterized creatine kinase from ox brain and had noted the difference in chemical properties and in electrophoretic mobility relative to the rabbit muscle isoenzyme before the discovery of the isoenzyme relationship. Now, very thorough studies on the enzyme from calf brain showed its physical, chemical and kinetic properties to be similar and yet distinctly different from those of the rabbit muscle enzyme (63-66). In comparative studies on the cytoplasmic isoenzymes from muscle and brain of rabbit and chicken, it was determined that different types of isoenzymes from one species are more dissimilar than the isoenzymes of the same type from different species (67,68). Properties of even the subunit of the rabbit muscle MM isoenzyme have been determined (69), but no comparable studies have yet been made on the BB isoenzyme subunit.

Since the description in 1973 of the myofibrillar isoenzyme by Scholte (59), who also proposed for its physiological function the degradation of creatine phosphate to yield ATP for muscular contraction, only one other study concerned with this isoenzyme has been published to this date (70). The kinetic parameters of crude preparations from myofibrils and mitochondria were compared and found to differ significantly only in the Michaelis constants for guanidino substrates which are larger by a factor of three for the myofibrillar enzyme.

Considerably more effort has been expended on the mitochondrial isoenzyme though almost only in relatively recent years. After its discovery by Jacobs et al. (71), Scholte et al. (72) and Jacobus and Lehninger (73) have localized the enzyme on the outside of the inner membrane, but Lipskaya and Goloveshkina (74) believe it to reside on both sides. Most of the investigations have been concerned with the relationship between the isoenzyme and the process of oxidative phosphorylation (70,73-80).

All workers agree that the forward reaction constitutes the physiological role of the enzyme. As substrate affinities and maximal velocities favor the reverse reaction, Saks and coworkers (70,76,78) have postulated a functional coupling of mitochondrial creatine kinase with the ATP-ADP translocase system.

The mitochondrial enzyme has been partially purified by Farrell et al. (81) and by Hall et al. (82) who also report the existence of two forms of the enzyme which differ in activity and are interconvertible by reduction and by an increase in concentration. A procedure has been developed in this laboratory which yields homogeneous enzyme (83) and physical and chemical studies have been carried out on such enzyme preparations (84-86). Parts of the present study have been submitted for publication and preliminary reports have been presented (87,88).

III. EXPERIMENTAL

A. Materials

Acetic acid (reagent grade) - Fisher

Adenosine 5'-diphosphate (sodium salt, grade III) - Sigma

Adenosine 5'-monophosphoric acid (sodium salt, from yeast, type II) - Sigma

Adenosine 5'-tetraphosphate (tri-monocyclohexylammonium salt, grade III) - Sigma

Adenosine 5'-triphosphate (disodium salt, from equine muscle) - Sigma

Adenylate kinase ($(NH_4)_2SO_4$ solution, from pig muscle, grade IV) - Sigma

β-Alanine - Eastman

β-Amino-n-butyric acid - Sigma

1,2,4-Aminonaphtholsulfonic acid (reagent grade) - J.T. Baker

Ammonium molybdate (reagent grade) - Fisher

Arginine - ICN Nutritional Biochemicals

Bovine serum albumin - Sigma

Creatine (reagent grade) - Fisher

Creatine phosphate (disodium salt) - Sigma

Cupric sulfate pentahydrate (reagent grade) - Matheson Coleman & Bell

Cytidine 5'-diphosphate (trisodium salt, from yeast, type I) - Sigma

Cytidine 5'-triphosphate (sodium salt, from muscle, type I) - Sigma

2'-Deoxycytidine 5'-diphosphate (sodium salt) - Sigma

Diacetyl - Sigma

β,β-Dimethylglutaric acid - Sigma

N,N-Dimethylglycine hydrochloride - Sigma

1,4-Dithioerythritol - Sigma

Ethylenediaminetetraacetic acid (disodium salt) - Sigma

Glycerol (spectroquality) - Fisher

Glycine (reagent grade) - J.T. Baker

Glycolic acid - Matheson Coleman & Bell

Guanidinoacetic acid - Mann

β-Guanidinopropionic acid - Sigma

Hydrochloric acid (reagent grade) - J.T. Baker

p-Hydroxymercuribenzoate (sodium salt) - Sigma

Inosine 5'-monophosphoric acid (disodium salt, from yeast, grade III) - Sigma

Magnesium acetate (reagent grade) - J.T. Baker

Magnesium sulfate (reagent grade) - J.T. Baker

Maleic anhydride - Matheson Coleman & Bell

Mallcosorb (analytical reagent, CO_2 absorbent) - Mallinckrodt

α-Naphthol (grade III) - Sigma

Nucleoside 5'-diphosphate kinase (from baker's yeast, grade III) - Sigma

Orcinol - J.T. Baker

Perchloric acid (60%) - Allied Chemical

Phenol reagent - Ingram & Bell

Polyethylene glycol (mol. wt. 15000-20000) - J.T. Baker

Potassium acetate (reagent grade) - J.T. Baker

Potassium chloride (reagent grade) - Matheson Coleman & Bell

Potassium dihydrogen phosphate (reagent grade) - Fisher

Potassium sodium tartrate (reagent grade) - Matheson Coleman & Bell

Pyruvate kinase (in 2.2M $(NH_4)_2SO_4$, from rabbit muscle, type II) - Sigma

Sarcosine hydrochloride - Sigma

Sodium acetate (reagent grade) - Matheson Coleman & Bell

Sodium bisulfite (reagent grade) - J.T. Baker

Sodium carbonate monohydrate (reagent grade) - BDH

Sodium chloride (reagent grade) - J.T. Baker

Sodium hydroxide (reagent grade) - Fisher

Sodium sulfite (reagent grade) - Fisher

Standard buffer pH 4.00 (25°) - Fisher

pH 6.50 (20°) - Radiometer

pH 7.00 (25°) - Fisher

pH 8.00 (25°) - Fisher

pH 10.00 (25°) - Fisher

Sulfuric acid - Matheson Coleman & Bell

Thymidine 5'-diphosphate (sodium salt) - Sigma

Thymidine 5'-triphosphate (sodium salt) - Sigma

Trichloroacetic acid (reagent grade) - J.T. Baker

Tris(hydroxymethyl)aminomethane (Sigma 7-9) - Sigma

Uridine 5'-diphosphate (sodium salt, from yeast, type I) - Sigma

Uridine 5'-triphosphate (trisodium salt, from yeast, type III) - Sigma

B. Methods

1. Enzyme Preparation

The isolation of creatine kinase from bovine heart mitochondria has been described by Jacobs et al. (83). The enzyme preparations which were used in the colorimetric assays (nos. 44, 38 and 43) were 0.2 M in potassium phosphate buffer which interfered with the colorimetric assay of the forward reaction. The phosphate was removed from preparation 44 by dialysis against several changes of a solution of pH 7.4 which was 0.002 M in DTE and 0.005 M in EDTA. Since this treatment resulted in a high degree of dilution of the dialysate, the other preparations were dialyzed against a buffer which contained as much as 13% polyethylene glycol of molecular weight 15000-20000. The use of this buffer in conjunction with dialysis tubing with a cut-off value of 3500 made it possible to control the volume of the dialysate quite accurately. There was no detectable detrimental effect on the activity of the enzyme.

The dialysate was diluted 1:1 with glycerol and stored at -20° in a vial of a size chosen to leave the smallest possible volume of air in contact with the solution. A slow continuous decrease in activity was observed during storage.

Enzyme preparation 48 which contained 0.2 M sodium chloride and 0.2 M bicarbonate buffer was also subjected to dialysis involving the use of polyethylene glycol, because the high salt concentration was expected to interfere with the enzyme activity and the pH-stat assay.

All these preparations were reactivated prior to use by making an aliquot 0.02 M in DTE, 0.01 M in EDTA and 0.4% in BSA and allowing to stand overnight at 4°. Such treatment raised the activity of the enzyme solution, but there was a tendency for the activity to drop off during the day's work.

The enzyme solution was diluted with 50% (w/v) glycerol immediately before use to a protein concentration of between 0.002 and 0.06 mg/ml depending on the pH and on the reaction which was investigated. From 0.1 to 2 μg of protein were used per colorimetric assay.

The last step employed in the purification of the enzyme preparations which were used in the pH-stat assay (nos. 53, 59, 64, 74/75 and 80/81/82) had involved affinity chromatography utilizing agarose-bound ATP. This procedure yields a highly concentrated enzyme solution which has a relatively low buffer concentration (0.001 M potassium phosphate) and can be stored in 50% (w/v) glycerol solution in the presence of 0.5 M sodium chloride, 0.001 M EDTA and 0.01 M DTE at -20^0 for long periods of time without appreciable loss of activity.

The enzyme was removed from the freezer in 25-μl portions, stored in a closed vial in ice and used undiluted within the hour. This method of handling the enzyme provides a constant level of activity throughout the day and avoids the reactivation procedure with its concomitant fluctuations in activity. Between 0.13 and 0.27 units of enzyme were used per pH-stat assay in the forward reaction and 0.19 and 0.45 units were used in the reverse reaction.

The enzyme was not pre-incubated under reaction conditions. Protein concentrations were determined by the method of Lowry et al. for precipitated proteins (89) using bovine serum albumin as standard.

2. Colorimetric Assays of Enzyme Activity

Two well established discontinuous assays for creatine kinase were chosen, one to measure the formation of creatine phosphate in the forward direction by the method of Fiske and Subbarow (90) as applied by Noda et al. (91), the other to measure the formation of creatine in the reverse

direction by the method of Eggleton et al. (92). Product formation was assayed by means of a Bausch and Lomb Spectronic 20 spectrophotometer equipped with a red-sensitive phototube.

These discontinuous assays offer the advantage that only properties of the enzyme under study are observed. Continuous coupled enzyme assays may well reflect properties of the coupling enzyme or enzymes in addition to those of the enzyme investigated. This is especially true for the present studies which have been conducted under widely varying conditions of pH, temperature, ionic strength and kind of buffer.

The forward reaction, phosphorylation of creatine by MgATP, employed substrate concentrations used by Noda et al. (91). It was studied at 30° in 0.1 M tris-maleic acid-NaOH buffer over the pH range 5.8 to 8.5 and in 0.1 M glycine-NaOH buffer between pH 8.2 and 10.7. The preparation of the buffer solutions was adapted from Gomori (93).

The relatively high buffer concentration of 0.1 M was chosen to provide adequate buffering capacity even at the limits of the useful buffer range. Also, this concentration had been used previously by Noda et al. (91).

With each buffer system covering a wide pH range, considerable differences in the ionic strengths of the individual buffer solutions were observed. To preclude the varying ionic strength from becoming a factor in the enzyme activity, sodium chloride was added to each buffer solution to a final ionic strength of 0.1. Specific conductance measurements were made with the Radiometer conductivity meter CDM2. Data relating specific conductance to moles NaCl/l were taken from the Handbook of Chemistry and Physics (94). Since chloride is known to inhibit the cytoplasmic MM isoenzyme from rabbit muscle (35), the experiment was repeated without added sodium chloride.

There appeared to be no inhibition of the color reaction by either of the two buffers used.

The reverse reaction, phosphorylation of MgADP by creatine phosphate, was studied at 30^0 in 0.1 M Tris-maleic acid-NaOH buffer of constant ionic strength over the pH range 5.5 to 8.3 and in 0.1 M β,β-dimethylglutaric acid-NaOH buffer of both constant and varying ionic strength between pH 5.1 and 7.7. The properties of the dimethylglutarate buffer have been described by Stafford et al. (95), and its preparation was adapted from Hems (96).

The Tris-maleate buffer was found to strongly interfere with the colorimetric assay for creatine as noted by Rosenberg et al. (97), whereas the dimethylglutarate buffer caused no adverse effects. On the other hand, dimethylglutaric acid precipitated out of the highly acidic medium in which inorganic phosphate is determined and was, therefore, unsuitable as buffer for the forward reaction. It also produced a precipitate with p-hydroxymercuribenzoic acid which had been originally added to quench the enzyme reaction but then was found to be unnecessary since the highly alkaline color mixture inactivated the enzyme completely by itself.

Substrate concentrations were 0.005 M creatine phosphate, 0.002 M ADP and 0.004 M $MgSO_4$ (98) in a total volume of 2 ml. Color development by the α-naphthol-diacetyl reaction was allowed to take place in 250-ml beakers to satisfy the oxygen requirement as noted by Ennor and Stocken (99).

The studies on enzyme activity as a function of temperature and of concentration of sodium chloride employed the same substrate concentrations as those used in the pH studies. A 0.05 M glycinate buffer of pH 8.6 was used in the forward reaction and a 0.05 M β,β-dimethylglutarate buffer of pH 6.3 was used in the reverse reaction. Because the optimal pH of the

forward reaction, 8.1, is located near the end of the useful range of the buffer system, a pH of 8.6 was used instead.

3. Potentiometric Assay of Initial Velocity

The pH-stat method has been used for assaying creatine kinase activity by the Kuby group (28, 63, 65, 66) and by Watts (34). In this study it was used for measurements of reaction velocity in all experiments which deal with the effects on initial velocity exerted by substrates, alternate substrates, substrate analogs, products and dead end inhibitors.

a) Instrumentation

The instrumental assembly from Radiometer included a TTT 11b titrator, an ABU 11a autoburette of 0.25 ml capacity, an SBR2c recorder, a PHM 28b pH meter and a TTA3 titration assembly which had been modified in several ways. The standard titration vessel had been replaced by a 20-ml thermostated cup which was connected to a K4R Lauda constant temperature bath accurate to $\pm$ 0.01^{o}C; the original stirrer had been exchanged for a more efficient propeller-type glass stirrer which was used at maximum speed; the end of the titrant delivery tube had been drawn out to a very small diameter in order to prevent mixing of solutions during periods when there was no titrant flow. The opening of the delivery tube was located directly above the blades of the stirrer to bring about the fastest possible mixing of titrant which was necessary to avoid adding an excess of titrant. The titration vessel was covered with a custom-made lid and water-saturated nitrogen was passed into the enclosed air space at such a rate that there was no net change in carbon dioxide tension.

b) Mode of Operation

The apparatus is depicted in schematic form in Figure 1. It functions in the following manner (100). The pH of the reaction mixture is

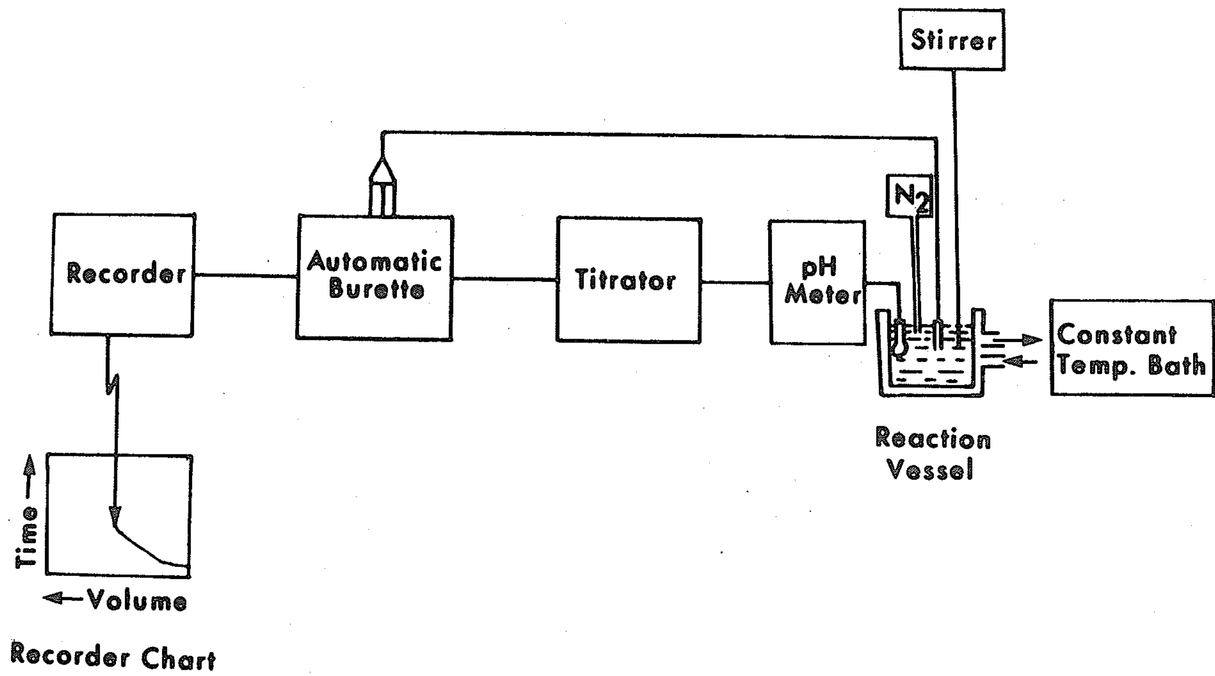


Figure 1. Schematic diagram of the pH-stat apparatus.

adjusted to the level at which the reaction is to take place. This value is preset on the titrator and constitutes the set end point. As hydrogen ions are produced or consumed in the reaction, the pH changes giving rise to an error signal which represents the difference between the actual meter current and the current which corresponds to the set end point. The error current is amplified and applied to a switch circuit which energizes an electromagnetic relay which in turn controls the flow of titrant from a motor-operated burette.

The average flow of titrant is proportional to the error current within a certain range called the proportional band. Within this band the titrant is added in increments which are reduced as the error current grows smaller, while the time interval between increments increases. The burette is mechanically linked to a recorder which records the volume of titrant dispensed as a function of time thus tracing out a curve whose slope at any given moment is equal to the reciprocal reaction rate in terms of minutes per volume %.

The titrator is not capable of quickly and accurately adjusting by itself the rate of titrant flow to the reaction rate. The operator has to set the proper proportional band on the titrator unit and the increment size labeled "speed" on the burette unit. The titration will follow the reaction in a manner which is determined by these settings.

A typical recorder trace of a reaction consists of two major parts. Initially the titrant is added at a rapid rate, causing a nearly horizontal line to be traced out, to make up for the displacement in the pH of the reaction mixture that is due to the addition of the reaction - initiating enzyme solution. If the pH is displaced in the other direction, no titrant is added at all during this early period, giving rise to a vertical trace, while the reaction is catching up to the set end point. Once the end point

has been reached, the titrant is added at such a rate as to maintain the pH of the reaction mixture at this level. It is the slope of the straight line drawn through the initial part of this proportional phase of the trace which is a measure of the reaction rate. The reliability of the assay depends upon the accuracy with which the titration follows the reaction which in turn depends upon the correctness of the control settings that have to be made by the operator. Improper settings will cause problems of various kinds.

The use of too narrow a proportional band allows the end point to be reached rapidly, but it will cause the end point to be overrun due to the lag in electrode response. When this happens no increments will be added during the time needed for the reaction to catch up and a trace resembling a step is obtained (Figure 2a). By the time the reaction has caught up, its rate may already have dropped significantly. The use of too wide a proportional band gives such a gradual approach to the end point that it is difficult to identify the point on the trace where the end point was in fact reached and the proportional phase begins (Figure 2b).

The speed setting controls the size of the volume increment for a given error signal. High settings lead to large increments, quick attainment of the end point and repeated overshoots which introduce a series of steps into the trace (Figure 2c). If such a step-profile is superimposed on a curve rather than a straight line, it is difficult to decide which of several possible tangents represents the reaction rate (Figure 2d). If, on the other hand, the speed setting is too low, the increments will be too small to enable the titration to keep up with the reaction with the result being that the slope of the initial proportional phase will be too small while the error signal gradually builds up and subsequently produces a trace with an increasing slope (Figure 2e).

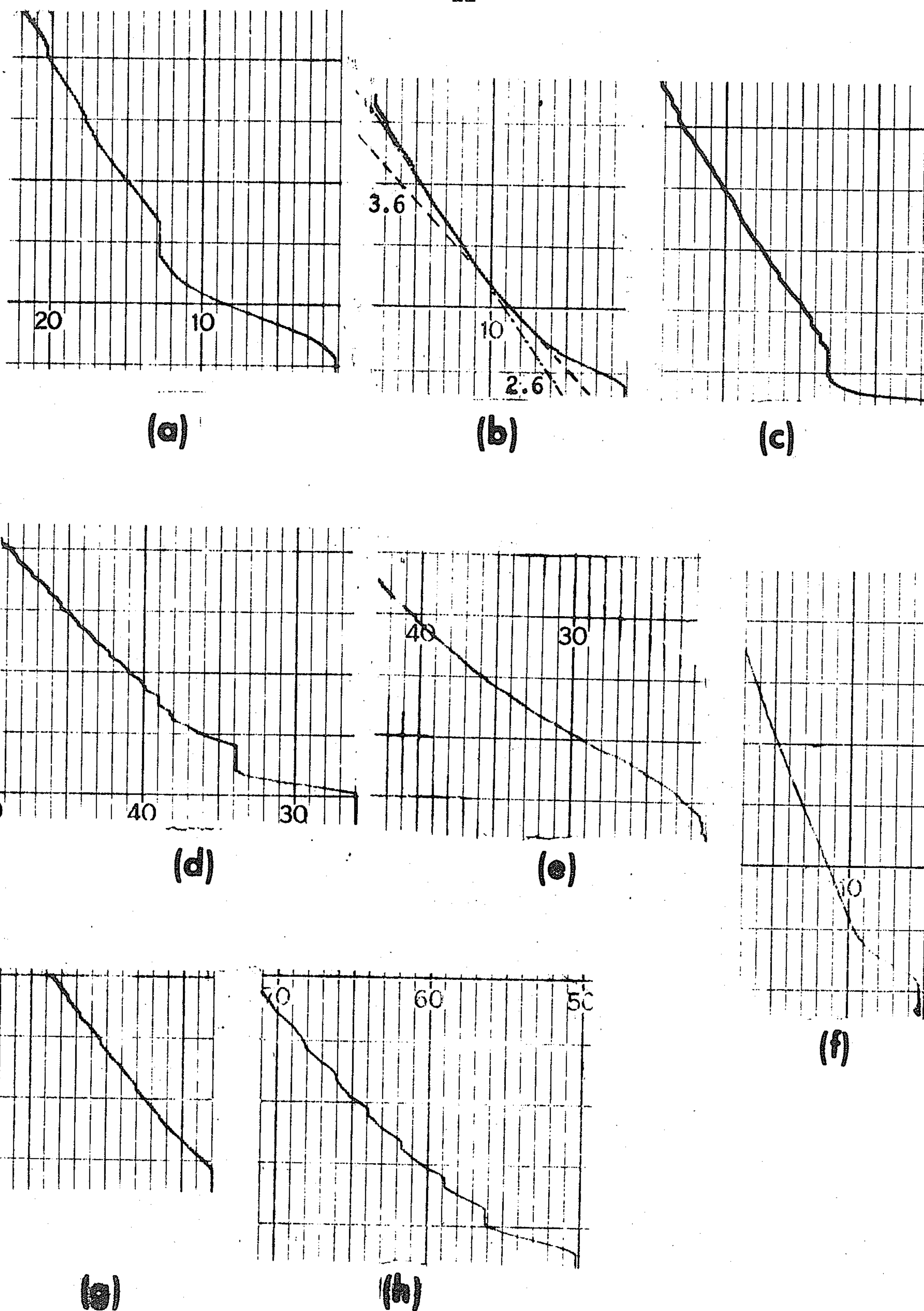


Figure 2. Actual recorder traces illustrating a variety of problems that may be encountered in the pH-stat assay. Details are given in the text.

The property of the apparatus which makes it even more difficult to obtain meaningful measurements is a certain "flexibility" in the end point which comes into play when the titration is not perfectly attuned to the reaction. When the proportional band and speed settings combine to add an excess of titrant, however slight, the pH of the reaction mixture will not be held constant at the set end point, but will be displaced somewhat in the direction of the titration giving rise to high velocity readings. Conversely, if the settings are conducive to a slow addition of titrant relative to the reaction rate, the pH of the mixture will not quite reach the set end point in the initial proportional phase; however, the lag will be taken up at a later stage when the reaction rate begins to drop. The resulting trace will possess a relatively long linear portion, but the velocity measurement will be low (Figure 2f).

This problem could be largely overcome by "pushing" the reaction, i.e. the end point was offset by the margin of drift and proportional band and speed settings were used which caused a slight excess of titrant to be delivered at all times thus pushing the actual end point beyond the set end point to the value originally intended. The trace obtained would look somewhat coarse and step-like and would possibly be more difficult to evaluate than a smooth trace, but the velocity measurement derived from it would be much less susceptible to random error caused by a drifting end point (Figure 2g).

To obtain a reliable recorder trace the proper control settings had to be selected prior to the start of the reaction and the titration had to be watched attentively so that any needed adjustments to these settings could be made at an early stage. It was necessary to anticipate any need for adjustment rather than to wait for it to arise, because by the time it did so the titration could usually not be brought into step with the react-

tion before the rate began to drop. It was thus normally possible to make the titration follow the reaction accurately within a period of about one minute which means that the velocity measurements can be regarded as representing true initial velocities.

c) Interferences

Superimposed upon the problems connected with the actual operation of the apparatus were the problems arising from the differences in the compositions of the reaction mixtures. High substrate concentrations allowed more time to attune the titration speed to the reaction velocity than did low substrate concentrations before product inhibition and decreasing substrate concentrations could take effect. In assays where the two substrate concentrations were most disproportionate, the relatively high reaction rate that was due to the high concentration of the one substrate reduced the time available for "tuning" into the reaction even more to something less than one minute.

In order to alleviate product inhibition by ADP several schemes for its removal were devised and tried out, but none proved to be suitable. Pyruvate kinase consumes the hydrogen ion generated by creatine kinase thereby interfering with the pH-stat assay for creatine kinase. Adenylate kinase produces AMP which was found to inhibit creatine kinase nearly as effectively as ADP. Although the stoichiometry of the reaction catalyzed by adenylate kinase should have led to a reduction in inhibition, no improvement in the shape of the reaction trace was detected. Nucleoside 5'-diphosphate kinase was found unsuitable, because mitochondrial creatine kinase reacts with all nucleoside 5'-triphosphates produced by the enzyme.

Yet another complicating factor is the buffering action of MgATP, MgADP, creatine phosphate, EDTA and BSA which affects the assay by decreasing

the sensitivity. The use of BSA was discontinued after it was discovered that it was not needed to maintain the activity of the enzyme. The effect of EDTA became negligible when the enzyme was used undiluted so that volumes of only one or two microliters were needed. The resulting low concentration of EDTA in the reaction mixture, however, caused no problems with oxidation of the enzyme (29). But each of the three substrates mentioned did adversely affect the sensitivity of the method to an extent which was proportional to their concentration. In product inhibition studies the buffering action of the product was seen in addition to that of the substrates, particularly when the inhibitory product was present at a saturating concentration. Thus, it can be said that each assay mixture exhibited its own distinctive behavior during titration which had to be taken into account when the trace was evaluated.

It is important to point out that due to the buffering action of substrates and to instrumental problems associated with titrating high-velocity reactions the assays involving the highest substrate concentrations do not yield the most reliable results. This is one reason for not subjecting data obtained by the pH-stat method to analysis by a computer program which weights assays according to substrate concentrations.

In early work a G 202 C glass electrode was used with a K 4312 calomel reference electrode and also the equivalent GK 2021 C combination electrode, all made by Radiometer. The dithioerythritol added to the reaction mixture with the enzyme solution, however, had a detrimental effect on the stability of the signal resulting in a great deal of noise and drift in the pH meter reading. This effect was cumulative and irreversible rendering a given electrode unusable within days or weeks depending on the extent of exposure to the reagent.

The problem was resolved by resorting to the use of the Beckman Combination Electrode 39183 which incorporates a silver-silver chloride reference electrode. The liquid junction is formed by the electrolyte, a silver-chloride saturated 4 M potassium chloride solution, flowing through an annular ceramic layer. This electrode showed no signs of poisoning during the entire period of use, approximately two years, and even more importantly generated consistently low noise readings.

The use of this electrode offers one major disadvantage, however, which is a relatively slow response. The delay in sensing the pH will cause an excess of titrant to be delivered with the result that the titration will pause until the reaction has not only caught up to but has in fact passed the end point. This cycle of overshoot and stop may repeat itself several times well past the initial proportional phase. The problem could be allayed by adjusting the initial pH of the substrate mixture to a value such that the addition of the enzyme solution would bring the pH of the reaction mixture very close to the set end point and also by carefully selecting that proportional band which, while allowing the titration to keep up with the reaction, would cause titrant to be added sufficiently slowly to avoid a large overshoot as the end point was approached. As such a cautious titration is more time-consuming than a more rapid one which is made possible by an electrode with a shorter response time, the evaluation of assays involving low substrate concentrations which entail short initial proportional phases remained problematical.

d) Evaluation

The purpose of this somewhat detailed discussion of sources of error affecting the recorder trace is to show that there is an appreciable number of both determinate and random errors which ultimately find their way into the experimentally determined variable, the initial velocity. In addition, the assignment of a numerical value to the slope of a trace can, on occasion, represent a difficult task. For example, the two tangents drawn to the trace in Figure 2b yield initial velocities of 3.6 and 2.6 volume %/cm. As only one of these values can be correct, a choice has to be made between them by the experimenter with the help of an understanding of how the instrument functions and of a knowledge of the exact circumstances under which the trace was recorded. Thus the accuracy of the data becomes to some extent a function of the worker's ability to extract the best values from the recorder traces. This necessarily subjective process further complicates the error structure of the data.

The term "experiment" as used in the initial velocity studies denotes the number of assays, each one performed with a different reaction mixture, sufficient to produce a family of double reciprocal plots. The binding constants and maximal velocity derived from such a plot constitute the results of the experiment. A typical experiment consists of 20 to 40 assays, all done on the same day. It was physically impossible to increase this number. When an experiment was continued on the following day, the assays were found to be reproducible only to such a degree as to render the additional data of little value in improving the accuracy of the results and of even less value in determining the reproducibility of replicate assays.

Askelöf et al. (101) consider a knowledge of the error structure of

a given set of experimental data a necessary prerequisite for incisive analysis. The absence of such knowledge makes the application of computer programs such as those by Cleland (102) which assume equal variances of the data appear to be of doubtful value. A methodical investigation into the variances of the data as a function of substrate concentration will yet have to be carried out to make possible a meaningful statistical analysis. For the present, the data in double reciprocal plots were subjected only to a linear least squares analysis.

The most effective safeguard against erroneous results and the most reliable method of obtaining estimates of uncertainty was considered to be the independent repetition of experiments. Whenever possible, more than one experiment were carried out and estimates of error are based upon the degree of precision achieved.

e) Advantages

The question may be raised why the pH-stat assay was used in spite of the numerous problems it presents. The answer is simply that it is deemed the best method available. As a continuous assay it is superior to discontinuous methods. The high degree of susceptibility of the reaction to product inhibition virtually prohibits the use of discontinuous methods for all experiments designed to investigate the effects of substrates and products on reaction velocity as all but the shortest assay times will yield data affected by such inhibition. By the same token, it is doubtful that the sensitivity of the known discontinuous methods would be sufficiently high to make meaningful kinetic measurements possible.

The only other applicable continuous assays, besides the pH-stat technique, would be coupled enzyme assays which are available for either

direction of the reaction under study and which are in widespread use for assaying cytoplasmic creatine kinase activity in the context of both clinical and research work. The coupling enzymes employed in the forward reaction are pyruvate kinase and lactate dehydrogenase (47) and hexokinase and glucose-6-phosphate dehydrogenase in the reverse reaction (103). The following points argue against the use of these assays in the present study.

i) Coupled enzyme assays require the use of a buffer, but mitochondrial creatine kinase activity has been found to be sensitive to most of the buffers used.

What would be the effect of a given buffer on the activity?

Would this effect be the same for both reactions?

Would the buffer interact with substrates or would it complex Mg^{2+} ion?

Would the kinetic mechanism be affected?

ii) Parts of this study have been conducted at different pH levels.

How would the change in pH affect the activity of the coupling enzymes?

iii) Coupling enzymes have their own specific requirements such as that of pyruvate kinase for K^{+} ion normally added in the form of the acetate or chloride.

Both these anions affect the activity of mitochondrial creatine kinase. So do most other anions.

iv) Some work was done with dinucleotides other than ADP as inhibitors of the forward reaction.

How would pyruvate kinase activity have been affected?

v) Product inhibition experiments were used to identify the kinetic mechanism.

The experiments involving the use of ADP and ATP as inhibitory products could not have been done, since these are the substrates of the coupling enzymes.

This list of problems which would have had to be resolved before coupled enzyme assays could have been used to carry out all the types of experiments required for this study is not meant to be complete. But it should suffice to show that the use of this method avoided more problems than it created. Finally, it afforded great savings in labor, time and materials.

f) Assay Conditions

Measurements of initial velocity as a function of the concentration of one substrate at various fixed concentrations of the second substrate were made in the presence of 0.001 M free Mg^{2+} ion which is sufficient to insure a high degree of complexation without likely causing appreciable inhibition (35). The concentrations used were between 9.9×10^{-4} M and 3.9×10^{-5} M for MgATP and between 1.8×10^{-4} M and 2.8×10^{-5} M for MgADP. Creatine concentrations of 4.0×10^{-2} M to 5.0×10^{-3} M were employed, while creatine phosphate concentrations ranged from 5.0×10^{-3} M to 3.0×10^{-4} M. The total volume was fixed at 8.0 ml.

In the product inhibition experiments the non-varied substrates were fixed at the following concentrations with values representing concentrations at which the enzyme was considered to be saturated given in parentheses: MgATP at 2.0×10^{-4} M (3.11×10^{-3} M), creatine at 1.6×10^{-2} M, MgADP at 9.3×10^{-5} M (1.83×10^{-3} M), and creatine phosphate at 1.0×10^{-3} M (4.04×10^{-2} M).

Inhibitory products were used at the following concentrations: creatine phsophate from 1.0×10^{-4} M to 3.7×10^{-4} M, MgADP from 3.7×10^{-5} M to 11.0×10^{-5} M, MgATP from 2.0×10^{-4} M to 9.9×10^{-4} M, creatine from 3.0×10^{-3} M to 25.0×10^{-3} M. Guanidinoacetate was used at 1.0×10^{-2} and 1.25×10^{-2} M concentrations, other creatine analogs at 1.0×10^{-3} M. Nucleoside 5'-mono- and diphosphates were used at 1.0×10^{-4} and 2.0×10^{-5} M, adenosine 5'-tetraphosphate at 5.37×10^{-4} M, and inorganic phosphate at 1.26×10^{-3} M concentrations.

In early experiments BSA was added to a concentration of 0.1 mg/ml as used by Mahowald et al. (28) to safeguard against inactivation possibly by the mechanism of adsorption of the enzyme to the glass surfaces or by denaturation induced by the injected but not yet diluted titrant. Also, the reaction mixture became approximately 12 μM in DTE and 6 μM in EDTA on the addition of enzyme volumes of the order of 50 μl.

In later work which produced most of the results of this study, enzyme volumes of 1 or 2 μl were added which resulted in even lower DTE and EDTA concentrations which, as mentioned above, had no apparent adverse effects on the initial reaction velocity. Likewise, it was established in careful experiments that the presence or absence of BSA produced no difference in initial velocity, whereas the cytoplasmic MM isoenzyme from rabbit muscle exhibited no activity at all in the absence of the protecting protein.

Titrants used were 3.62×10^{-3} M HCl and NaOH solutions which were prepared weekly by dilution of 2.0 N stock standard solutions with CO_2 free double distilled deionized water and which were carefully protected from air. Glycolic acid (35) was used to repeat some of the experiments done with HCl to check if the chloride ion introduced with the titrant

affected results, but differences were found in neither the overall results nor the shape of the trace.

The use of titrants of low normality was necessitated by the high affinity of the enzyme for its nucleotide substrates. Fromm (104) considers a substrate range of 1/2 K_m to 5 K_m as adequate for a Michaelis-Menten type enzyme. For mitochondrial creatine kinase this means a range of 5×10^{-5} M to 5×10^{-4} M with regard to MgATP which is very close to the range that was actually used. Even at the low titrant concentration employed it would require only 44% of the total burette volume to titrate the entire sample with the lowest substrate concentrations assuming that the hydrogen ion coefficient is 1.0 and the reaction goes to completion. If initial velocity conditions are considered to prevail until 10% of the total substrate has been consumed, the initial velocity would have to be determined over a volume range of only 5%. The sensitivity of the assay is taxed to the limit in the reverse reaction with MgADP possessing a K_m value of around 20 μM. An even lower titrant concentration of 2×10^{-3} M was tried out but not adopted, because high velocity assays could no longer be accurately titrated.

The enzyme concentration had to be adjusted to give reaction velocities no higher than about 12 vol. %/cm at a recorder speed of 2 cm/min and a pH change of 0.5 pH/cm, because at higher activities the titration would follow rather than lead the reaction and low readings would be obtained. Also, the slope of the resulting trace would become so small that the relative uncertainty in determining it would become quite large. On the other hand the enzyme activity had to be sufficiently high to make adequate control over the titration possible, i.e. the reaction had to use up titrant at a sufficient rate to keep the trace coherent. The threshold velocity was near 2.5%/cm, but a more convenient value was about 4%/cm at

the stated recorder speed.

Thus, a velocity range of only about three to five-fold was accessible by the technique. This restriction was accepted in preference to the use of different aliquots of enzyme. Such a procedure would have required not only the reproducible dispensation of 1 or 2 μl of enzyme solution, but also the measurement of these small volumes at the 1%-level of accuracy which was not quite attainable with the 5-place Mettler balance which was available. But the more important reason for not adopting such a procedure was the fact that differing enzyme concentrations would have led to yet greater variety in the appearance of the recorder traces and would thus have further complicated their evaluation.

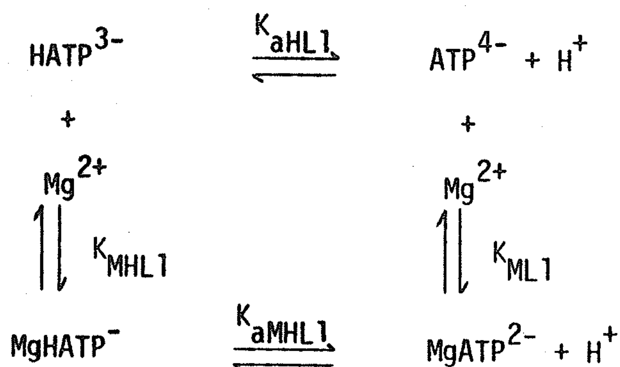
g) Assay Procedure

The reagents except the enzyme solution were pipetted into the clean dry cup in such an order as to prevent as much as possible the pH from dropping below 7 and from rising above 8.5. Specifically, creatine phosphate was not added to acidic mixtures, nucleotides were not added to strongly basic mixtures. Small volumes dispensed by micropipettes were added last to allow rinsing of the pipette. The electrode(s) and delivery tube were immersed in the mixture, the cover was put in place and nitrogen swept over the liquid surface. The pH of the mixture was adjusted by the addition of acetic acid or NaOH of the appropriate concentration by means of a wire loop. Conditions were allowed several minutes to equilibrate. At the end of this period a blank trace was recorded for all substrate mixtures assayed at pH 6.8 and all mixtures assayed at pH 7.4 and containing creatine phosphate. The reaction was started by blowing the enzyme solution into the reaction mixture from a pipette protected by CO_2 absorber.

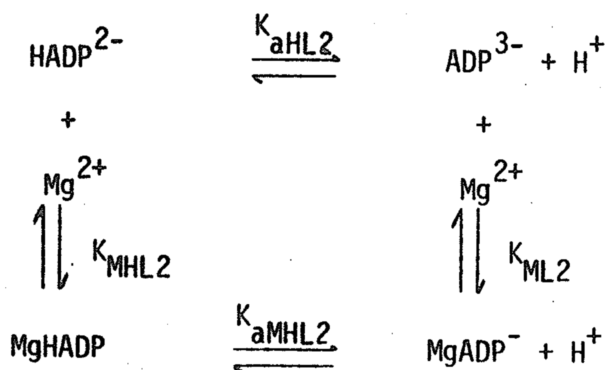
The substrates were stored frozen. Creatine solutions were prepared by warming just sufficiently (@ 40° C) to dissolve all solid in about 24 hours, since heating accelerates the rate of conversion to creatinine (105). ATP and ADP solutions were brought to a pH of 7.4 by the addition of 2 N NaOH immediately upon dissolution and before being made up to volume.

4. Calculation of Substrate Concentrations

As first proposed by Kuby et al. (16) the true substrates of creatine kinase are the magnesium complexes of the nucleotides, whereas at least two of the uncomplexed species act as inhibitors (33). For the forward reaction the equilibria depicted in Scheme 1 have to be considered for the pH range 6 to 9 (35,106). Scheme 2 shows the pertinent equilibria for the reverse reaction (35).



SCHEME 1



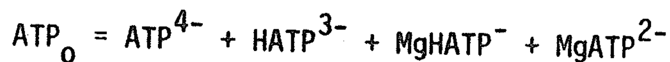
SCHEME 2

Obviously, the proportions of the various species depend on the pH and on the magnitude of the pertinent ionization and complex stability constants. The scarcity of reliable values hampered early kinetic investigations as previously mentioned. Kuby and Noltmann (22) used the values of Burton (107), O'Sullivan and Perrin (108), Smith and Alberty (109,110), Melchior (111) and Martell and Schwarzenbach (112). Noda et al. (19) and Morrison et al. (20) also referred to these authors.

Although becoming somewhat dated, these values have continued to be used, for example, in the review article by Watts (35) and even as recently as 1977 by Altschuld and Brierley (79). This is surprising in view of the fact that more recent data by Phillips et al. (113) and also

by Mohan and Rechnitz (114) have become available. Saks et al. (76) have used the pK functions of Phillips et al. to calculate stability constants, but their derived values cannot be reproduced. It was decided to use in this study the data of Phillips et al. for the nucleotide complexes and those listed by Kuby and Noltmann for the creatine phosphate species for which no new values have since been proposed. The values recalculated for the prevailing experimental conditions are shown in Table 1.

$MgATP^{2-}$ concentrations were calculated for a free Mg^{2+} ion concentration of 0.001 M using the following conservation equations where the subscript zero denotes total concentrations.

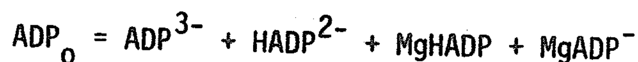


$$ATP_0 = ATP^{4-} + HATP^{3-} + MgHATP^{-} + MgATP^{2-}$$

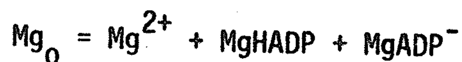
$$Mg_0 = Mg^{2+} + MgHATP^{-} + MgATP^{2-}$$

Kuby and Noltmann (22) consider additional species such as $NaATP^{3-}$, Mg_2ATP and $Mg(ATP)_2^{6-}$, but calculations have shown that the concentration of $NaATP^{3-}$ is negligible, while the two Mg^{2+} complexes are not taken into account by Alberty (115) at Mg^{2+} ion concentrations as high as 0.1 M or as low as 10^{-7} M. No complexation or ionization reactions are considered for creatine.

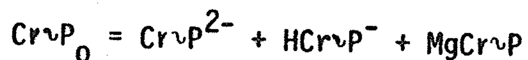
Proportions of the various substrate species were calculated for the reverse reaction on the basis of the following conservation equations.



$$ADP_0 = ADP^{3-} + HADP^{2-} + MgHADP + MgADP^{-}$$



$$Mg_0 = Mg^{2+} + MgHADP + MgADP^{-}$$



$$Cr{\sim}P_0 = Cr{\sim}P^{2-} + HCr{\sim}P^{-} + MgCr{\sim}P$$

TABLE 1

Ionization and Mg^{2+} Complex Stability Constants for ATP, ADP and Cr∿P at 30°

Constant	Species	Value	Ref.
K_{ML1}	$MgATP^{2-}$	7.28×10^{5}	113[a]
K_{ML2}	$MgADP^{-}$	2.00×10^{4}	113
K_{ML3}	MgCr∿P	2.4×10^{1}	22[b]
K_{MHL1}	$MgHATP^{-}$	4.12×10^{3}	113
K_{MHL2}	MgHADP	2.78×10^{2}	113
K_{aHL1}	$HATP^{3-}$	2.09×10^{-8}	113
K_{aHL2}	$HADP^{2-}$	6.36×10^{-8}	113
K_{aHL3}	HCr∿P^{-}	3.16×10^{-5}	22
K_{aMHL1}	$MgHATP^{-}$	3.75×10^{-6}	113
K_{aMHL2}	MgHADP	5.01×10^{-6}	113

[a] Recalculated from pK functions of Phillips et al. (113) for 30° and zero ionic strength.

[b] As selected by Kuby and Noltmann (22) from other sources.

The relative proportions of the various species calculated on the basis of both the older and the more recent values of the ionization and complex stability constants are listed in Table 2. It might be worth noting that the effective substrate concentrations for the forward reaction do not change greatly when a different set of constants is used, whereas the difference for the reverse reaction is quite large. This has to be taken into account when binding constants based on different sets of values are compared. A Michaelis constant reported for MgADP based on the older values has to be considered low by about 22% relative to a constant based on the newer data.

The creatine phosphate concentration can be regarded as being independent of pH.

TABLE 2

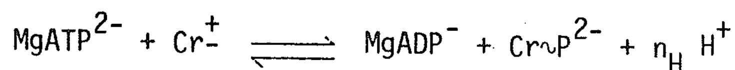
Substrate Distribution Among the Various Species as a Function of pH at 30° and a Free Mg^{2+} Ion Concentration of 1 mM[a]

Substrate Added	Species Present	Percentage of the Total Substrate at pH 8.1	pH 7.4	pH 6.8	pH 6.3
ATP	ATP^{4-}	0.1 (1.4)	0.1 (1.4)	0.1 (1.4)	0.5
	$HATP^{3-}$	0.1 (0.1)	0.3 (0.5)	1.0 (1.8)	12.4
	$MgHATP^{-}$	0.2 (0.1)	1.1 (0.3)	4.1 (1.0)	51.0
	$MgATP^{2-}$	99.6 (99.9)	98.5 (99.3)	94.8 (97.2)	36.1
ADP	ADP^{3-}	4.7 (24.7)	4.6 (23.8)	4.1 (20.7)	3.2
	$HADP^{2-}$	0.6 (1.0)	2.9 (4.7)	10.3 (16.4)	25.4
	MgHADP	0.2 (0.04)	0.8 (0.2)	2.9 (0.6)	7.0
	$MgADP^{-}$	94.5 (74.2)	91.7 (71.3)	82.7 (62.2)	64.4
Cr∿P	$HCr{\sim}P^{-}$	0.02	0.1	0.5	
	MgCr∿P	2.3	2.3	2.3	
	$Cr{\sim}P^{2-}$	97.7	97.6	97.2	

[a] Values in parentheses represent proportions calculated on the basis of the constants used by Kuby and Noltmann((22).

5. Determination of the Hydrogen Ion Coefficient

In the forward reaction H^+ ion is liberated and in the reverse reaction it is consumed.



$$MgATP^{2-} + Cr^{\pm} \rightleftharpoons MgADP^{-} + Cr{\sim}P^{2-} + n_H\ H^+$$

n_H, the number of moles of H^+ ion used up or produced per mole of nucleotide and guanidino substrate, depends on the conditions of pH, temperature and ionic strength. It was experimentally estimated in the forward reaction by a combination of the pH-stat assay as used by Kuby (116) and the colorimetric method for creatine phosphate described above.

The results obtained at pH values of 8.1 and 7.4 were 0.98 ± 0.01 (average of 3 trials) and 0.86 ± 0.03 (average of 5 trials), respectively, and were thus in good agreement with the coefficients determined at 30° and 0.12 ionic strength by Dyson and Noltmann (117) for the phosphofructokinase reaction and also with those of Wedler and McClune (118) determined at 25° for the glutamine synthetase reaction. Alberty (119) has calculated n_H values for the hydrolysis of ATP as a function of pH and pMg which appear to be in fairly good agreement with the values determined experimentally at the two higher pH levels. Since no reproducible results could be obtained at pH 6.3 due to the low value of n_H, and the resulting low sensitivity of the pH-stat assay, and due to the high proportion of ATP being present in the form of protonated species (cf. Table 2), the results of Dyson and Noltmann were used in this study to calculate initial velocities.

These values are 0.99, 0.89 and 0.64 for pH levels of 8.1, 7.4 and 6.8, respectively. It was decided at this point to use the pH of 6.8 in preference to 6.3 for initial velocity studies for these same two reasons

and also because of the high rate of spontaneous hydrolysis of creatine phosphate at the lower pH value.

6. Linearization of the pH Meter Scale

Since the pH-stat assay was performed at different pH values but at constant temperature, it was desirable to linearize the meter scale over the pH range used. Since several electrodes were poisoned by thiol compounds in the early stages, the linearization was performed repeatedly. The normally recommended procedure involves repeated immersions of the electrodes in two standard buffer solutions in an alternating fashion while making small adjustments to the "Sample Temperature" and "Buffer Adjust" controls. It was found cumbersome and time-consuming and to take its place a procedure was developed which proved to be far more efficient and accurate. If such a procedure has been described elsewhere, the author is not aware of it.

Two standard buffers are selected according to the range over which the scale is to be linearized. The electrodes are placed into one of the solutions and with the sample temperature set to some low value such as 0 or 10, that buffer adjust setting is determined which makes the meter read exactly the pH of the buffer specified for the operating temperature. These measurements are repeated at other sample temperature settings and the results are plotted as shown in Figure 3. The resulting straight line represents all those sample temperature/buffer adjust settings that produce the correct meter reading for the buffer used.

The procedure is repeated with the second buffer and a straight line plot is obtained which intersects the other one. The coordinates of the point of intersection represent those sample temperature and buffer adjust settings which produce accurate meter readings for both buffers.

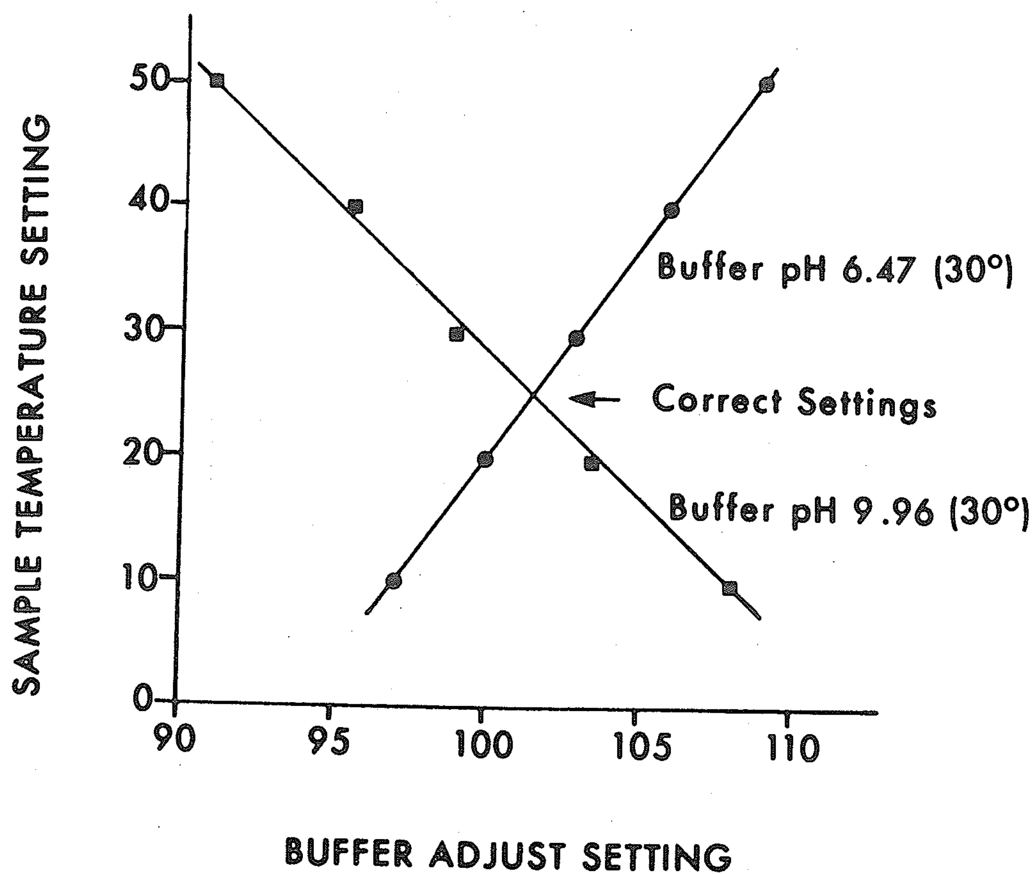
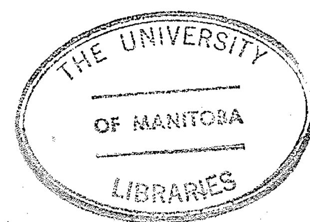


Figure 3. Linearization of the pH meter scale over the range 6.5 to 10 at 30^{0}.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It appears reasonable to assume that meter readings made within the range between the two buffers are accurate as well; this was found to be the case when the meter setting was checked against other standard buffers.

IV. RESULTS

The experimental work can be divided into two major sections: the preliminary studies and the initial velocity studies. Experiments belonging to the first category utilized discontinuous colorimetric assays involving saturating or near-saturating substrate concentrations. They yielded data which represent apparent enzyme activities, i.e., reaction rates which are subject to the particular set of experimental conditions and which derive their significance only from their relationship to other data obtained under the same circumstances.

The purpose of the preliminary investigations was to define, and also to some extent establish, the best experimental conditions for the initial velocity studies. With these variables determined, initial velocity experiments were conducted by means of the pH-stat assay which yields true initial velocity measurements as has been shown in section III of this report. The main purpose of these experiments and, in fact, the focal point of the entire project was the determination of the kinetic mechanism of mitochondrial creatine kinase. It was accomplished by means of experiments conducted in the absence and presence of products.

Other parts of this section deal with the effect of pH on the kinetic parameters of the enzyme and with the Haldane relationship for the reaction, and also with the use of various compounds as inhibitors to gain some insight into the properties of the catalytic locus.

A. Preliminary Studies

1. pH-Activity Profiles.

Apparent enzyme activity as a function of pH is shown in Figure 4. In the forward reaction the enzyme is active over a wide pH range with the

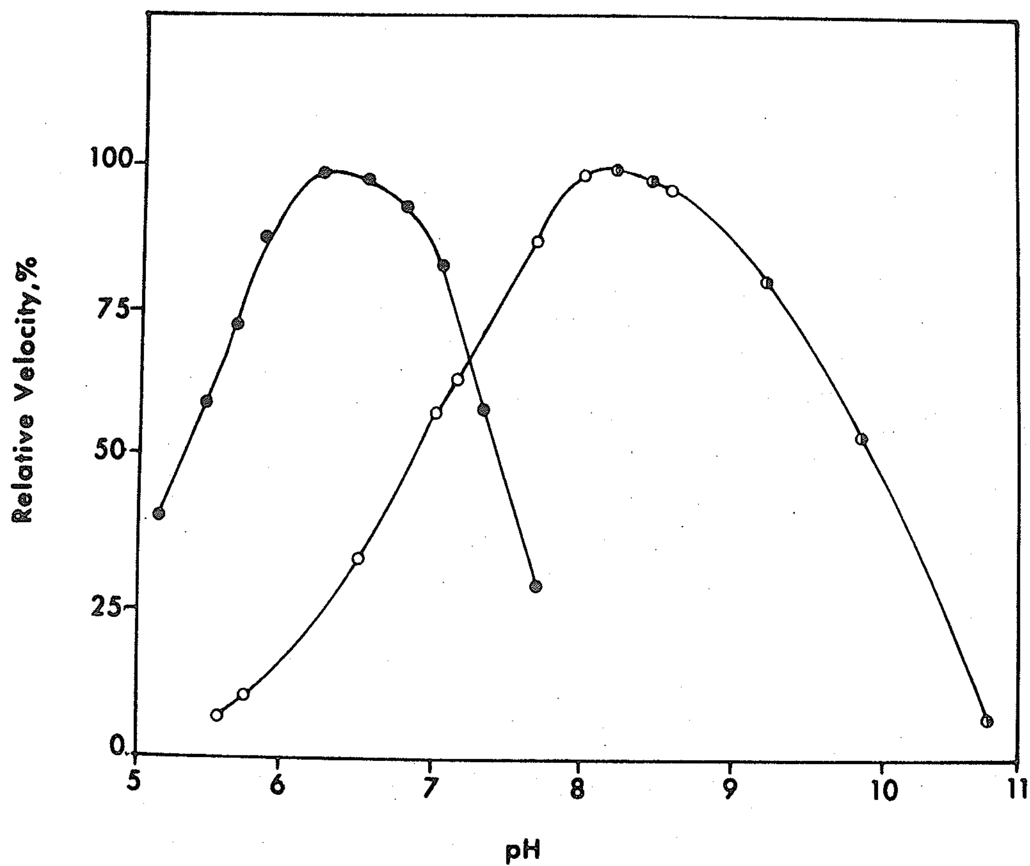


Figure 4. Effect of pH on the apparent activity of mitochondrial creatine kinase at 30° in the forward reaction in 0.1M Tris-maleic acid-NaOH (o-o-o) and glycine-NaOH buffers (◐-◐-◐) and in the reverse reaction in 0.1M β,β-dimethylglutaric acid-NaOH buffer (●-●-●).

optimum occurring near 8.1, a value which is in close agreement with that observed for creatine kinase in rat heart mitochondria by Jacobus and Lehninger (73). The same result was obtained in buffer systems of constant ionic strength. While a certain degree of inhibition was observed in the Tris-maleate buffer relative to the glycinate buffer, the two segments of the curve could be easily fitted together by overlapping the two pH ranges. The true substrate of the enzyme is the Mg^{2+} complex of ATP, $MgATP^{2-}$. Since the protonated complexes $HATP^{3-}$ and $MgHATP^{-}$ also form, the concentration of $MgATP^{2-}$ is pH dependent (see Table 2). It decreases by approximately 5% as the pH of the assay medium is changed from 8.6 to 6.8, but is virtually constant above pH 8.6. This change in true substrate concentration will not affect significantly the value of the pH optimum, but it will affect to some degree the slope of the ascending limb of the curve. As $HATP^{3-}$ has been identified as an inhibitor of the cytoplasmic MM isoenzyme from rabbit muscle (19), it could also have an effect on the shape of this portion of the plot. The high-pH side of the plot does not involve significant changes in the concentration of any species and can thus be considered with greater justification as reflecting the progressive denaturation of the enzyme at the various pH levels under assay conditions.

The optimum for the reverse reaction is located near pH 6.3, a result which is also observed in a buffer system of constant ionic strength (0.1 M in terms of NaCl concentration) as well as in a tris-maleate buffer. The maximum thus appears to be independent of ionic strength and of the identity of the buffer species. There are two major sources of error which could well significantly affect the value of the pH optimum. One is the concentration of the true substrate $MgADP^{-}$ which is also dependent on pH, since protonated species such as $HADP^{2-}$ and MgHADP are

formed. In contrast to the situation involving MgATP, the concentration of MgADP decreases by about 27% as the pH is lowered from 7.4 to 6.3. This variation must have significantly affected the shape and particularly the slope of the plot and caused a displacement of the optimum towards a higher pH value. The other major source of error is the spontaneous hydrolysis of creatine phosphate, whose rate increases steeply as the pH drops below 7. Thus, at pH 6.3 the non-enzymic blank represents 20% of the total reaction, a proportion which increases to 83% at pH 5.1. The observed decrease in apparent activity below pH 6.3 consequently is due at least in part to a lower effective creatine phosphate concentration. This effect is probably combined with denaturation of the enzyme at pH values below 6.0 which has been observed in stability studies (83). $HADP^{2-}$ is not an effective inhibitor of the cytoplasmic MM isoenzyme, and nothing is known about the inhibitory properties of MgHADP (35).

Even with these limitations placed upon the reliability of the pH-activity profiles, it is tempting to observe that the descending limb of the plot for the reverse reaction and the ascending limb of the plot for the forward reaction have inflection points near pH 7.2 which suggests that an ionizable group with this pK might be involved in the rate-determining step of the catalytic mechanism. For the cytoplasmic isoenzyme from rabbit muscle such a group, the imidazole group of histidine, has been proposed to be involved in the mechanism (120).

2. Temperature-Activity Profiles.

Figure 5 shows the dependence of apparent activity on temperature. Optimum temperatures are 34° for the forward reaction at pH 8.6 and 44° for the reverse reaction at pH 6.3. Stability studies conducted in the absence of substrates indicate that the enzyme is stable at pH 6.3 between

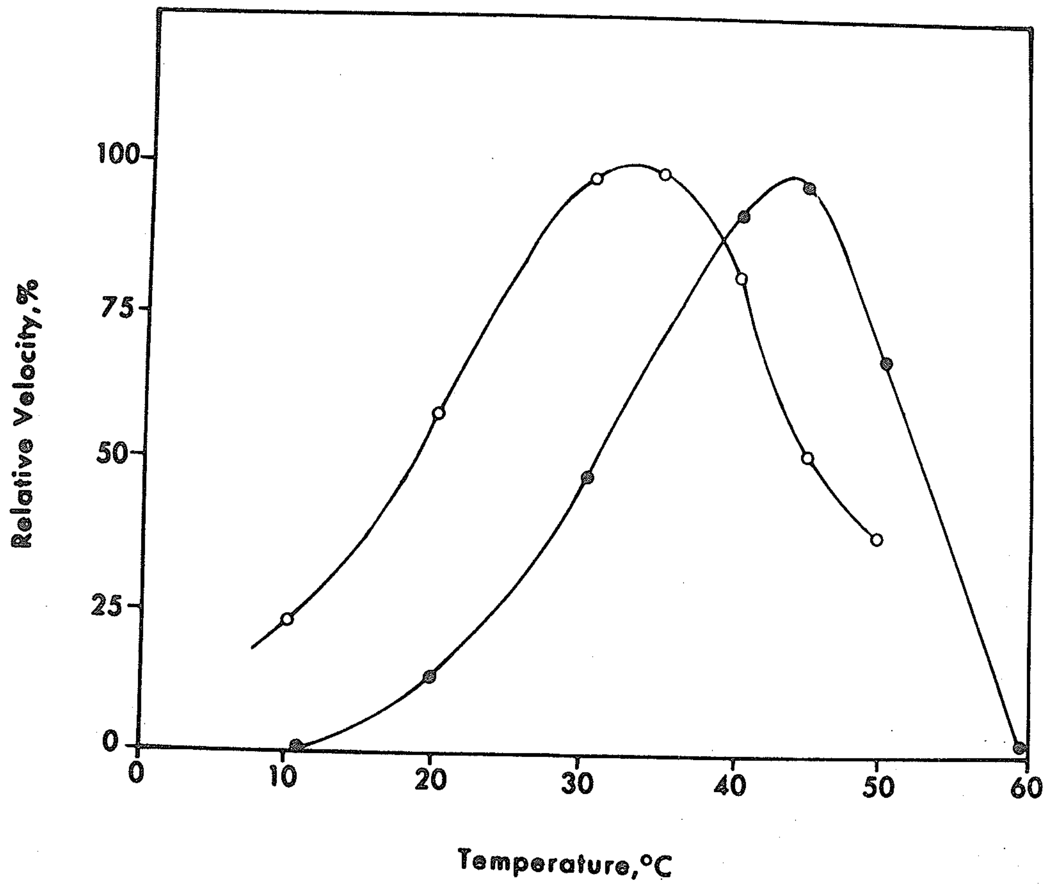


Figure 5. Effect of temperature on the apparent activity of mitochondrial creatine kinase in the forward reaction in 0.05M glycine-NaOH buffer of pH 8.6 (○-○-○) and in the reverse reaction in 0.05M β,β-dimethylglutaric acid-NaOH buffer of pH 6.3 (●-●-●).

0^{0} and 50^{0} and at pH 8.6 between 0^{0} and 35^{0} (83). These observations correlate well with the optimum temperatures determined here and suggest that the difference in the optimum temperatures is chiefly due to the difference in stability of the protein at different pH values rather than to the presence of different sets of substrates.

Figure 6 represents the Arrhenius plots of the data utilized in Figure 5. In the forward reaction at lower temperatures the plot is linear with a slope that yields an apparent energy of activation of 15.6 kcal $mole^{-1}$ deg^{-1}. Around 25^{0} there is a discontinuity which seems to signify a change in the rate-controlling step which in turn likely reflects a change in the physico-chemical state of the enzyme. The assay temperature of 30^{0} is contained within a non-linear portion of the plot where a more or less continuous temperature-induced change in the rate-controlling step occurs. Consequently, an assay temperature of 25^{0} would appear to be more suitable than one of 30^{0} for the forward reaction at pH 8.6. In the reverse direction at pH 6.3 the discontinuity occurs at approximately 33^{0}. The temperature of 30^{0} is located within the linear region where no temperature-induced changes affect the enzymic mechanism and thus appears to be a suitable assay temperature under the prevailing conditions. The apparent activation energy amounts to 22.0 kcal $mole^{-1}$ deg^{-1}. Nealon and Henderson (121) have obtained similar results for the cytoplasmic isoenzymes. They found that all three enzymes, the MM, MB and BB forms, undergo some change of state near 30^{0}.

Assay conditions employed in these studies differ with regard to pH, ionic strength, buffer species and substrates present. Moreover, the effects of temperature on the degree of complexation of the nucleotide substrates and on the affinity of the enzyme for the substrates are ignored.

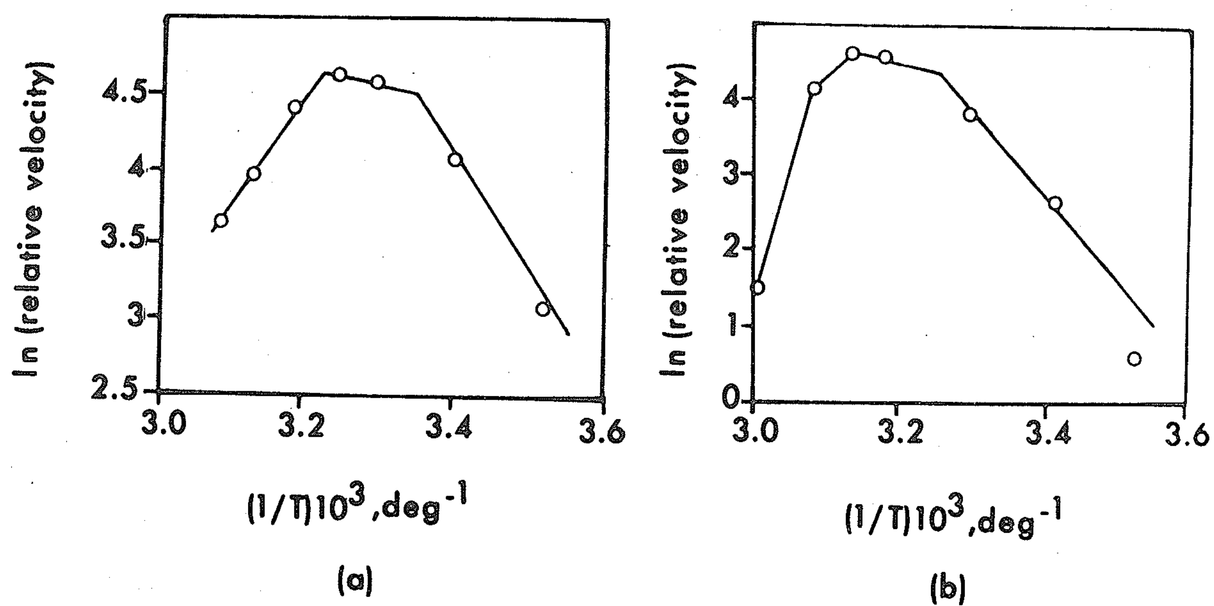


Figure 6. Arrhenius plots for mitochondrial creatine kinase in the forward reaction at pH 8.6 (a) and in the reverse reaction at pH 6.3 (b).

Consequently, the two profiles should not be directly compared to each other. This caution appears justified when, for example, apparent energies of activation in the denaturation zones are considered. They are15 and 35 kcal $mole^{-1}$ deg^{-1} for the forward and reverse reactions, respectively.

3. Effect of Sodium Chloride on Apparent Activity.

When increasing amounts of sodium chloride are added to the reaction mixture, enzyme activity falls off rapidly as is shown in Figure 7. The forward reaction appears to be particularly susceptible with the inhibition becoming almost complete at chloride concentrations above 0.6 M.

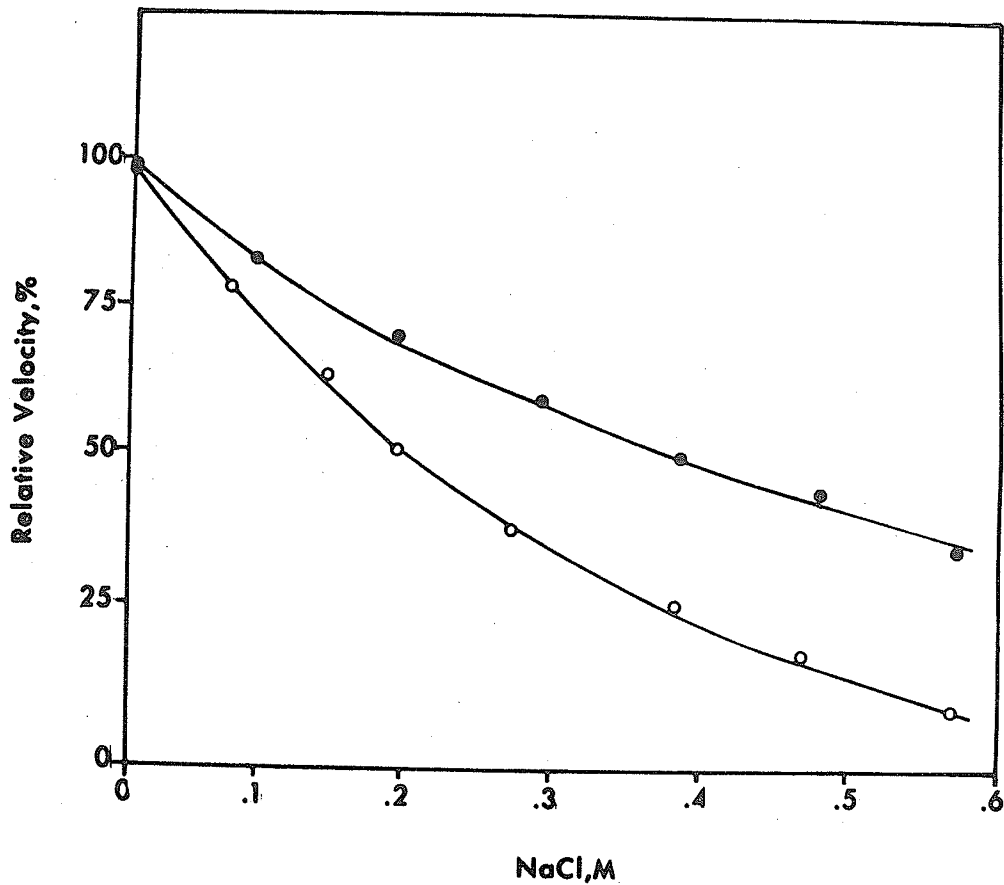


Figure 7. Effect of the concentration of NaCl on the apparent activity of mitochondrial creatine kinase in the forward reaction in 0.05M glycine-NaOH buffer of pH 8.6 (o-o-o) and in the reverse reaction in 0.05M dimethylglutaric acid-NaOH buffer of pH 6.3 (●-●-●) at 30°.

B. Initial Velocity Studies

1. Initial Velocity as a Function of Substrate Concentration at Various pH Values

a) Kinetic Constants

Initial velocity measurements were made as a function of substrate concentration at various fixed concentrations of the second substrate for both the forward and reverse reactions at pH values of 8.1, 7.4 and 6.8. The data were processed in the form of double reciprocal plots according to Lineweaver and Burk (122). Figures 8 to 13 show representative plots.

All plots were found to be linear and to intersect at a point to the left of the ordinate and are thus indicative of a sequential mechanism.

Analysis of these plots was based on the initial velocity rate equation shown by Alberty (123) to apply to the rapid-equilibrium Random, the Ordered and the Theorell-Chance mechanisms, written in Cleland's notation (124)

$$v = \frac{V_1 AB}{K_{ia}K_b + K_b A + K_a B + AB}$$

where V_1 is the maximum velocity of the forward reaction,

K_a and K_b are the Michaelis constants of substrates A and B, respectively, and K_{ia} is the inhibition constant for substrate A.

The reciprocal forms of this equation written with A and B as the variable substrates are

$$\frac{1}{v} = \frac{K_a}{V_1}\left(1 + \frac{K_{ia}K_b}{K_a B}\right)\frac{1}{A} + \frac{1}{V_1}\left(1 + \frac{K_b}{B}\right)$$

$$\frac{1}{v} = \frac{K_b}{V_1}\left(1 + \frac{K_{ia}}{A}\right)\frac{1}{B} + \frac{1}{V_1}\left(1 + \frac{K_a}{A}\right)$$

The intercepts and slopes of the primary plots were replotted according to

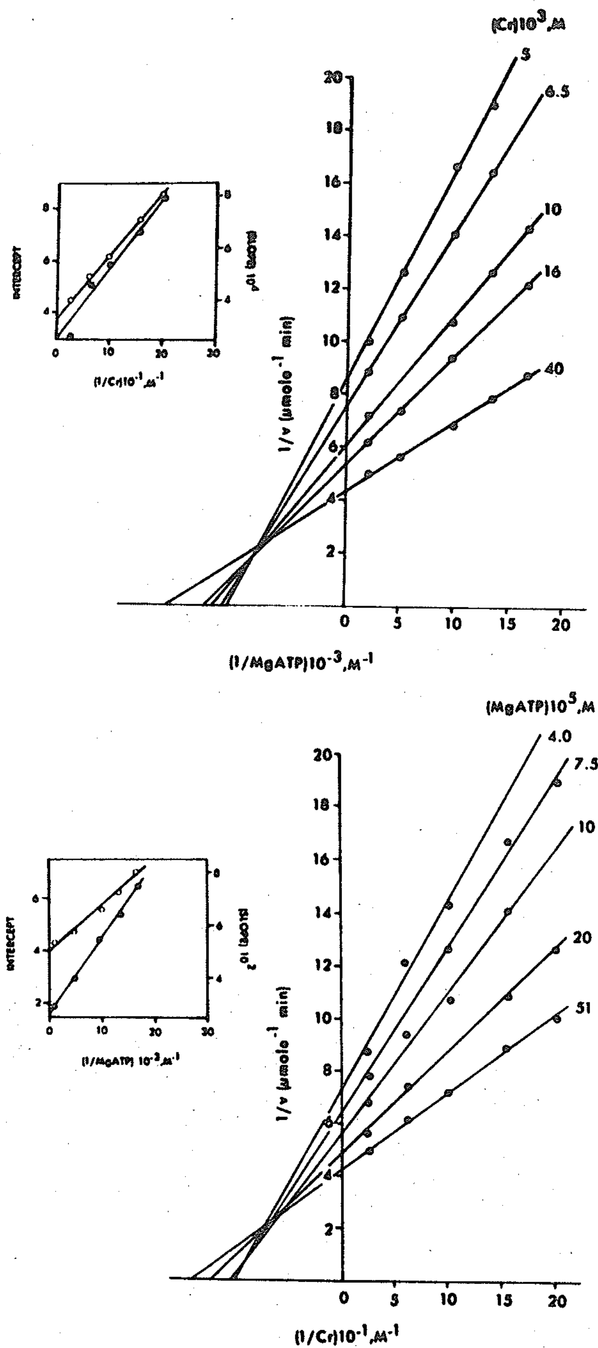
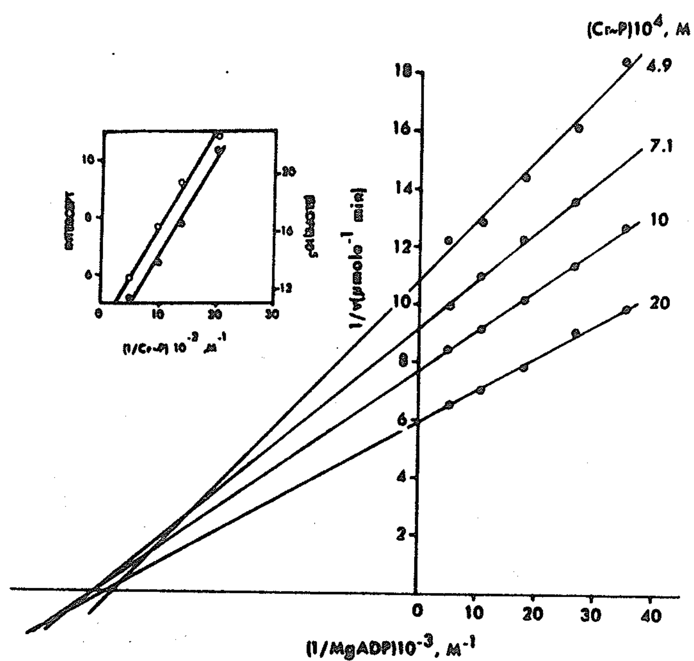


Figure 8. Kinetics of the forward reaction catalyzed by mitochondrial creatine kinase at pH 8.1 and 30°. The concentrations of the fixed substrates are as indicated. The insets show the slope (●-●-●) and intercept (o-o-o) replots.



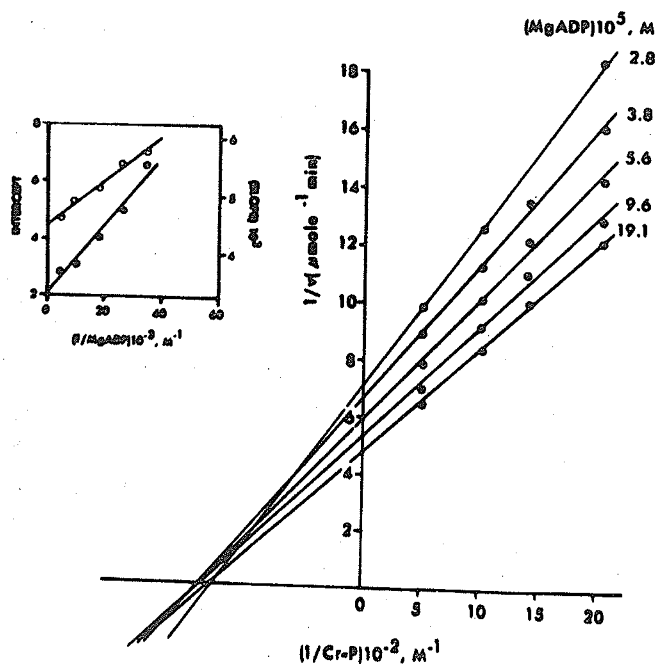
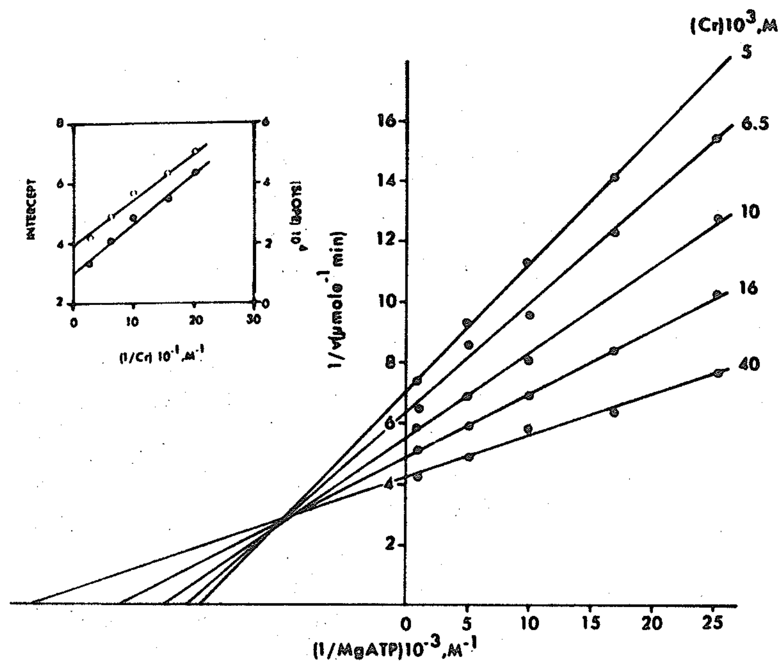


Figure 9. Kinetics of the reverse reaction at pH 8.1 and 30^0. (●-●-● slope, o-o-o intercept replot)



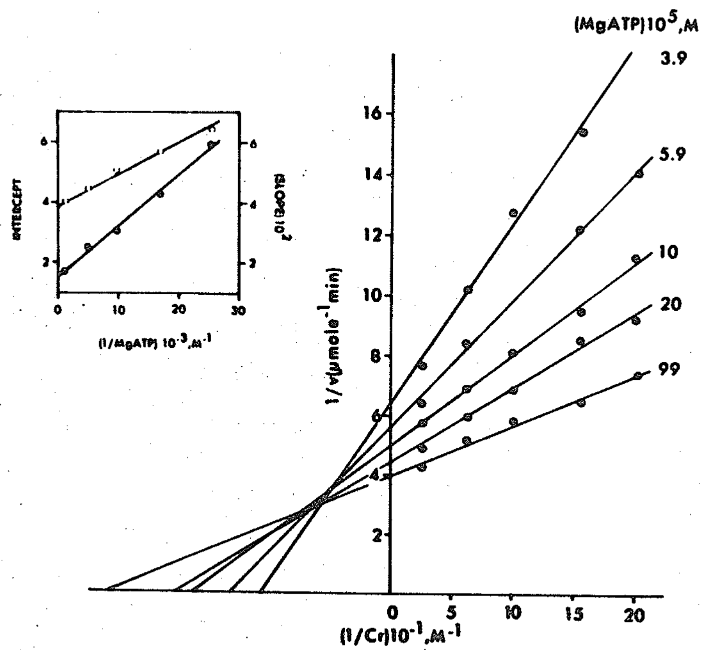


Figure 10. Kinetics of the forward reaction at pH 7.4 and 30°. (●-●-● slope, o-o-o intercept replot)

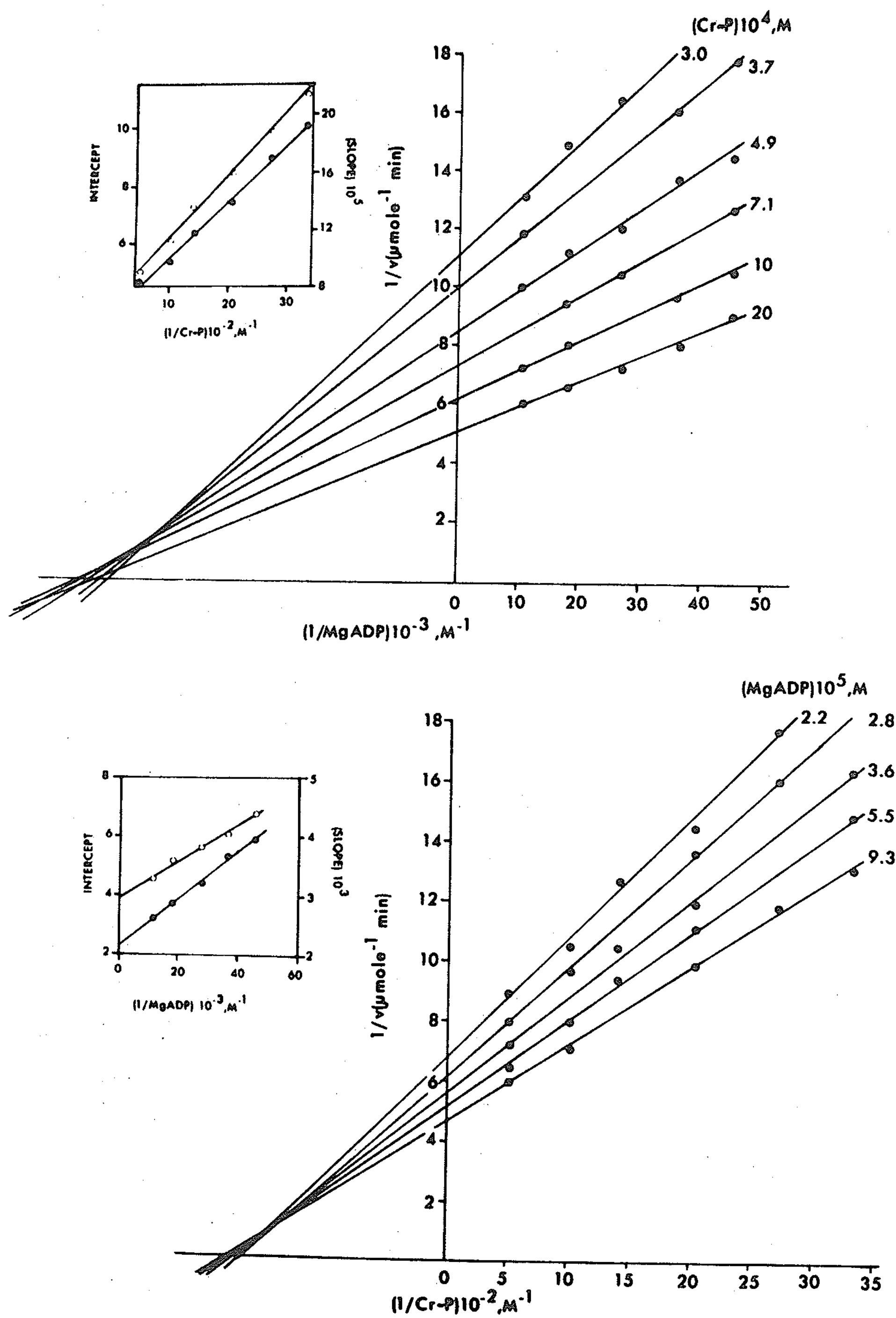


Figure 11. Kinetics of the reverse reaction at pH 7.4 and 30°. (●-●-● slope, o-o-o intercept replot)

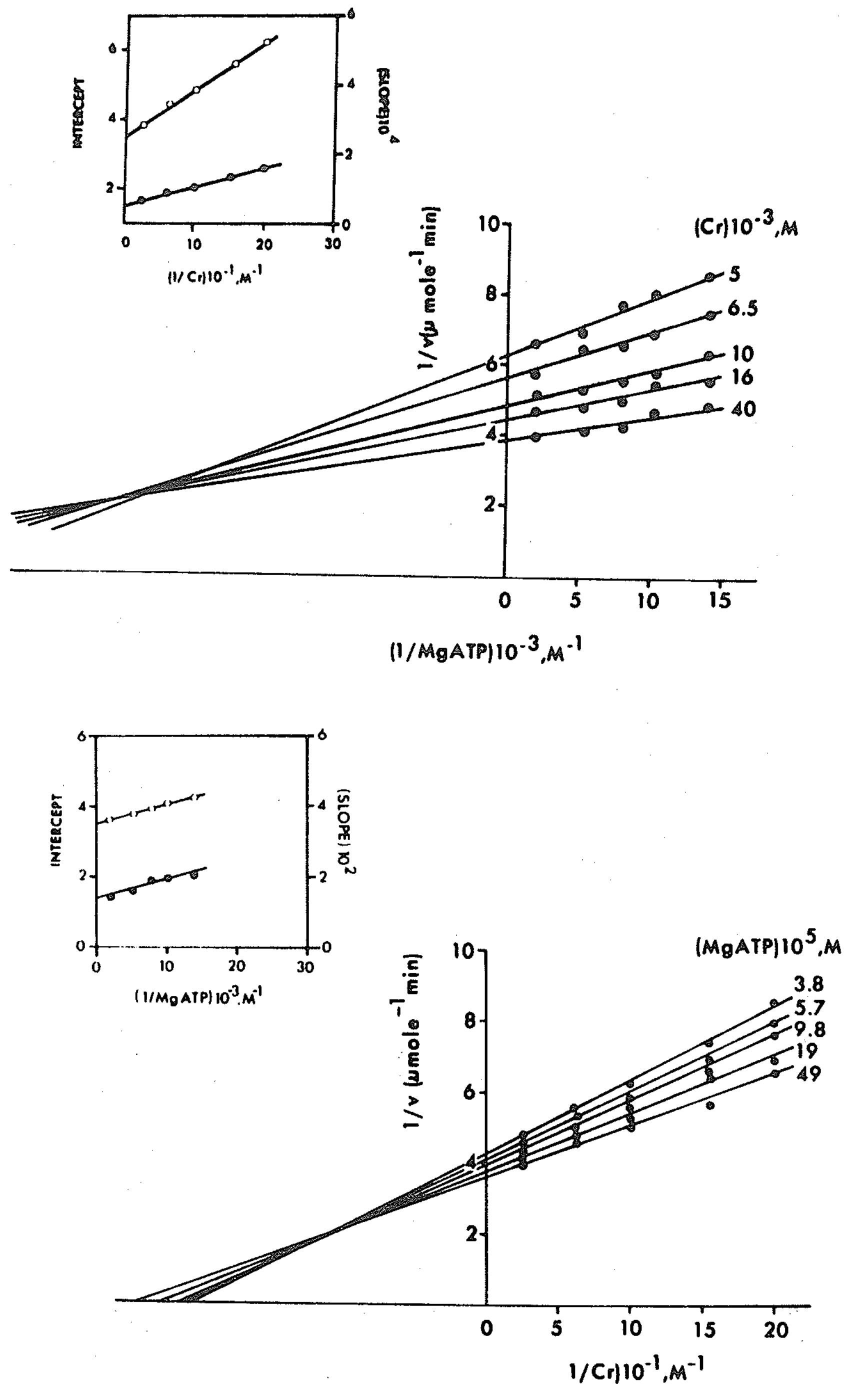


Figure 12. Kinetics of the forward reaction at pH 6.8 and 30^0. (●-●-● slope,o-o-o intercept replot)

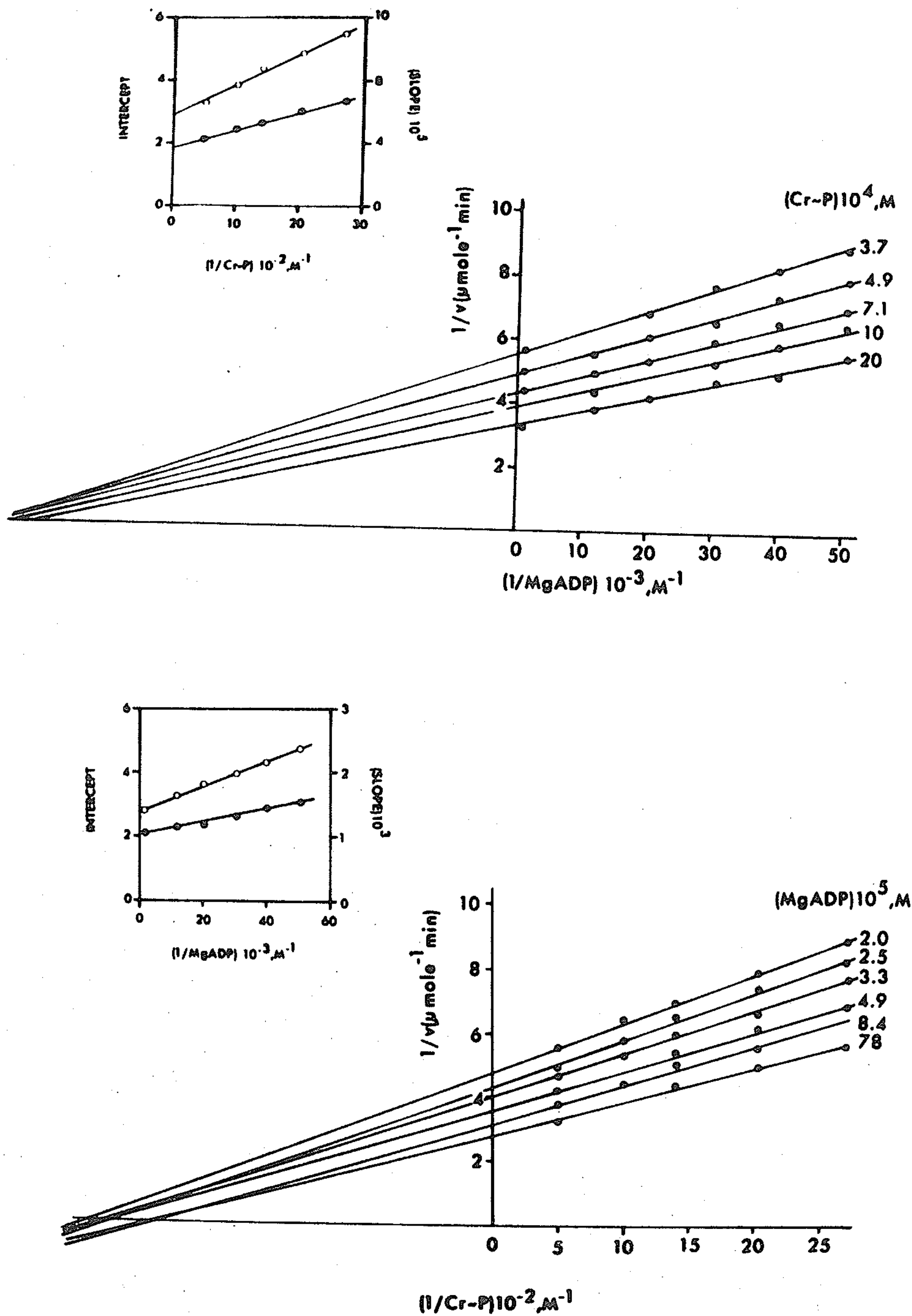


Figure 13. Kinetics of the reverse reaction at pH 6.8 and 30°. (●-●-● slope, o-o-o intercept replot)

Florini and Vestling (125) and the kinetic parameters were evaluated from the secondary replots thus obtained. By this procedure one set of experimental data yields two values for every parameter which should be identical so that the agreement between them may be regarded as a measure of the internal consistency of the data. The average of the two values was used as the experimental result.

All experiments were performed at least twice, in random order, with repetitions done at different times, and were evaluated without reference to previously completed experiments. Whereas one particular enzyme preparation (no. 59) was used at least once in a given experiment, other preparations were utilized as well. Average results from a number of experiments are given in Table 3.

In general terms, the magnitude of the kinetic parameters varies with pH and the changes follow quite regular patterns. All Michaelis and inhibition constants increase with increasing pH in both reactions. Their values are approximately two times larger at pH 8.1 than at 6.8. Since this trend is evident for all substrates, the mechanism from which it arises does not appear to be a localized change such as the protonation, exposure or displacement of a specific group, but rather a gross change in the conformation of the catalytic site such as might result from a general tightening up of the entire molecule.

The factor of 2 to 3 between K_{ia} and K_a values in the forward reaction persists at all pH levels which can also be said for the factor of near-unity between the same constants in the reverse reaction. The magnitude of these parameters does not depend on the identity of the enzyme preparation nor on its specific activity.

Maximal velocities conform to the trend exhibited by the pH-

TABLE 3

Kinetic Constants of Mitochondrial Creatine Kinase

at Various pH Values and 30°

Kinetic Constant	Substrate	Units	Average Values at pH 8.1	pH 7.4	pH 6.8
			(2)[a]	(3)[a]	(2)[a]
K_a	MgATP	mM	0.052±.005	0.031±.004	0.021±.007
K_{ia}	MgATP	mM	0.119±.011	0.097±.010	0.046±.006
K_b	Cr	mM	6.1±.3	4.6±.6	4.3±.6
V_1		$\frac{\mu mole}{min\ mg}$	364± 7	309± 9	264± 37
			(2)[a]	(5)[a]	(2)[a]
K_q	MgADP	mM	0.023±.005	0.019±.003	0.013±.001
K_{iq}	MgADP	mM	0.017±.002	0.020±.003	0.010±.001
K_p	Cr∿P	mM	0.73±.03	0.50±.09	0.37±.02
V_2		$\frac{\mu mole}{min\ mg}$	180[b]	486[b]	776[b]

[a]The number of experiments is given in parentheses.

[b]V values stated without standard deviation represent the highest single result obtained for a particular experiment. All V values reported were obtained with enzyme preparation 59 in optimal condition.

activity profiles described in part IV. A. With increasing pH they increase in the forward reaction and decrease in the reverse reaction with the rate of change being much greater in the reverse reaction. As the Michaelis and inhibition constants are also increasing in the forward reaction, the increase in the maximal velocity cannot simply be due to an increase in the affinity of the enzyme for its substrates; rather, there must be a change in the rate-controlling step as the pH changes.

The specific activity of the enzyme varied appreciably from one preparation to another. The short-term activity of a given preparation was observed to be dependent upon three factors: presence of reducing agent, temperature and dilution. While it was possible to increase the activity by adding more reducing agent to an aliquot of the stock enzyme solution, it was found that the degree of activation achieved was not entirely reproducible and that the resulting level of activity could not be strictly maintained. Therefore, it was preferred not to reactivate the enzyme in order to ensure that its activity was as nearly as possible the same for all experiments. Towards this end experiments in which the maximal velocities were to be determined and compared to one another were performed within the shortest possible period of time with a recently prepared, fully active enzyme preparation, which contained functional reducing agent, which had not been diluted for the purpose of making volume measurements easier, and which was exposed to ice-bath temperature for no longer than one hour.

Standard deviations for the average values of the maximal velocities obtained in the reverse reaction are in the 10% to 20% range. Since the highest individual results yield better values for the equilibrium constant by the Haldane relationship than the averaged results, they are included

in the table rather than the average values. The justification for such selective treatment of data lies in the fact that the experiments could be repeated only at appreciable time intervals during which a gradual decrease in the activity of the enzyme occurred and that no attempt was made for the aforementioned reasons to regain full activity by reactivation.

b) Haldane Relationships

The thermodynamic equilibrium constant for the multi-substrate system of creatine kinase is given by

$$K_{eq} = \frac{(MgADP^{-})(Cr{\sim}P^{2-})}{(MgATP^{2-})(Cr)}\,(H^{+})$$

The equilibrium has been studied by Noda et al. (126) and Nihei et al. (33). The findings have been critically evaluated by Kuby and Noltmann (22) who have determined that the equilibrium constant is pH dependent and have assigned to it a value of 2.81 ($\pm$.41) x 10^{-10}.

The Haldane equation relates the thermodynamic equilibrium constant to the parameters of a kinetic mechanism. While the studies reported so far have indicated a sequential mechanism, subsequent product inhibition studies (section IV. B.2.) have allowed to identify it as Ordered Bi Bi for which the following Haldane relationships exist (124)

$$K_{eq} = \frac{V_1 K_p K_{iq}}{V_2 K_{ia} K_b} = \left(\frac{V_1}{V_2}\right)^2 \frac{K_{ip} K_q}{K_a K_{ib}}$$

Values for the equilibrium constant calculated from results displayed in Tables 3 and 9 are given in Table 4.

Whereas the experimentally determined values of the equilibrium constant may appear to be in poor agreement with the accepted value, they agree nevertheless within the range of estimated error as determined from

the standard deviations associated with each of the parameters involved in its calculation, taking into account that standard deviations for V_2 have not been considered. The fact that the estimated error is very high is not so much the fault of high uncertainties in the individual constants, although these admittedly do exist, but rather is due to the large number of parameters used to calculate the equilibrium constant.

TABLE 4

Values of K_{eq} Obtained from the Haldane Relationships for the Ordered Mechanism of the Reaction Catalyzed by Mitochondrial Creatine Kinase at 30° and Various pH Values

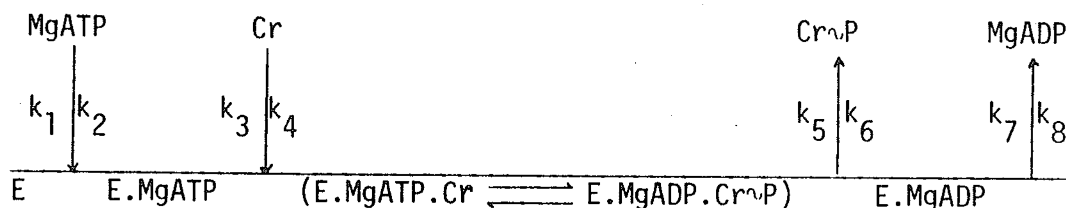
Haldane Expression	Value of K_{eq} at pH 8.1	pH 7.4	pH 6.8
$\frac{V_1 K_p K_{iq}}{V_2 K_{ia} K_b} (H^+)$	$2.8(\pm.9)\times10^{-10}$	$5.7(\pm3.4)\times10^{-10}$	$10.1(\pm5.7)\times10^{-10}$
$(\frac{V_1}{V_2})^2 \frac{K_{ip} K_q}{K_a K_{ib}} (H^+)$		$3.4(\pm2.2)\times10^{-10}$	

Consequently, the Haldane relationship of a two-substrate reaction appears to be of limited value in checking the accuracy of experimentally determined kinetic constants. This limitation to its usefulness is expressed by Kuby and Noltmann (22), and Cleland (124) points out that Haldanes are useless for this same reason for the purpose of distinguishing between mechanisms. It can thus be said that the agreement that is achieved is satisfactory considering the appreciable uncertainties contained in the

kinetic parameters and the sensitivity of the Haldane equations to such uncertainties.

c) Rate Constants

With a knowledge of the values of the kinetic constants and the identity of the mechanism, it is possible to calculate the rate constants for each step in the mechanism shown in Scheme 3. The values calculated with the aid of the relationships given by Cleland (124) for the Ordered Bi Bi mechanism are given in Table 5. An equivalent weight per catalytic center of 42,560 has been used (86).



SCHEME 3

At pH 7.4 the dissociations of the nucleotides from the enzyme, denoted by the rate constants k_7 and k_2, represent the rate-controlling steps in both the forward and reverse reactions. As all unimolecular steps in one direction are faster than the turnover number, V/E_t, for that reaction and the equilibrium constant is fairly close to the accepted values, no isomerizations are indicated at pH 7.4. This is a significant finding, because isomerizations would affect the results of product inhibition studies.

At pH 8.1 the value of k_7, one of the unimolecular steps in the forward reaction, is lower than the turnover number. This means that the E.MgADP complex isomerizes and renders the value of k_7 invalid.

TABLE 5

Rate Constants for the Reaction Catalyzed by Mitochondrial Creatine Kinase at 30° and Various pH Values

Constant	Units	pH 8.1	pH 7.4	pH 6.8
V_1/E_t	min^{-1}	$1.5x10^4$	$1.3x10^4$	$1.1x10^4$
k_1	$min^{-1}M^{-1}$	$3.0x10^8$	$4.2x10^8$	$5.4x10^8$
k_2	min^{-1}	$3.6x10^4$	$4.1x10^4$	$2.5x10^4$
k_3	$min^{-1}M^{-1}$	-	$6.4x10^6$	-
k_4	min^{-1}	$9.8x10^3$	$4.2x10^4$	-
k_5	min^{-1}	-	$3.3x10^4$	$2.0x10^4$
k_6	$min^{-1}M^{-1}$	-	$7.4x10^7$	-
k_7	min^{-1}	$5.7x10^3$	$2.2x10^4$	$2.5x10^4$
k_8	$min^{-1}M^{-1}$	$3.0x10^8$	$1.1x10^9$	$2.5x10^9$
V_2/E_t	min^{-1}	$6.9x10^3$	$2.1x10^4$	$3.3x10^4$

Also, k_3, k_5 and k_6 cannot be calculated. In the reverse reaction the rate-controlling step has changed from the k_2 step at pH 7.4 to the dissociation step involving the central complex with the rate constant k_4.

Likewise, the dissociation of the central complex with rate constant k_5 becomes the rate-controlling step in the forward reaction at pH 6.8. Since the value of k_2 is less than that of the turnover number for the reverse reaction, isomerization of E.MgATP probably occurs, the value of k_2 becomes invalid, and k_3, k_4 and k_6 cannot be calculated.

The values of k_1 and k_8 which reflect the rate of formation of the enzyme-nucleotide complexes increase with a decrease in pH indicating that nucleotide binding is aided by protonation of the enzyme. The differences in k_2 between pH 8.1 and 7.4 and in k_7 between pH 7.4 and 6.8 are not considered significant. The dissociation of creatine from the central complex is hindered by raising the pH from 7.4 to 8.1, while the dissociation of creatine phosphate from the central complex is aided by raising the pH from 6.8 to 7.4.

d) Comparison with Published Work

Michaelis constants determined in this study for the isoenzyme from bovine heart mitochondria and those reported for the enzyme in whole rat heart mitochondria by Jacobus and Lehninger (73) and by Saks et al. (76) are listed in Table 6. The agreement between the results from all three studies is good for the guanidino substrates but less satisfactory for the nucleotides. Whether the differences between the results from this study and the published ones are due to differences in the species, in the molecular environment or in experimental procedure is an open question. The published studies are alike in each of these three criteria and unlike the present study. Yet, the differences in results between them

TABLE 6

Kinetic Constants of Mitochondrial Creatine Kinase at

pH 7.4 and 30°

Constant	Substrate, Units	Bovine Heart In Vitro	Rat Heart In Situ	Rat Heart In Situ
K_a	MgATP,mM	0.031	.100	0.730
K_b	Cr,mM	4.6	6.0	5.0
V_1	$\frac{\mu mole}{min\ mg}$	309	0.62	0.76
K_q	MgADP,mM	0.019	0.035	0.052
K_p	Cr∿P,mM	0.50	0.72	0.49
V_2	$\frac{\mu mole}{min\ mg}$	486		3.5
Reference		this study	73	76

are as great as the differences between each of them and the present study. Therefore, it seems reasonable to conclude that the differences in the results are due to experimental uncertainty and that within the resultant range of error the three studies support rather than contradict one another. If this is correct, then it must be concluded that at least the Michaelis constants are not subject to change upon the removal of the enzyme from its native environment.

Kinetic constants of mitochondrial creatine kinase as determined in this study are compared and contrasted to those of various cytoplasmic creatine kinases in Table 7. The difference between the mitochondrial and the cytoplasmic MM forms is pronounced with the mitochondrial isoenzyme possessing much smaller Michaelis and inhibition constants. There exists a difference of roughly an order of magnitude among nucleotide substrates, while the difference for the guanidino substrates is variable.

The similarity between the enzyme under study and the cytoplasmic BB type from calf brain is remarkable. At pH 7.4 the parameters differ only by a factor of 2, at a higher pH value by a somewhat larger factor particularly in the case of the inhibition constants. As the kinetic mechanism of the BB isoenzyme has never been determined, it would be most interesting to do so in view of the enzyme's relationship with regard to subcellular localization to the MM form for which a rapid-equilibrium Random mechanism has been established and in view of its resemblance to the mitochondrial form whose kinetic mechanism is shown in this study to be Ordered.

TABLE 7

Kinetic Constants of Creatine Kinases from Various Sources at 30°

Constant	Substrate, Units	Mitochondrial CK from Bovine Heart		Cytoplasmic Creatine Kinase from			
				Calf Brain(BB)		Muscle(MM)	
		pH 7.4	pH 8.1	pH 7.4	pH 8.8	Calf pH 8.8	Rabbit pH 8.0
K_a	MgATP,mM	0.031	0.052	0.047	0.13	0.78	0.48
K_{ia}	MgATP,mM	0.097	0.119	0.18	0.93	0.97	1.2
K_b	Cr,mM	4.6	6.1	3.0	3.7	21.0	6.1
V_1	$\frac{\mu mole}{min\ mg}$	309	364	120	200	220	
K_q	MgADP,mM	0.019	0.023	0.030	0.010	0.094	0.05
K_{iq}	MgADP,mM	0.020	0.017	0.039	0.120	0.17	0.17
K_p	Cr∿P,mM	0.50	0.73	0.52	2.0	23.0	2.9
V_2	$\frac{\mu mole}{min\ mg}$	486	161	510	70	130	
Reference		this study		65	65	65	25

2. Product Inhibition Studies

a) Fixed Substrate Present at a Non-Saturating Concentration

Initial velocity measurements were made as a function of the concentration of one of the substrates with the other substrate fixed at one non-saturating concentration of one of the products. The double reciprocal plots and the secondary replots are shown in Figures 14 to 17.

The inhibition patterns obtained in the forward reaction are competitive for the inhibition of MgATP by MgADP and noncompetitive for all other substrate-product combinations. In the reverse reaction the only competitive pattern is again observed with the two nucleotide compounds present, while the other three patterns are noncompetitive. All slope and intercept replots are found to be linear.

The inhibition patterns are indicative of an Ordered Bi Bi mechanism in which the magnesium complexes of the nucleotides form the A-Q substrate-product pair and the guanidino compounds represent the B-P pair. Due to the linearity of the replots all inhibitions can be termed linear according to Cleland's nomenclature (127). Consequently, these inhibition experiments were evaluated on the basis of the rate equation for the Ordered Bi Bi mechanism in which the denominator terms containing the inhibiting product either alone or together with one or both of the substrates had been retained.

With the guanidino product P present, the initial rate equation takes the form

$$v = \frac{V_1 AB}{K_{ia}K_b + K_bA + K_aB + AB + \frac{K_qK_{ia}K_bP}{K_pK_{iq}} + \frac{K_bK_qAP}{K_pK_{iq}} + \frac{ABP}{K_{ip}}}$$

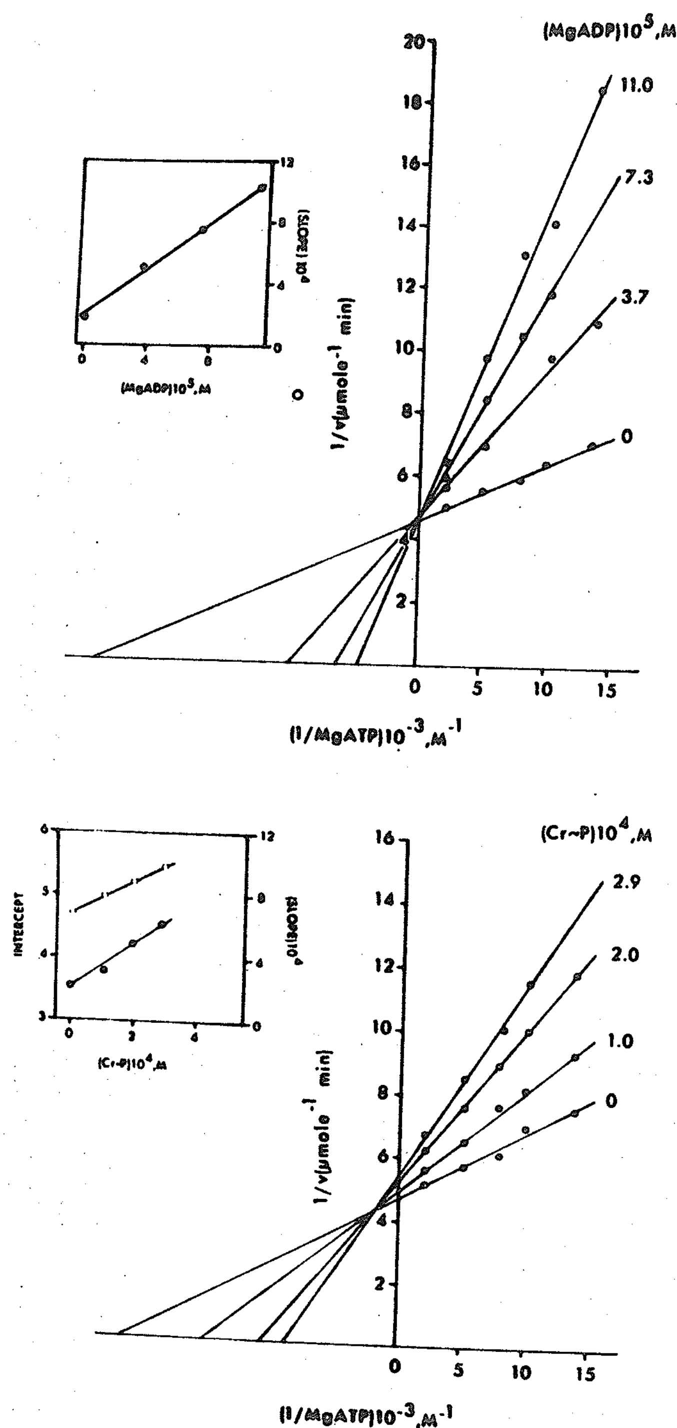


Figure 14. Product inhibition of the forward reaction catalyzed by mitochondrial creatine kinase at pH 7.4 and 30° with MgATP as the variable substrate and creatine present at the non-saturating concentration of 1.6 x 10^{-2} M. Inhibitory product concentrations are as indicated. The insets show the slope (●-●-●) and intercept (o-o-o) replots.

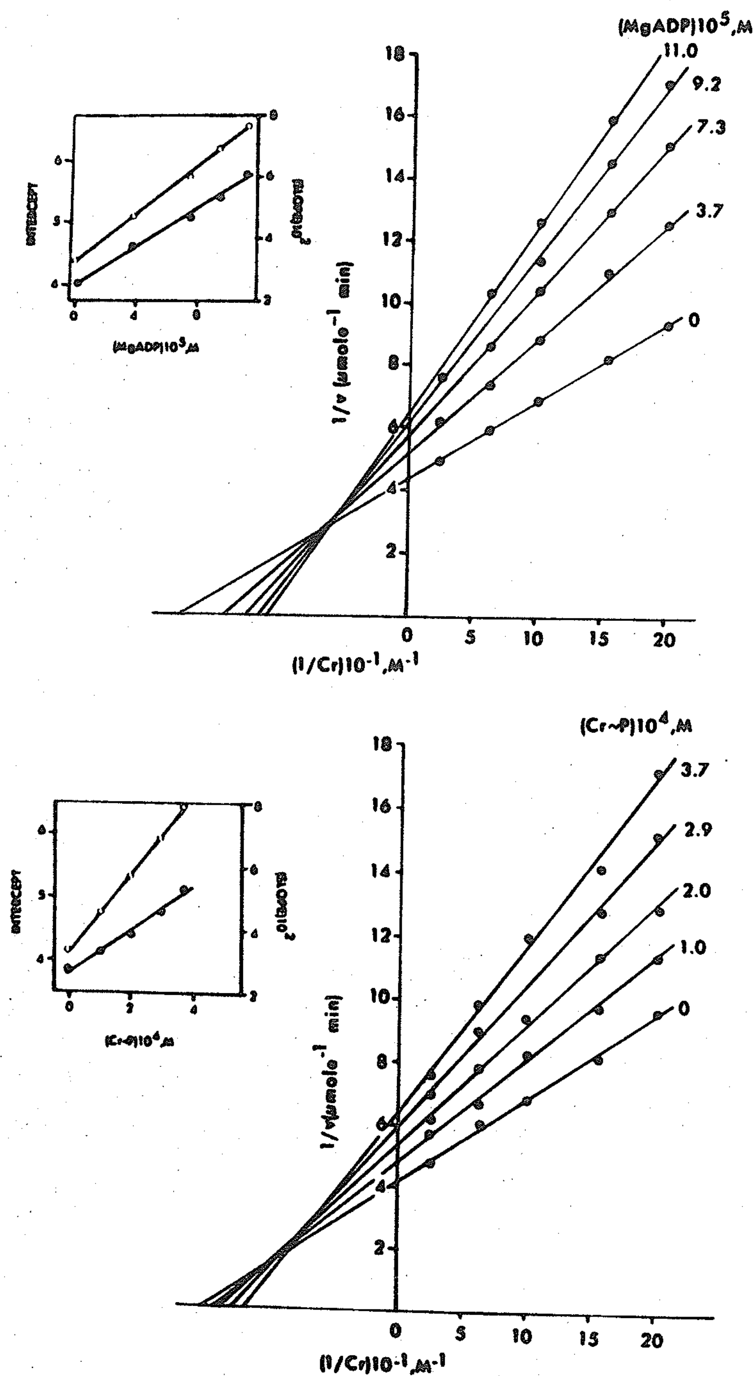
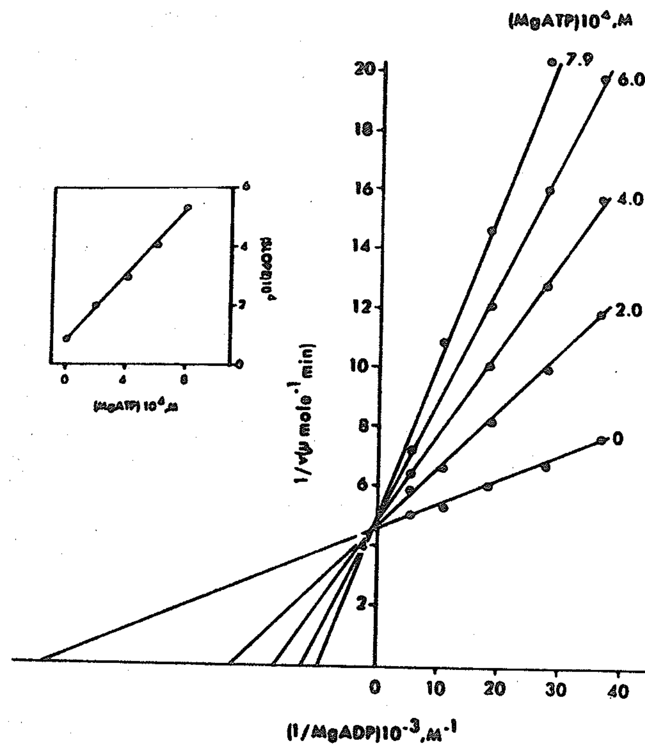


Figure 15. Product inhibition of the forward reaction at pH 7.4 and 30^0 with creatine as the variable substrate and MgATP present at the non-saturating concentration of 2.0×10^{-4} M. Inhibitory product concentrations are as indicated. (●-●-● slope, o-o-o intercept replot)



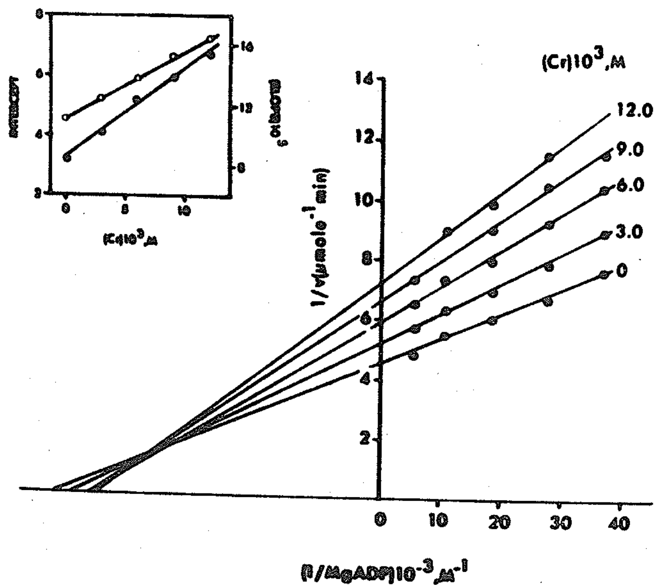


Figure 16. Product inhibition of the reverse reaction at pH 7.4 and 30^{0} with MgADP as the variable substrate and creatine phosphate present at the non-saturating concentration of 1.0 x 10^{-3} M. Inhibitory product concentrations are as indicated. (●-●-● slope, o-o-o intercept replot)

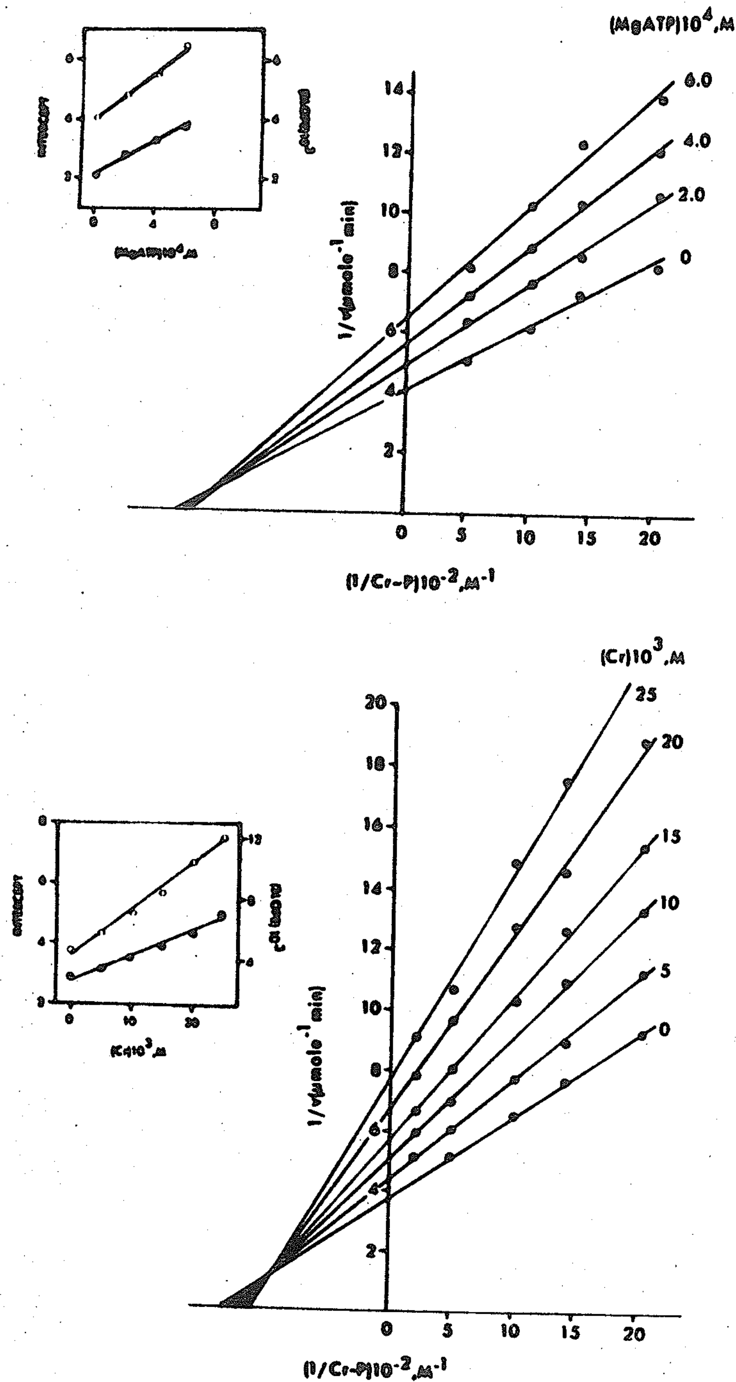


Figure 17. Product inhibition of the reverse reaction at pH 7.4 and 30^{0} with creatine phosphate as the variable substrate and MgADP present at the non-saturating concentration of 9.3 x 10^{-5} M. Inhibitory product concentrations are as indicated. (●-●-● slope, o-o-o intercept replot)

When A, the nucleotide substrate, is variable, the reciprocal rate equation is

$$\frac{1}{v} = \frac{K_a}{V_1}\left[1 + \frac{K_{ia}K_b}{K_a B}\left(1 + \frac{K_q P}{K_p K_{iq}}\right)\right]\frac{1}{A} + \frac{1}{V_1}\left[1 + \frac{K_b}{B}\left(1 + \frac{K_q P}{K_p K_{iq}}\right) + \frac{P}{K_{ip}}\right]$$

The primary plot will indicate noncompetitive inhibition and produce replots which are unsuitable for the determination of either of the product inhibition constants K_{ip} or K_{iq}, because the experiments were performed at only one concentration of the fixed substrate, while the inhibition constants are functions of the fixed substrate concentration.

When B, the guanidino substrate, is variable, the reciprocal rate equation becomes

$$\frac{1}{v} = \frac{K_b}{V_1}\left(1 + \frac{K_{ia}}{A}\right)\left(1 + \frac{K_q P}{K_p K_{iq}}\right)\frac{1}{B} + \frac{1}{V_1}\left(1 + \frac{K_a}{A} + \frac{P}{K_{ip}}\right)$$

Product P is a noncompetitive inhibitor relative to B and the intercept replot is useful in determining K_{ip}. The slope replot was used to assign a value to K_p with the aid of a K_{iq}/K_q ratio determined in the absence of products (section IV. B.1.).

With the nucleotide product Q present the initial rate equation is

$$v = \frac{V_1 AB}{K_{ia}K_b + K_b A + K_a B + AB + \frac{K_{ia}K_b Q}{K_{iq}} + \frac{K_a BQ}{K_{iq}}}$$

The reciprocal form written with the nucleotide A as the variable substrate is

$$\frac{1}{v} = \frac{K_a}{V_1}\left(1 + \frac{Q}{K_{iq}}\right)\left(1 + \frac{K_{ia}K_b}{K_a B}\right)\frac{1}{A} + \frac{1}{V_1}\left(1 + \frac{K_b}{B}\right)$$

The primary plot will reflect competitive inhibition and the abscissa intercept of the slope replot will yield a value for K_{iq}.

When B is the variable substrate, the reciprocal rate equation becomes

$$\frac{1}{v} = \frac{K_b}{V_1}\left[1 + \frac{K_{ia}}{A}\left(1 + \frac{Q}{K_{iq}}\right)\right]\frac{1}{B} + \frac{1}{V_1}\left[1 + \frac{K_a}{A}\left(1 + \frac{Q}{K_{iq}}\right)\right]$$

The primary plot will indicate noncompetitive inhibition and the slope and intercept replots can be used to calculate K_{iq}.

The numerical results of these experiments are listed in Table 8. The values obtained from slope replots for the inhibition constant of MgADP are in good agreement with those obtained from initial velocity measurements in the reverse reaction in the absence of products. The agreement for the value derived from intercept replots is less good, but the average value 0.026 $\pm$ .006 mM is still easily within the range of experimental uncertainty in the average value of 0.020 $\pm$ .003 mM obtained in the absence of products.

The results for the inhibition constant of MgATP are consistently higher than those obtained from experiments in the forward direction done in the absence of products. The average value of 0.15 $\pm$ .02 mM does not agree within the standard deviation with the K_i value of 0.097 $\pm$ .010. The discrepancy cannot be removed by evaluating the recorder traces with a bias towards its elimination. It could be an experimental artifact, since the assay mixtures contain MgATP which exhibits some buffering activity in addition to MgADP and creatine phosphate which also buffer and are thus the most highly buffered reaction mixtures. As buffering activity would be expected to affect low-velocity assays to a greater degree

TABLE 8

Kinetic Constants (in mM) of Mitochondrial Creatine Kinase at pH 7.4 and 30° Obtained from Product Inhibition Experiments Utilizing Non-Saturating Fixed-Substrate Concentrations[a]

Type of Replot	Variable Substrate	Inhibiting Product	$K_{i(MgATP)}$	$K_{i(MgADP)}$	$K_{i(Cr)}$	$K_{i(Cr\sim P)}$	$K_{(Cr)}$	$K_{(Cr\sim P)}$
Slope	A	Q	0.145	0.023				
				0.019				
Slope	B	Q	0.145	0.026				
			0.139	0.022				
Intercept	B	Q	0.162	0.028				
			0.176	0.037				
Intercept	B	P			16.0	0.58		
					14.2			
					19.7			
Slope	B	P					4.8	0.41
							5.2	
							5.9	
	Average Values		$0.15 \pm .02$	$0.026 \pm .006$	16.6 ± 2.8		$5.3 \pm .6$	

[a] Replicate values listed for a given type of replot were obtained from replicate experiments.

than higher-velocity reactions, the overall effect would be a decrease in the slope of the secondary replots and thus an increase in K_i making this line of reasoning appear plausible.

Substrate inhibition of the dead end EA_2 type could be postulated to provide another explanation. If it did occur, it would give rise to low apparent inhibition constants (128) which means that the K_i value of $0.097 \pm .010$ would have to be considered low. No indication of this type of substrate inhibition in the forward reaction was ever observed for MgATP, but some curvature in double reciprocal plots for the reverse reaction was detected at high concentrations of MgADP.

The inhibition constants of the guanidino substrates have not been determined in previous experiments and consequently no assessment of their accuracy can be made at this point.

b) Fixed Substrate Present at a Saturating Concentration

Initial velocity data were collected in experiments in which the concentration of one of the substrates was varied and that of the other was held constant at a saturating, or near-saturating, level (MgATP at 32 times its K_i, MgADP at 91 times its K_i, Cr∿P at 81 times its K_b) in the absence and in the presence of one of the products fixed at one concentration (Figures 18,19). Experiments involving creatine as the saturating substrate were not possible due to the low solubility of the compound relative to its Michaelis constant. The other three substrates exhibit some buffering activity at pH 7.4 which causes no serious problems at normal concentrations but which requires special operating techniques to be employed when concentrations are very high; more specifically, the titration had to be "pushed" very hard in order to insure that even the slightest change in pH would activate the titrator. The double reciprocal

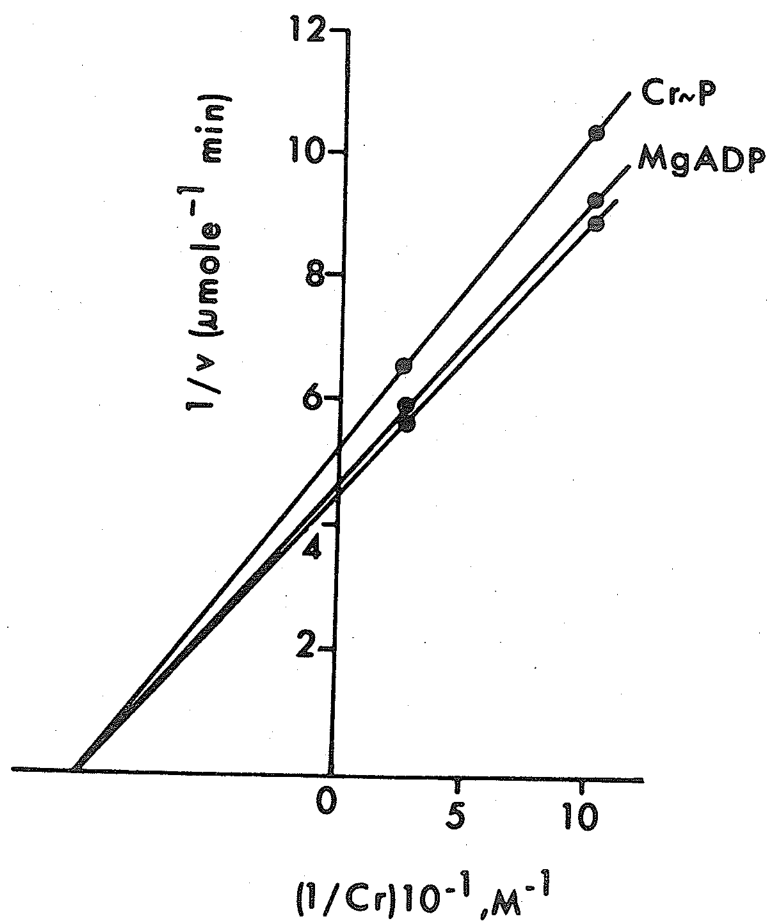
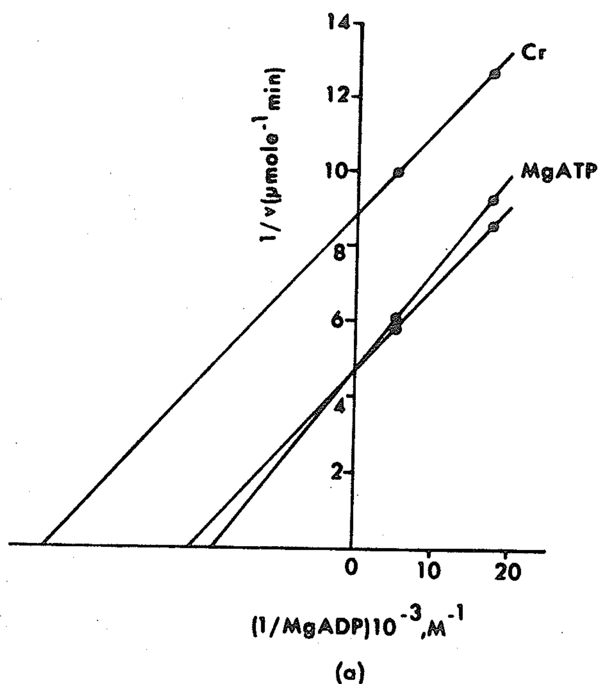


Figure 18. Product inhibition of the forward reaction at pH 7.4 and 30° with MgATP present at a saturating concentration of 3.11×10^{-3} M. The concentrations of the inhibitory products MgADP and Cr~P are 4.73×10^{-5} M and 1.95×10^{-4} M, respectively.



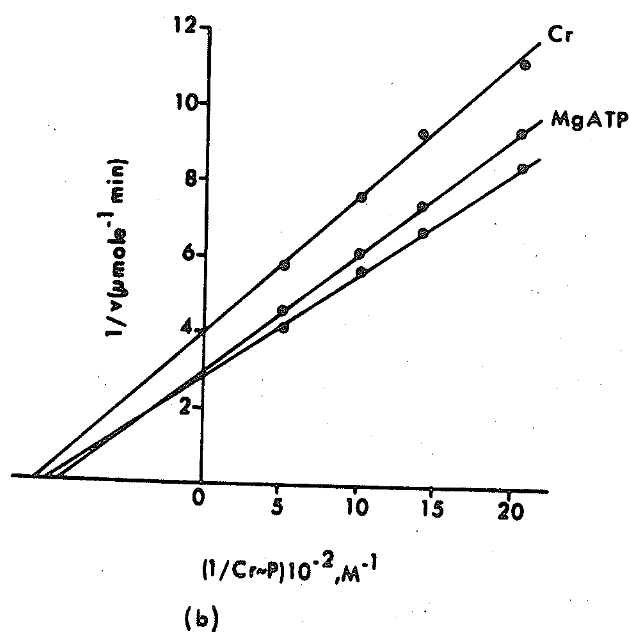


Figure 19. Product inhibition of the reverse reaction at 30° and pH 8.1 (a) and 7.4 (b) with the fixed substrate present at a saturating concentration (Cr∿P at 4.04×10^{-2} M, MgADP at 1.83×10^{-3} M). The concentrations of the inhibitory products MgATP and Cr are 3.94×10^{-4} M and 6.1×10^{-3} M, respectively. The pH of 8.1 was used due to the high rate of spontaneous hydrolysis of Cr∿P at pH 7.4.

plots obtained in this fashion are not directly comparable to those obtained under non-saturating conditions. For example, the slope of a given plot was often found to be greater than would have been expected on the basis of results from experiments utilizing subsaturating substrate levels. This means that the evaluation of these experiments had to be self-contained.

To this purpose expressions were derived which relate the slope or intercept of the plot which involves inhibition, designated m_i and b_i, respectively, to the slope or intercept of the plot obtained in the absence of product, designated m_o and b_o.

For example, when the slope term of the reciprocal initial rate equation for A as the variable substrate with product P present is divided by the slope term of the corresponding form of the rate equation which applies in the absence of products and the term containing the high value for the concentration of B in the denominator is cancelled, the following expression is obtained

$$1/K_{ip} = \frac{b_i/b_o - 1}{P}$$

The slope ratio m_i/m_o for this experiment reduces to 1 so that inhibition by P with B saturating will produce no slope effect making the predicted inhibition pattern uncompetitive.

For A, B, and P present, A saturating, the following expressions are obtained

$$1/K_{ip} = \frac{b_i/b_o - 1}{P} \quad \text{and} \quad 1/K_p = \frac{(m_i/m_o - 1)K_{iq}}{K_q P}$$

With both the slope and the intercept being affected, the inhibition pattern should be noncompetitive.

For A, B, and Q present, B saturating, an experiment which like the one described first could be conducted only in the reverse direction, the inhibition pattern is predicted to be competitive, since only a slope expression is obtained

$$1/K_{iq} = \frac{{}^{m}i/m_o - 1}{Q}$$

Finally, for A, B, and Q present, A saturating, another noncompetitive pattern is predicted

$$1/K_{iq} = \frac{({}^{b}i/b_o - 1)A}{K_a Q} \quad \text{and} \quad 1/K_{iq} = \frac{({}^{m}i/m_o - 1)A}{K_{iq} Q}$$

Inhibition and Michaelis constants calculated by means of these relationships are given in Table 9. The K_i values obtained for the nucleotides are in good agreement with results obtained in previous experiments, although the variance among results involving MgATP is very high. The K_i values for the nucleotides agree well with those obtained under non-saturating conditions and the same can be said for their Michaelis constants.

3. Inhibition Studies with Substrate Analogs

Additional experiments were performed which provided some information on the substrate specificity of the enzyme and on its molecular mechanism. Some of these experiments were designed to aid in the assignment of the kinetic mechanism, while others were ancillary to attempts to improve the activity assay.

a) Studies with Creatine Analogs

i) Inhibition by Guanidinoacetate

Guanidinoacetate is a close structural analog to creatine but is

TABLE 9

Kinetic Constants (in mM) of Mitochondrial Creatine Kinase at pH 7.4 and 30° Obtained from Product Inhibition Experiments Utilizing Saturating Fixed-Substrate Concentrations

Type of Replot	Varied Substrate	Inhibiting Product	$K_{i(MgATP)}$	$K_{i(MgADP)}$	$K_{i(Cr)}$	$K_{i(Cr \sim P)}$	$K_{(Cr)}$	$K_{(Cr \sim P)}$
Slope	A	Q	2.51[a,b]					
			0.312[a,b,c]					
Slope	B	Q	0.101	0.005				
			0.029	0.033				
Intercept	B	Q	0.063	0.027				
			0.115	0.022				
Intercept	B	P			14.1	0.48		
					13.4			
Intercept	A	P			13.9			
					6.8[a,b]			
Slope	A	P					–[b,d]	
							6.3	
Slope	B	P					6.8	0.43
							–[d]	1.13[a]
Average values			$0.077 \pm .039$	$0.022 \pm .012$	$13.8 \pm .36$		$6.6 \pm .4$	
Average values including results from Table 8			$0.119 \pm .048$	$0.024 \pm .009$	15.2 ± 2.4	$0.53 \pm .07$	$5.8 \pm .8$	$0.42 \pm .01$

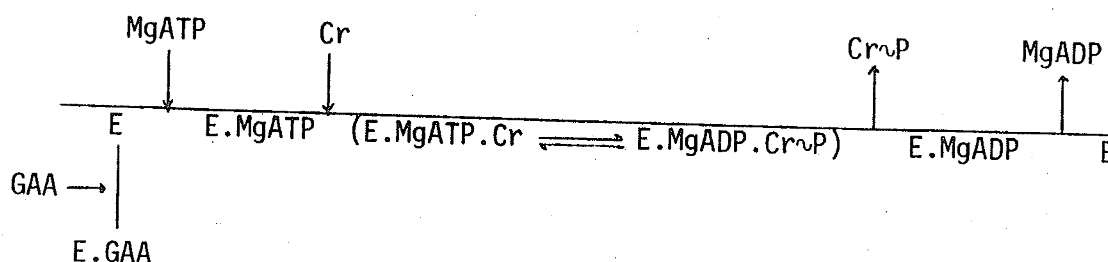
[a] Rejected on the basis of the Q test (138).

[b] Done at pH 8.1 due to high rate of hydrolysis of Cr∿P at pH 7.4.

[c] Replicate values are from replicate experiments.

[d] The observed inhibition effect was too small to yield a meaningful result.

completely inactive as an alternate substrate under normal assay conditions. These properties should make it a competitive inhibitor relative to creatine and a noncompetitive inhibitor relative to MgATP. The results shown in Figure 20 are contrary to these predictions. The compound produces competitive inhibition relative to the nucleotide substrates and noncompetitive inhibition in relation to the guanidino substrates which means that the analog can bind only to the free enzyme as shown in Scheme 4.



SCHEME 4

To check out these unexpected observations inhibition experiments were performed with the fixed substrates at saturating concentrations. The results support the previous findings. Saturation with the nucleotide substrates eliminates the inhibition relative to the guanidino substrates. When the creatine phosphate concentration was raised to a saturating level, the inhibition relative to MgADP remained competitive.

To contrast the fundamental difference in the action as inhibitors of the reverse reaction between creatine and guanidinoacetate, each was added to an assay mixture which contained both MgADP and creatine phosphate at saturating concentrations. The results were unequivocal: there was no inhibition by guanidinoacetate, whereas the inhibition by creatine appeared undiminished.

There are interesting implications arising from the different

behavior of the two guanidino compounds. The one structural feature which can account for the difference is the N-methyl group of creatine. It prevents the binding of creatine to the free enzyme, while its absence in guanidinoacetate allows this latter compound to bind. Various mechanisms could possibly account for its action.

An obstruction on the enzyme could interfere with the N-methyl group and prevent proper orientation of the substrate molecule for reaction to occur. Such a mechanism would explain why guanidinoacetate can bind to the free enzyme, but it cannot account for the fact that it does not bind to the E.MgATP complex.

Alternately, an attachment site into which the N-methyl group could fit might be required for creatine to bind at all. Such a site would be opened up only upon binding of MgATP. On the basis of this mechanism guanidinoacetate should bind neither to the free enzyme nor to the E.MgATP complex. Thus, the N-methyl group cannot by itself account for the difference in the affinity of the enzyme for the two analogs.

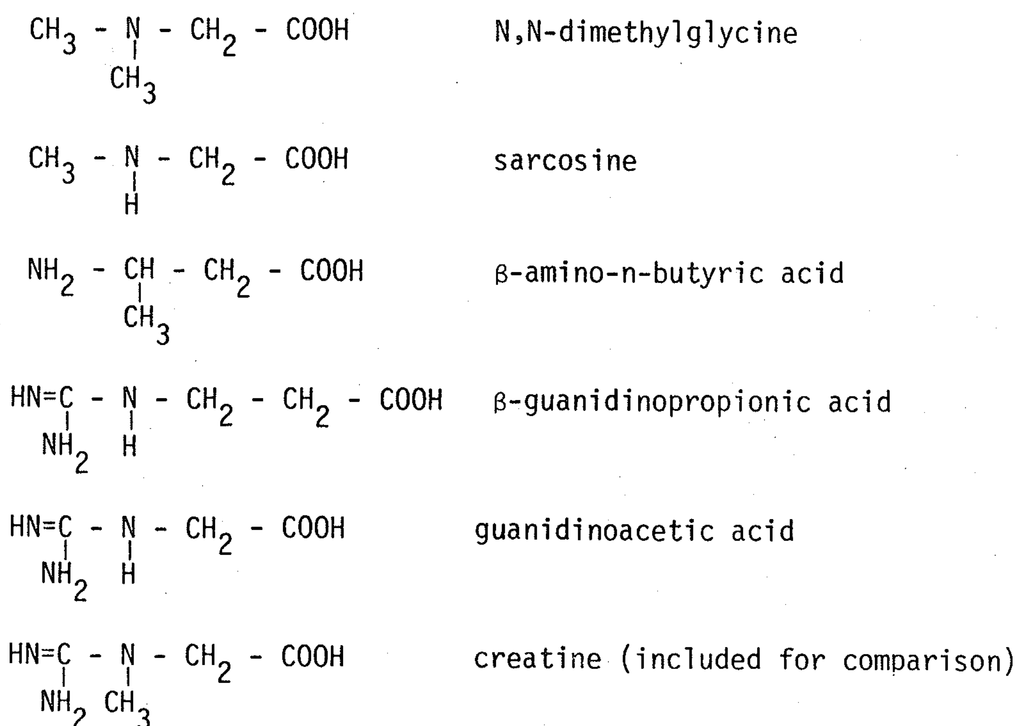
However, no uncompetitive substrate inhibition by creatine is observed which would indicate the formation of E.MgADP.Cr. This complex would produce an intercept versus reciprocal creatine concentration replot which is hyperbolic concave up (104, p. 147). The most likely reason why this complex does not form is a conformational change occurring in the enzyme upon binding of MgADP which would prevent subsequent binding of creatine. It does not appear unreasonable to postulate this adverse conformational change to occur also upon binding of MgATP which would prevent guanidinoacetate from binding to the binary complex, while creatine would be able to overcome the hindrance by virtue of its N-methyl group. The γ-phosphoryl group of MgATP appears to enable the N-methyl

group to function in this specific manner as MgADP which lacks this group does not allow creatine to bind.

The evaluation of the dissociation constant of guanidinoacetate was carried out in the same way as described for inhibiting products with the fixed substrate at a saturating concentration. The results are shown in Table 10.

ii) Inhibition by Other Analogs

To learn more about the function of the N-methyl group of creatine, experiments were conducted in which the reverse reaction with creatine phosphate as the variable substrate was inhibited by the following structural analogs:



As can be seen, the structural modifications incorporated in these compounds include replacement of the N-amidino group by a methyl group (N,N-dimethylglycine) and by an amino group accompanied by the replacement of α-nitrogen by methylidene (β-amino-n-butyric acid), elimination

of the N-methyl group with and without simultaneous replacement of the guanidino group by a methyl group (sarcosine and guanidinoacetic acid, respectively), and an increase in the parent carboxylic acid chain length (β-guanidino-propionic acid). A compound representing an increase in the size of the N-side group such as N-ethylguanidinoacetic acid is not commercially available.

All compounds tested caused the same type and very nearly the same degree of inhibition with respect to creatine phosphate as did guanidinoacetate and it appears likely that they bind to the enzyme in a manner analogous to that of guanidinoacetate. Arginine was found not to inhibit the forward reaction at all.

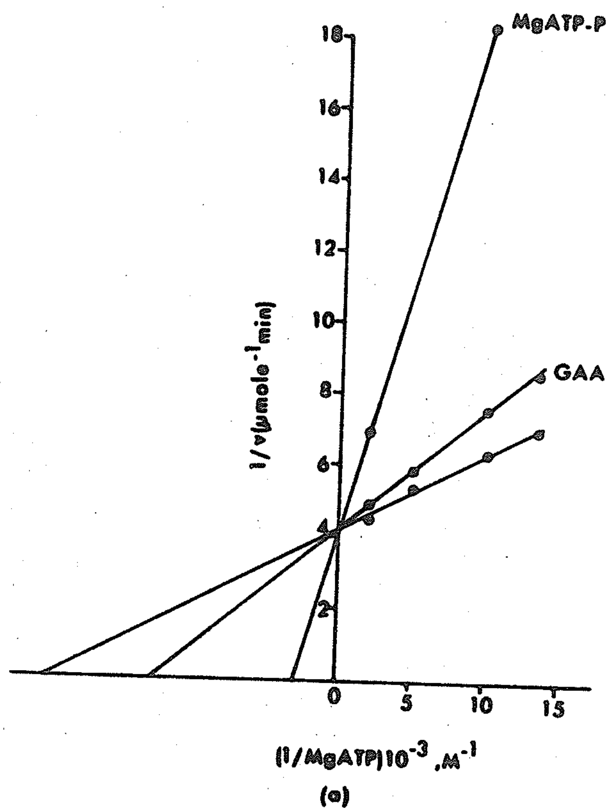
It can be concluded from these results that the binding site for creatine is extremely specific, that regardless of how slight a structural change is it will prevent binding in a manner exactly equivalent to that which is characteristic for creatine. This specificity correlates well with the fact that the alternate substrates which are known for the cytoplasmic MM isoenzymes are so few in number and so low in activity.

b) Studies with Nucleotide Analogs

i) Inhibition by Adenosine 5'-Tetraphosphate

In contrast to the very high specificity which characterizes binding of the guanidino substrate, the locus at which the nucleotides attach to the enzyme can bind species other than the normal substrates. MgATP-P inhibits competitively relative to MgATP and noncompetitively relative to creatine phosphate (Figures 20,21). The dissociation constant is very close to the inhibition constant of MgATP (Table 10).

The results show that the nucleotide-binding portion of the active site is spacious and capable of accommodating an additional negative charge.



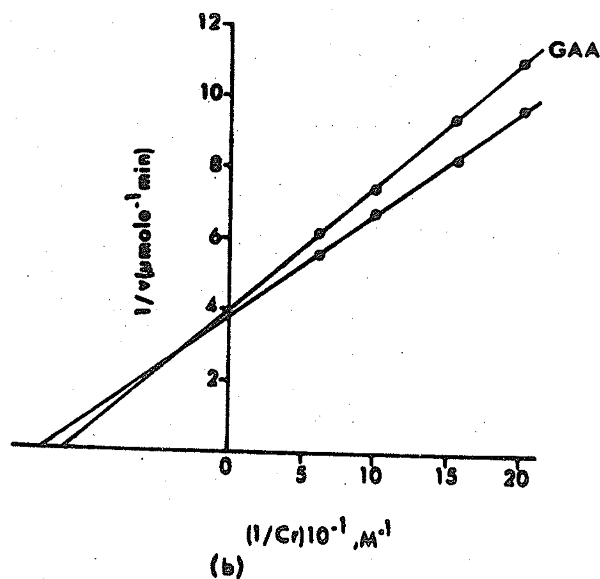


Figure 20. Inhibition of the forward reaction at pH 7.4 and 30° by substrate analogs. Fixed substrate concentrations are 1.6×10^{-2} M for creatine and 2.0×10^{-3} M for MgATP. The concentration of MgATP-P is 5.4×10^{-4} M and that of guanidinoacetate is 1.0×10^{-2} M in (a) and 1.25×10^{-2} M in (b).

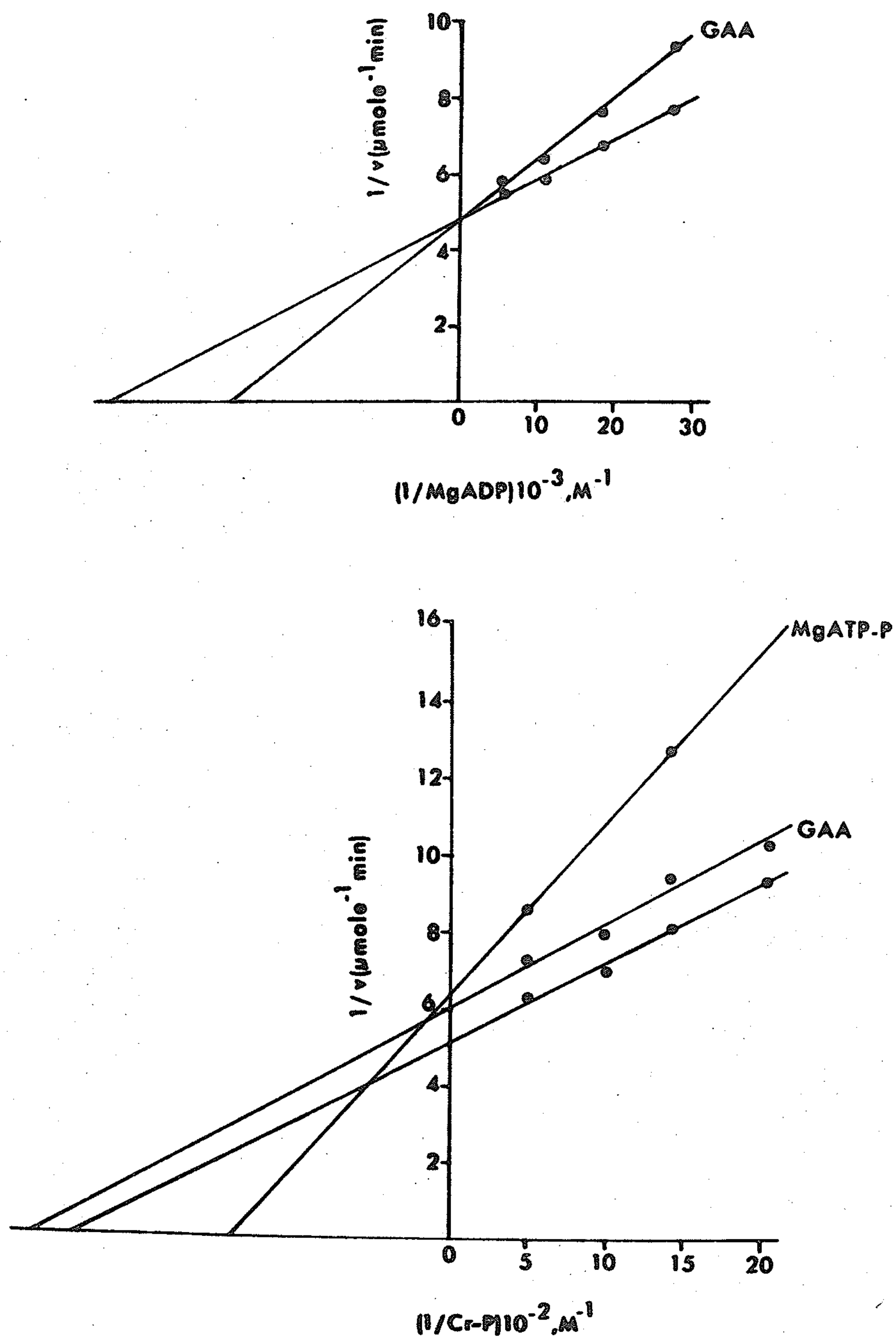


Figure 21. Inhibition of the reverse reaction at pH 7.4 and 30° by substrate analogs. Concentrations are 1.0×10^{-3} M for Cr∿P and 9.3×10^{-5} M for MgADP as the fixed substrates. The concentrations of MgATP-P and guanidinoacetate are 5.4×10^{-4} M and 1.25×10^{-2} M, respectively.

TABLE 10

Dissociation Constants (in mM) of Substrate Analogs at pH 7.4 and 30°

Effect Used	Variable Substrate	$K_{i(GAA)}$	$K_{i(MgATP-P)}$
Slope	MgADP	32.2	
Slope	Cr∿P	41.5	0.090
Intercept	Cr∿P	12.4	0.239[a]
Slope	MgATP	15.2	0.094
		17.2	
Slope	Cr	31.6	
		37.1	
Intercept	Cr	24.9	
		24.8	
		24.0	
Average results		26.1 ± 9.5	$0.092 \pm .003$

[a] Rejected on the basis of the Q test (138).

The inhibition patterns are consistent with an Ordered mechanism.

ii) Inhibition by Other Nucleotides

The same lack of specificity that was observed in conjunction with MgATP-P was evident when various other nucleotides were employed to inhibit the forward reaction. The approximate dissociation constants are given in Table 11. The constants are of the same order of magnitude as the one of MgADP. As can be seen, both mono-and diphosphates act as inhibitors as well as both purine and pyrimidine compounds.

Also, there was some activity observed with thymidine 5'-triphosphate and cytidine 5'-triphosphate used as substrates in place of ATP.

Nihei et al. (33) and James and Morrison (51) observed a similarly wide specificity relative to nucleotide substrates in the case of the cytoplasmic MM isoenzyme.

c) Studies with Inorganic Phosphate

With no substrate analogs of creatine phosphate being commercially available and after an attempt to obtain glycocyamine phosphate from workers in France had failed, inorganic phosphate was tried as a substitute. It was found to inhibit both reactions noncompetitively relative to all substrates. This type of inhibition can be explained on the basis of a non-specific interaction with the enzyme such as a conformational change occurring upon binding of phosphate. However, the interaction could also take place at the active site. At pH 7.4 and 30^{0} and in the presence of 1 mM Mg^{2+} ion, phosphate, at a total concentration of 1.26 mM, exists as $MgHPO_4$ (22%), $H_2PO_4^-$ (29%), and HPO_4^{2-} (49%). If the complexed species had an affinity for that part of the nucleotide-binding site which normally interacts with the phosphate side chain and if one or both of the uncomplexed ions interacted with the creatine phosphate binding site, then noncompetitive inhibition would also result.

TABLE 11

Dissociation Constants of Various Nucleotides Determined in the Forward Reaction Catalyzed by Mitochondrial Creatine Kinase at pH 8.1 and 30°

Nucleotide	K_i,mM
ADP	0.017[a]
AMP	0.021
IMP	0.012
UDP	0.022
	0.032
TDP	0.027
	0.044
CDP	0.030
	0.054
dCDP	0.015
	0.026

[a]Each value represents the result of a single assay.

V. DISCUSSION

A. Preliminary Studies

When the apparent pH optima of 8.1 for the forward and 6.3 for the reverse reaction are compared to values of 8.8 to 9.0 and 6.9 to 7.0, respectively, which were reported by Kuby et al. (16) for the cytoplasmic MM isoenzyme from rabbit muscle, the constant difference of about 1.8 pH units between the peaks for each enzyme is noted. Also, the relative peak widths are similar. It can thus be said that the apparent activities of the two isoenzymes exhibit similar responses to changes in pH except for a displacement of about 0.7 units on the pH scale. Moreover, Nihei et al. (33) observe inflection points at pH 6.6 for both reactions catalyzed by the cytoplasmic isoenzyme which compares to our value of pH 7.2 (see IV.A.1).

It has been suggested that the low pH optimum of the reverse reaction represents a physiological adaptation in the sense that the drop in pH which accompanies contraction and utilization of ATP in muscle shifts the cytoplasmic enzyme toward its pH optimum for the reverse reaction in which ATP is regenerated (35). The physiological function of the mitochondrial enzyme which is located on the outer surface of the inner mitochondrial membrane (72) is to convert creatine to creatine phosphate utilizing the ATP generated on the matrix side of the inner membrane by oxidative phosphorylation (73). During this process H^+ ions are taken up into the matrix leaving the outside more alkaline and moving the enzyme toward its optimum for the forward reaction, its physiologically important reaction. The lower value of the optimum, relative to that of the cytoplasmic enzyme, aids the mitochondrial enzyme in performing this function.

In its natural state the enzyme is bound to the inner mitochondrial membrane which means that the environment *in vivo* is very

different from the reaction media used in the activity assays. Yet, there is excellent agreement between the results of the present study and the data obtained by Jacobus and Lehninger (73). Their pH optimum of 8.0 for the forward direction of the rat enzyme in whole mitochondria compares to our value of 8.1 for the isolated pure bovine mitochondrial enzyme. In the reverse reaction Jacobus and Lehninger found an optimum of pH 6.7 as compared to our observation of 6.3. The difference may be due to the different animal species. Another reason might be differences in assay techniques, particularly in the correction for non-enzymic hydrolysis of creatine phosphate. The temperature optimum of 34° would indicate that the mitochondrial enzyme is functioning _in vivo_ near its optimal temperature.

Much of the early work on the cytoplasmic MM isoenzyme has been done with buffer systems incorporating chloride ions at relatively high concentrations. Since inhibition by inorganic ions has been recognized, (19,33) chloride and other small ions have been excluded as much as possible from assays for the cytoplasmic isoenzymes. This restriction must certainly also be placed on all assays for the mitochondrial enzyme as a result of this study. Watts (35) has discussed the inhibition of cytoplasmic creatine kinases in considerable detail. There exists much evidence that the small planar and halide anions inhibit the enzyme by stabilizing an abortive complex formed by the enzyme and its two smaller substrates, i.e., the E.MgADP.Cr complex (34,36,37). Such a mechanism does not appear likely for mitochondrial creatine kinase in view of its Ordered mechanism which does not comprise the E.MgADP.Cr complex. NMR studies would be suitable for testing the correctness, not only of this prediction, but also of the assignment of the Ordered mechanism.

B. The Kinetic Mechanism

It has been shown that for both reactions the double reciprocal plots obtained in the absence of products are linear and intersect at a point to the left of the ordinate and that the secondary plots are also linear and do not pass through the origin. These patterns eliminate as possible mechanisms the Ping Pong mechanism which would give rise to parallel primary plots and the rapid-equilibrium Ordered mechanism in which the primary plots with creatine or creatine phosphate as the variable substrate would intersect at a point on the ordinate due to the missing K_a/A term in the rate equation and the secondary slope replot would pass through the origin (104, p. 81). The steady-state Random mechanism can be largely ruled out on the basis of the linearity of the primary plots.

Product inhibition studies were required to distinguish between the sequential mechanisms remaining as possibilities. Three noncompetitive and one competitive pattern with linear secondary replots were observed in both directions. These data are in accord with a steady-state Ordered mechanism in which the nucleotide substrate binds first. Substrate inhibition by formation of the abortive complex EBQ is not indicated for either reaction as primary plots with B as the variable substrate in the absence of products and intercept $_{1/A}$ versus 1/B are linear rather than hyperbolic concave up (104, p. 147). The failure of the E.MgATP.Cr∿P complex to form is not surprising as it involves the two bulky substrates which possess the transferable phosphoryl group, but the fact that the E.MgADP.Cr complex does not form is unusual. It is conceivable that the complex might form without being observed due to low concentrations of creatine. However, product inhibition studies confirm the results obtained in the absence of products. The abortive product EBQ would make the intercept $_{1/A}$ versus P

replot and the slope $_{1/B}$ versus P replot parabolic concave up (104, p. 131). As all replots were found to be linear, the E.MgATP.Cr∿P and E.MgADP.Cr complexes can be ruled out.

The Theorell-Chance mechanism would yield competitive inhibition patterns with one guanidino compound as the variable substrate and the other as the inhibitory product. Since the inhibitions for this pair of compounds are noncompetitive in both reactions, this scheme is also eliminated as a possible mechanism.

Neither are the inhibition patterns consistent with the rapid-equilibrium Random mechanism unless three dead end complexes are postulated which are E.MgADP.Cr, E.MgATP.Cr∿P and E.Cr.Cr∿P. Whereas the first complex has been shown to exist in the case of the cytoplasmic isoenzyme from rabbit muscle, and at least an indication for the existence of the second has been obtained in a product inhibition experiment (25), there has never been evidence of any kind for the existence of the third complex.

Product inhibition studies with the fixed substrate saturating have produced results which would be predicted for the Ordered mechanism. The inhibition by creatine relative to MgADP with creatine phosphate saturating is the dominant pattern coming out of these experiments: it is strong and uncompetitive.

The results could also be regarded as being consistent with the rapid-equilibrium Random mechanism provided it included the three abovementioned dead end complexes, if it were not for this very inhibition pattern. The inhibition by creatine is not eliminated by saturation with either MgADP or creatine phosphate as would be predicted if only the complexes E.MgADP.Cr and E.Cr.Cr∿P could form. Instead, the inhibition is undiminished which can be explained on the basis of this mechanism only

by postulating yet another complex, the one that would be formed if creatine combined with the central ternary complex to give E.MgADP.Cr~P.Cr. The complete mechanism which would result is shown in Figure 22.

Guanidinoacetate, finally, inhibits competitively relative to the nucleotide substrate and noncompetitively relative to the guanidino substrate. Such behavior can be rationalized in terms of the Ordered mechanism by postulating a conformational change upon binding of the nucleotide as discussed in section IV. B.3.

The data are more difficult to reconcile with the rapid-equilibrium Random mechanism. The competitive inhibitions relative to the nucleotides could be rationalized on the basis of the failure of the E.MgadN.GAA complexes to form for similar reasons as were postulated for the Ordered mechanism, although there would be the added difficulty of having to allow identical behavior of creatine and guanidinoacetate towards the free enzyme while disallowing it towards the E.MgadN complex. However, the greatest problem would lie in finding an explanation for the noncompetitive inhibition relative to creatine. The pattern would require that guanidinoacetate bind to the E.Cr complex, an unlikely occurrence in view of the close structural relationship between the two compounds which would make it appear probable that they share a common binding site.

The reason for presenting the arguments for and against the two mechanisms in so much detail lies, of course, in the fact that the mechanism of the cytoplasmic MM isoenzyme has been established as rapid-equilibrium Random with one ternary dead end complex at pH 8 (25,39), and as rapid-equilibrium Random in the reverse reaction and rapid-equilibrium Ordered with MgATP adding first in the forward reaction at pH 7 (41).

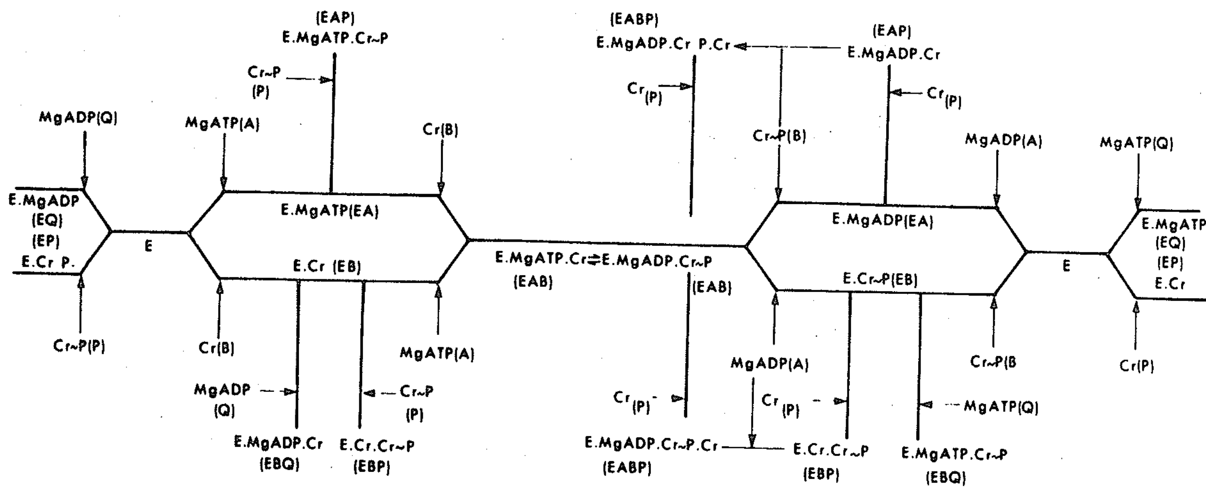


Figure 22. A rapid-equilibrium Random mechanism for mitochondrial creatine kinase.

Although the present work deals with a different isoenzyme, the kinetic mechanism was nevertheless anticipated to be rapid-equilibrium Random. A complete set of kinetic constants was worked out on the basis of the complex mechanism shown in Figure 22. The set of results from the forward reaction was not fully consistent with that from the reverse reaction. One of the inconsistencies which could not be resolved was the large slope effect produced by creatine phosphate as a product inhibitor of the forward reaction which was out of proportion to its affinity to the free enzyme. Another problem was the large intercept effect caused by creatine as inhibitor of the reverse reaction which could only be rationalized by postulating the quaternary complex whose existence is difficult to accept for mechanistic reasons. Thus conceptual difficulties as well as lack of quantitative correlation contribute to the body of evidence against the assignment of the rapid-equilibrium Random mechanism to the mitochondrial isoenzyme of creatine kinase.

The identification of the mechanism as Ordered Bi Bi should be checked by other methods such as isotope exchange. Its confirmation would raise a number of interesting points. As the majority of the kinases appear to possess Random mechanisms, an Ordered mechanism would set mitochondrial creatine kinase clearly apart, although exceptions are known, such as carbamate kinase which possesses a steady-state Ordered mechanism (129) and nucleoside diphosphokinase for which a Ping Pong mechanism has been demonstrated (130).

Another point is that not only can the rapid-equilibrium mechanism of the cytoplasmic MM isoenzyme change from Random to Ordered but that two isoenzymes of creatine kinase may possess completely different mechanisms. This is an important result as there appears to be a tendency among

researchers to assume that the mechanism is the same for different isoenzymes. Thus Saks et al. (76) have only on the basis of initial velocity measurements in the absence of products assumed the rapid-equilibrium Random mechanism for mitochondrial creatine kinase, have developed what they call a mathematical model of the reaction catalyzed by the enzyme and have designed a computer program to describe its kinetic behavior.

C. Physical Properties

While this study is concerned with catalytic and kinetic properties of mitochondrial creatine kinase, there arise from it a few implications with regard to the physical properties of the enzyme.

The observation that bovine serum albumin is not required to maintain activity, presumably because the enzyme has no tendency to be adsorbed to the polar glass surface of the reaction vessel, correlates well with a number of observations made elsewhere which attest to the lipophilic nature of the enzyme. Thus, the enzyme could not easily be removed from a hydrophobic resin (Jacobs, unpublished results), and the partial specific volume indicates a certain lipid content (86).

At a relatively high concentration the enzyme forms an octamer which dissociates into monomers upon dilution (86). In the pH-stat assay the enzyme is used at very low concentrations and should consequently be present in monomeric, possibly dimeric, form. The absence of a quaternary structure fits in with the linear double reciprocal plots which are characteristic for the enzyme's kinetic behavior under *in vitro* conditions at low concentrations and which are indicative of the absence of allosteric effects. Although it is conceivable that the situation with regard to allostery might be different *in vivo*, the data of Jacobus and Lehninger (73) and Saks et al. (76) indicate that this is not the case.

D. Kinetic Constants and the Directionality of the Reaction Catalyzed

Mitochondrial creatine kinase shares the intermembranous space with other enzymes which have the adenine nucleotides as substrates. Their K_m values and other data are given in Table 12.

Creatine kinase has to compete for common substrates with nucleoside diphosphokinase and adenylate kinase. Its Michaelis constant for MgATP which is much lower than that of nucleoside diphosphokinase and its location on the inner membrane enable it to perform a channeling function: ATP can be utilized in the formation of creatine phosphate in preference to that of other nucleoside 5'-triphosphates which means that the immediate energy needs of the cell and survival of the organism have precedence over anabolic reactions.

A similar situation exists with regard to the relationship of creatine kinase with adenylate kinase. Its lower K_m value and its greater proximity to the mitochondrial matrix favor the formation of creatine phosphate over the conversion of AMP to ADP, i.e., the upgrading of the energy charge of the cell. Only that portion of ATP which is not used by creatine kinase becomes available to adenylate kinase.

The adN translocating enzyme system probably uses as substrates the uncomplexed adenine nucleotides (references in 133) and, therefore, does not compete directly for substrates with creatine kinase. Since the ADP-ATP exchange is asymmetrical, i.e. ADP is preferentially taken up in the intermembrane space, such competition would only involve the reverse reaction of creatine kinase which is the non-physiological reaction. In the physiological reaction the two enzymes work in tandem, and the relative magnitudes of the Michaelis constants for the nucleotides are of little consequence.

TABLE 12

Selected Properties of Several Mitochondrial Enzymes

Property	Nucleoside Diphosphokinase	Adenylate Kinase	Creatine Kinase	adN Translocator
Source	Bovine heart	Bovine liver	Bovine heart	Rat liver
Location	Outer membrane and intermembranous space	Intermembranous space	Outer surface of inner membrane	Inner membrane
$K_{m(ATP)}$,mM	1.4	1.8	0.03	0.001-0.150[a]
$K_{m(ADP)}$,mM	0.1	1.8	0.02	0.001-0.010
Requirement for Mg^{2+}	Yes	Yes	Yes	No
Function	Synthesis of NTP's	Storage and use of high energy in adN's	Synthesis of Cr~P	Asymmetric ATP-ADP exchange across inner membrane
Reference	131 and references in 131	132 and references in 132	this study	133 and references in 133

[a]Depends on the energy state of the mitochondria.

If the specificity of the adN translocator for the free nucleotide does exist, then the enzyme can, of course, only be active if the ADP in the intermembranous space is present in non-complexed form. This means that the concentration of non-compartmentalized Mg^{2+} ion must be sufficiently low for much of the ADP to be present as the free nucleotide, yet sufficiently high to complex much of the ATP present, if the three enzymes which require MgATP are to function in this direction at a significant level of activity. This will be the case when the Mg^{2+} ion concentration is less than or equal to the ATP concentration so that ATP with its significantly larger complex stability constant will complex in preference to ADP with the result that the concentration of MgADP is low.

This line of reasoning provides one answer to the problem of the directionality of the reaction catalyzed by creatine kinase. Since all kinetic parameters favor the reverse reaction, the question arises how the enzyme can efficiently catalyze the forward reaction _in vivo_. If a large proportion of ADP remains non-complexed, then the forward reaction is favored in spite of lower K_m and V_{max} values by virtue of higher substrate concentrations.

The Ordered mechanism may represent an adaptation which helps to prevent the reverse reaction even in the presence of creatine phosphate at a high concentration. Heart cells contain creatine at a relatively high concentration, approximately 10 mM according to Jacobus and Lehninger (73). In the present study a high degree of inhibition of the reverse reaction by 6 mM creatine was observed even at saturating concentrations of MgADP and creatine phosphate. This may well constitute another mechanism of control of the reverse reaction in addition to the regulatory effects ascribed by Saks et al. (76) to Mg^{2+} ion and to the functional coupling of creatine kinase and the adN translocator.

E. Conclusion

Mitochondrial creatine kinase has been identified as an isoenzyme of the earlier known cytoplasmic forms. It catalyzes the same reactions and performs a similar function (73); it is present in the same cell and displays the same tissue distribution (71,73). It possesses a similar subunit weight, but the particle weight at high concentration is different (86). The fact that the enzyme is membrane bound and possesses appreciable hydrophobic character may well account for the high degree of subunit association. Additional studies probing into the nature and steric orientation of subunit interaction and the possible existence of a dimeric molecule are still required at this stage. Chemical modification experiments used in conjunction with physical methods such as sedimentation analysis and spectral methods including UV absorption, fluorescence and ORD would be a suitable approach to solving these problems, but kinetic experiments could also be of help. For example, the activity and even the kinetics of the enzyme in modified or unchanged form could be determined by means of the pH-stat assay using reaction media of varying polarity and reducing potential. Also, active site studies by these methods need yet be done.

Differences typical of isoenzymes have also been observed. Electrophoretic mobility (71), isoelectric pH (137) and amino acid composition (86) are clearly different. There remain to be done sequencing studies at least of the active site. Such data are available for the section containing the essential cysteine residue for the cytoplasmic isoenzyme from rabbit muscle (134,135) and brain (136) and also for some other sequences of the rabbit muscle enzyme.

The present study contributes to the establishment of the isozymic

relationship in several ways. Although this has not been mentioned previously in this report, the mitochondrial enzyme, like the cytoplasmic forms, has been found to be inactive with the unchelated nucleotides. No activity was observed in the absence of Mg^{2+} ion, while nearly full activity was reached at a Mg/ATP ratio of 1 at all pH values investigated in the forward reaction and at pH 8.1 and 7.4 in the reverse reaction. At pH 6.8 the reverse reaction required a ratio of 2 for apparent maximal velocity.

A broad specificity with regard to nucleotide substrates has been established for the rabbit muscle isoenzyme (33,51). The inhibition by the diphosphates of various nucleosides as well as by adenosine tetraphosphate observed in this study ahs been interpreted as signalling a similar lack of specificity in the mitochondrial isoenzyme. This finding is in contrast to Jacobus' and Lehninger's (73) observation that other nucleoside di- and triphosphates are poor alternate substrates or are essentially inactive. Thus, much kinetic work remains to be done in this area. In addition, chromium nucleotides and nucleotides modified in the phosphate side chain might shed further light on the molecular mechanism, while little could be learned from such studies with regard to the kinetic mechanism that has not already been or could not be observed with the tetraphosphate.

Specificity studies into the guanidino binding site using glycocyamine phosphate are still needed to round off the present kinetic investigation. An urgent question is whether the compound would exhibit a behavior analogous to that of guanidinoacetate. Equally rewarding could be studies with cyclocreatine as substrate. It might turn out to be the competitive inhibitor of creatine that could not be found for the present study.

The specificity of the enzyme in relation to the metal ion has not

yet been touched. Will it be active with nucleotides complexed with manganese? If so, will NMR studies point to the existence of an E.MnADP.Cr.anion complex which has been observed in the case of the rabbit muscle enzyme and which would place in doubt the Ordered mechanism as developed in this study?

The results which have been brought forward here show conclusively that the mitochondrial isoenzyme differs in kinetic properties from its cytoplasmic counterparts, more so when compared to the muscle enzyme than when compared to the brain enzyme. This takes us to the problem of subunit structure. Does the mitochondrial enzyme consist of only one type of subunit or of two or even more? Will the subunit(s) be similar to the B or/ and the M subunit? On the basis of the kinetic constants one would predict a greater resemblance to the cytoplasmic B unit.

Up to this point only one form of mitochondrial creatine kinase has been recognized. Could there be more than one type? If so, would these forms be subject to ontogenic evolution and pathological de-differentiation? Even if separate forms were not found to exist, then there are still the two forms of different activity seen by Hall et al. (82) which require to be characterized.

When working with creatine kinase a researcher can never afford to forget that the enzyme has at least one essential sulfhydryl group per subunit and that there is a continuous spectrum of activity states between zero and maximum. An attempt has been made in this study to correlate the state of activation of the enzyme with the values of its kinetic parameters, but no dependence could be discerned with regard to binding constants, only the obvious one involving maximal velocity. Because it utilizes such a simple reaction mixture, the pH-stat assay is well suited not only for monitoring the development of ways in which the enzyme can be reproducibly

reactivated but also for long-term studies into the effects of storage conditions on kinetic properties. Another feasible project would be to repeat with the mitochondrial enzyme the experiments by Maggio et al. (46) on CH_3S-blocked rabbit muscle enzyme.

The Ordered mechanism cannot be considered established while it has not been confirmed by isotope exchange studies. Work in this area would have been undertaken next, if it had been possible to expand the scope of this project. Whereas the application of this technique represents the minimum requirement, other methods such as equilibrium binding could be used in addition to it.

There are pH-dependent changes in the rate-controlling steps as the relative magnitudes of the rate constants governing the various steps in the reaction mechanism have shown. Hence, it would be a worthwhile project to look into the possibility of an associated change in the kinetic mechanism which has been demonstrated by Schimerlik and Cleland (41) for the MM isoenzyme.

Finally, it might be noted that no kinetic investigation is complete as long as it is restricted to steady-state experiments. Only rapid reaction techniques allow a glimpse at the occurrences which take place during that brief span of time which begins when all substrates are in place and ends when the conversion to products is complete. The results would provide another check on the kinetic mechanism.

This enumeration of ideas about what could yet be done to enrich our knowledge of the enzyme under study is not meant to be complete nor to be a detailed proposal for further research, it is more an expression of this writer's realization that there are more questions at the end of a project than at its beginning.

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