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A creatine-based affinity label for creatine kinase

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A CREATINE-BASED AFFINITY LABEL FOR CREATINE KINASE

by

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DISSERTATION

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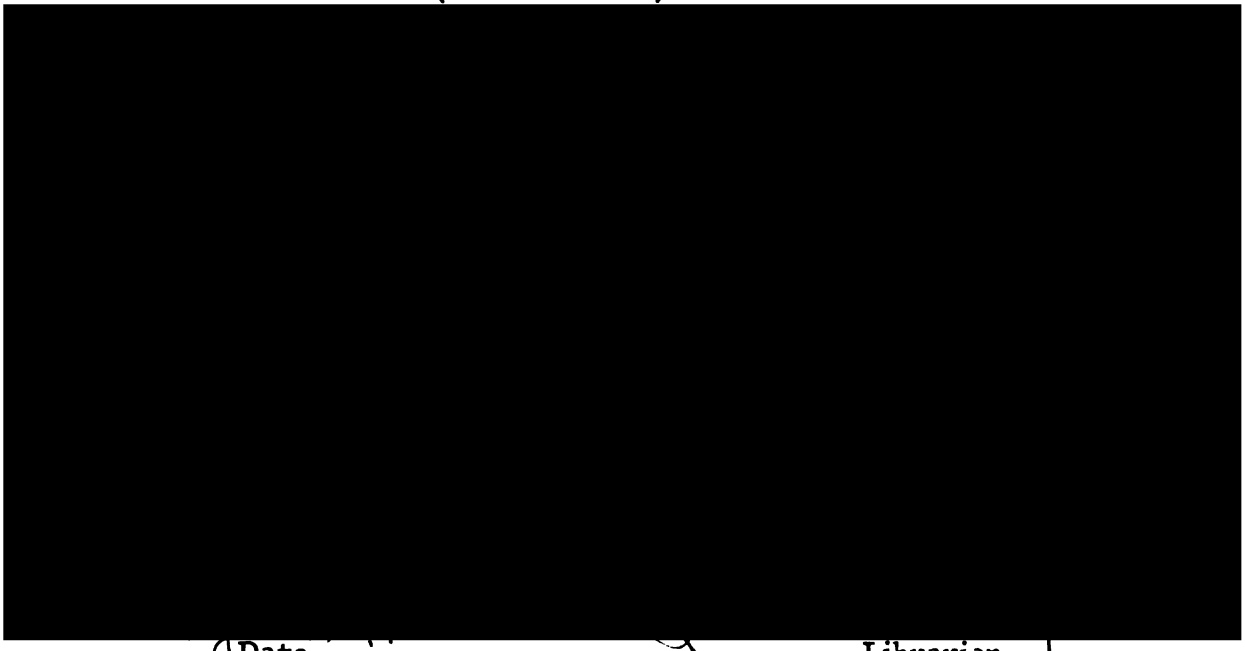

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In memory of my mother

and

To my father

A Creatine-Based Affinity Label for Creatine Kinase

Abstract

The compound N-(2,3-epoxypropyl)-N-amidinoglycine (epoxycreatine) was synthesized to probe the active site of rabbit muscle creatine kinase because of its structural similarity to the normal substrate creatine and because of the reactivity of its epoxide moiety toward nucleophiles. In the presence of epoxycreatine and in the absence of other substrates complete inhibition of enzymatic activity is observed which does not return after exhaustive dialysis. The inhibition follows saturation kinetics. At 0^{o} C (pH 7.45) K_{inact} is 417 mM compared to a K_s value of 24 mM for creatine at 30^{o} C. The inactivation half-time at infinite epoxycreatine concentration is 3.44 minutes and therefore the pseudo first-order rate constant is 3.36×10^{-3} $sec.^{-1}$ In a competitive manner, creatine protects the enzyme from inactivation by epoxycreatine. Using polyethylenimine-cellulose thin layer chromatography, according to the method of G.L. Rowley and G.L. Kenyon, Anal. Biochem., 58, 525 (1974), epoxycreatine was also shown to be a substrate for the enzymatic reaction in the presence of both Mg^{2+} and ATP. The inactivation half-time in the presence of MgATP is slightly greater than that when MgADP is present which also is slightly greater than that of epoxycreatine alone. The so-called transition state complex of $MgADP^{2-}$, NO_3^- and creatine provides strong protection against inactivation by epoxy-

creatine. The stoichiometry of the reaction, determined with [^{14}C]-labeled epoxycreatine, is one site modified per subunit of enzyme. These results indicate that epoxycreatine is binding at the active site and that it is therefore an affinity label.

Basic hydrolysis cleaves the label from the enzyme and this hydrolysis rate is accelerated in the presence of hydroxylamine, results which suggest that a carboxylate group of an aspartate or glutamate residue on the enzyme is being modified by epoxycreatine.

Preliminary experiments with perfused cat hearts indicate that epoxycreatine will be useful in in vivo type investigations concerning the physiological function of the enzyme.

ACKNOLEDGMENTS

I would like to thank Professor George Kenyon for his advice, patience and for teaching me to have a sound appreciation for careful and exact scientific experimentation. He has been a fine mentor and friend, a rare combination.

I am also grateful to the faculty of the Department of Pharmaceutical Chemistry and especially to Professors Neal Castagnoli, Roger Ketcham and Norman Oppenheimer.

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I. INTRODUCTION

Creatine kinase (adenosine 5'-triphosphate-creatine phosphotransferase, EC 2.7.3.2) catalyzes the reaction shown below with the reaction proceeding from left to right being arbitrarily designated the forward direction.

$$\text{creatine}^{\pm} + \text{MgATP}^{2-} \rightleftharpoons \text{phosphocreatine}^{2-} + \text{MgADP}^{-} + \text{H}^{+}$$

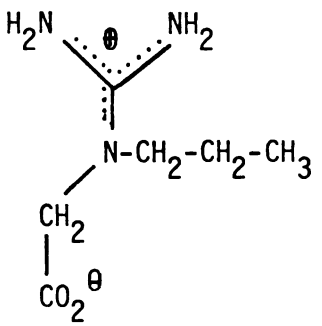
The enzyme is found in abundant supply in muscle tissue, both soluble and bound, and is generally considered to be important in maintaining constant ATP levels in the tissue. Creatine kinase consists of two identical subunits with the molecular weight of the dimer being 82,000. It requires a divalent metal ion for catalysis.

Investigations involving creatine kinase range from chemical modification studies to clinical measurements of the enzyme's plasma level as an indicator of myocardial infarctions. The phosphotransferase has been under intense investigation for a number of decades; however, in all of the chemical modification experiments which have been carried out none have been active -site directed. In other words,except for our work, no report of an active-site directed reagent based on a substrate analog, such as a creatine analog, has appeared in the literature.

The initial report of such a reagent has been published (Marletta and Kenyon, 1977) and this dissertation will detail the rationale

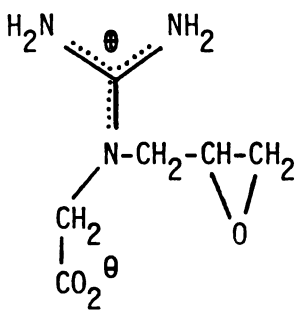
for design, synthesis and testing (both in vitro and in vivo) of this molecule as a creatine-related affinity label.

Investigations in the Kenyon laboratory with substrate analogs of creatine began to map out the active site. These prior experiments lent invaluable information as to the bulk tolerance of the active site for creatine analogs. Thus, it was found that a propyl homolog of creatine was a substrate in the enzymatic reaction (1% V_{max} relative). The structure of this analog is shown below:



N-propyl-N-amidinoglycine

Based on that information the following molecule was designed



N-(2,3-epoxypropyl)-N-amidinoglycine (epoxycreatine)

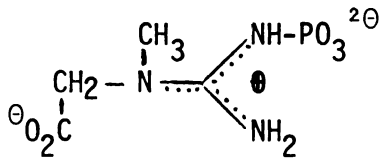
It was felt that since the propyl analog was a substrate, the epoxide analog of that might also serve as one.

The subsequent investigations concerning epoxycreatine and creatine kinase have indicated that indeed the molecule does conform to criteria designed to show that its reaction with the enzyme is both at the active site and is highly specific. The experiments described in this report serve to establish epoxycreatine as the first creatine-based affinity label for creatine kinase.

II. HISTORICAL BACKGROUND

A. Creatine Kinase

Fifty years ago, Eggleton and Eggleton (1927) and Fiske and Subbarow (1927) simultaneously reported the isolation of a very unstable phosphate derivative from muscle tissue. The compound was called "phosphagen" by Eggleton and Eggleton, so named because of its apparent instability. Neither of the two groups mentioned above identified the compound chemically; however, they did show that the breakdown of the phosphagen was stimulated by muscle contraction and that re-synthesis took place by the muscle during a resting period which followed a series of contractions. The compound thus appeared to be linked to muscle contraction and was the first discovered to contain a high phosphoryl transfer potential and to be involved in energy transfer. Hill and Meyerhof (1923) had established the link between muscle contraction and lactic acid formation. Meyerhof, now intrigued with this newly discovered compound, investigated it further. Meyerhof and Lohmann (1927) isolated the compound and identified it as a derivative of creatine and from electrotitration data determined that the phosphoryl group was attached to one of the guanidine nitrogens. The compound, phosphocreatine, is shown below:



phosphocreatine

They determined the heat of hydrolysis by calorimetry and found it to be very high relative to other phosphate derivatives known at the time.

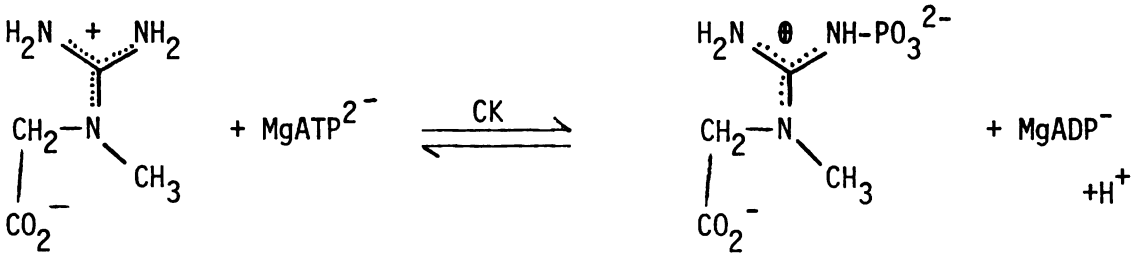
Lundsgaard (1932) had been carrying out experiments with iodoacetate poisoned muscle and found that injection of iodoacetate into rats caused no reaction for a brief period and then their muscles entered a state of rigor. Lundsgaard expected to find high levels of lactic acid and the negative result led him to investigate more thoroughly. He found that glycolysis was totally inhibited by iodoacetic acid. From his studies on isolated iodoacetic acid-treated muscles he found that contraction continued until the supply of phosphocreatine was exhausted.

Two years later Fiske and Subbarow (1929) isolated adenosine 5'-triphosphate (ATP) from muscle. Lohmann (1929) independently reported isolation of ATP and his report contained the finding that it also was capable of energy transfer because of high phosphoryl transfer potential.

Lohmann (1934) continued the investigation further and discovered that in dialyzed muscle extracts phosphocreatine was not hydrolyzed directly but transferred its phosphoryl group to adenosine 5'-diphosphate (ADP). The reaction came to be known as the Lohmann reaction.

Some ten years elapsed before it was definitively determined that ATP was the chemical source of energy utilized by the myofibrillar proteins to contract. The evidence at the time indicated that ATP was consumed by an ATPase which was located on the myosin portion of the contractile apparatus and that phosphocreatine served as a reservoir of energy capable of generating ATP when needed.

The enzyme which catalyzes the reversible transfer of the phosphoryl group from ATP to creatine was named creatine kinase (adenosine 5'-triphosphate-creatine phosphotransferase EC 2.7.3.2). The reaction is shown below:



and is written (left to right) in what has been arbitrarily called the forward direction.

The enzyme is composed of two identical subunits, each with a molecular weight of about 41,000 as determined by Yue et al. (1967) and Dawson et al. (1967). The enzyme requires a divalent metal ion such as Mg^{2+}, Mn^{2+}, Ca^{2+} or Co^{2+}. The divalent metal in solution is complexed with the nucleotide substrate and these complexes (e.g., MgATP or MgADP) may be considered to be the substrates in the enzymatic reaction (Cohn, 1970; Hammes and Hurst, 1969). Kinetic studies carried out under steady-state conditions by Morrison and James (1965) together with isotope exchange studies of Morrison and Cleland (1966) and Morrison and White (1967) and temperature-jump relaxation kinetic measurements by Hammes and Hurst (1969) led to the conclusion that at pH 8 the reaction catalyzed by the rabbit skeletal muscle enzyme follows a rapid equilibrium, random, bimolecular, bimolecular kinetic scheme as described by Cleland (1963). It was also determined that the rate-determining process in the reaction was the interconversion of the enzyme-creatine-MgATP complex to the enzyme-phosphocreatine-MgADP complex. Morrision and James (1965) also observed synergism of creatine and MgATP binding at pH 8.

There have been many reports of chemical modifications of creatine kinase. The enzyme has one unique, highly reactive sulfhydryl group per

subunit (Mahowald et al., 1962). This reactive group has been treated with numerous reagents such as iodoacetic acid (Mahowald et al., 1962; Ennor and Rosenberg, 1954), iodoacetamide (Watts and Rabin, 1962), p-chloromercuribenzoate (Ennor and Rosenberg, 1954), 2,4-dinitrofluorobenzene (Mahowald et al., 1962), iodine (Trundle and Cunningham, 1969), dansyl chloride (Brown and Cunningham, 1970), and the spin-label N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide (Taylor et al., 1969). In all of these studies the stoichiometry of the reaction showed one mole of sulfhydryl blocking reagent per subunit of enzyme, together with complete inhibition of enzymatic activity. Recently, new sulfhydryl reagents have been developed which introduce the relatively small, neutral, non-hydrogen bonding CH_3S-group (Smith and Kenyon, 1974; Smith et al., 1975; Markham et al., 1977). This group cam be introduced into creatine kinase with the same stoichiometry stated above; however, the enzyme retains a substantial amount of activity ($\sim$ 20%). This latest work implies a secondary role for this reactive sulfhydryl group.

Modification of amino acid side chains other than the sulfhydryl group have also been reported. A lysine residue was modified by Clark and Cunningham (1965), an arginine by Borders and Riordan (1975), a histidine by Pradel and Kassab (1968) and a threonine and serine by Henkin (1977).

The enzyme was first crystallized from rabbit skeletal muscle by Kuby et al. (1954). Since then creatine kinase has been isolated from a variety of skeletal muscle sources. In all such cases it is considered to occur in the cell cytoplasm and which is readily extracted at low ionic strength.

Creatine kinase with properties distinct from the skeletal muscle form has also been found associated with the mitochondria of various tissues, especially where the tissue is rich in mitochondria such as the heart. The enzyme occurs in three forms readily distinguishable by their electrophoretic mobility. These three forms have been designated as the muscle type (M·M), the brain type(B·B) and the hybrid form (M·B). Recently it has been shown by Jacobus (unpublished results) that the M·M form from cardiac tissue, although electrophoretically the same as the skeletal M·M form, differs significantly in its hydrophobic amino acid composition, its tryptic digest map and its isoelectric point. Controversy concerning the physiological function and the location (bound) of creatine kinase has recently arisen and will be discussed later.

B. Affinity Labeling

The general term affinity labeling refers to the method of specifically labeling a target molecule by using a reactive molecule which binds to the target and which exhibits a degree of specificity and affinity for binding to that target. The technique has found wide application in biochemical research of enzymes, transport proteins, immunoproteins, receptors and contractile proteins.These proteins all bind molecules as a key step in their biological function: i.e.,enzymes bind, in most cases, with a high degree of specificity, their substrate or substrates prior to the catalytic event.Similiarly, receptors bind with a hormone prior to the biological response. A specific region on the protein molecule which is small and well-defined, the so-called binding site is responsible for this selective, high-affinty interaction with a specific ligand. A thorough

understanding of the biological function of these proteins can only be accomplished when the factors involved in this interaction and the structure of the binding site are elucidated.

During the late 1950's and early 1960's considerable research effort was initiated to investigate these protein-ligand interactions. The initial investigations studied the topology of the binding regions with the use of competitive inhibitors. A second area of research involved the study of the amino acid residues responsible for the specific interaction by utilizing chemical reagents which were developed to react with specific amino acids. A serious drawback to the chemical modification technique was that reaction at some area on the protein other than the binding site was always a possibility. Significant then was the combination of the two approaches, producing a molecule which resembled the natural ligand and which also contained a group capable of reacting with an amino acid residue either at or very close to the binding site. Such a molecule therefore is defined as an affinity label. The first substrate analog discovered which could irreversibly inhibit an enzyme was azaserine (Levenberg et al., 1957). Azaserine inactivated a series of enzymes utilizing glutamine as an NH_2 donor. This discovery was a very important stimulus to research in this field. Work in this area has been reviewed by Shaw (1970), Baker (1967) and Singer (1967).

The initial investigations using affinity labels provided elegant proof that this approach would yield valuable information regarding the protein structure-function relationship (Baker et al., 1961; Schoellmann and Shaw, 1962; Lawson and Schramm, 1962; Wofsy et al., 1962; Singh et al., 1962; Gundlach and Turba, 1962).

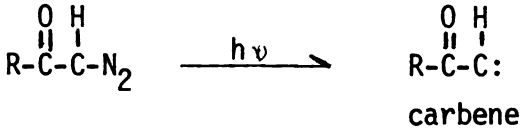
The binding site on enzymes has been called the active site (Koshland, 1960). The active site therefore is the region of the enzymic surface in contact with the substrate and which contains the structural features responsible for both specificity and catalysis. Baker (1967) described these affinity label molecules in the case of enzymes as active-site directed reagents, and his descriptively useful term has had wide acceptance. Active-site directed reagents therefore are substrate analogs which are capable of forming a reversible complex with the enzyme in the same specific manner as natural substrate and then by virtue of the built-in reactivity form a covalent bond with the enzyme, thereby irreversibly inhibiting its catalytic function. The process therefore contains two distinct steps, (1) selective and reversible binding and (2) irreversible covalent bond formation. Meloche (1967) has treated this two step (shown below) in a concise and elegant fashion:

$$E + I \rightleftharpoons E \cdots I \longrightarrow E - I$$

where E is the enzyme, I the active-site directed reagent, E···I the reversible enzyme-inhibitor complex, and E - I the covalent adduct formed upon reaction of the active-site directed reagent with the enzyme. Baker (1967) has called this method of irreversible inhibition of the enzyme an endo reaction mechanism because the reaction takes place in the active site.

Reactive functional groups which have had wide appeal are those which contain an electrophilic type carbon atom and which therefore are capable of reacting with nucleophiles present at or near the active site,

such as carboxylate group of aspartic or glutamic acid or the sulfhydryl group of cysteine. Therefore halomethyl, halomethyl keto, epoxy and aziridinyl analogs of substrates have been quite successful. Another approach has been the use of what are called photoaffinity labels. The pioneering work in this field was carried out by Westheimer and co-workers. While the initial work was carried out on enzymes which during the catalytic process generate an acyl-enzyme intermediate, it has been extended to include actual substrate analogs. A general model is illustrated below:



The reactive group is unmasked when activated by ultraviolet light generating a highly reactive carbene which is capable of insertion reactions. This enables the labeling of the relatively inert hydrophobic amino acids. In other cases nitrenes are generated.

Another type of affinity label is that which has been termed by Rando (1974) to be k_{cat} inhibitors or mechanism-based irreversible inhibitors. This approach has recently been reviewed by Rando (1977). These reagents have also been termed suicide substrates because they require enzymatic activation to generate the reactive group. This type of inactivation is interesting because the reactive moiety is generated by the enzyme at the active site and lessens the probability that some random, i.e., other than the active-site, alkylation will take place. This approach, however, is not always possible, as the enzyme may not be able to convert the substrate from an unreactive one to a

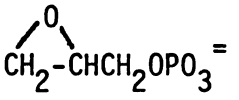
reactive one. A functional group which has been successful is the acetylene moiety which can be activated as shown below:

$$R\text{-}CH_2\text{-}C{\equiv}CH \longrightarrow \underset{\text{allene}}{R\text{-}CH{=}C{=}CH_2}$$

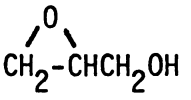
The *in situ*-generated allene is highly reactive toward nucleophilic attack at the central carbon atom. In order to produce the allene the enzyme must of course be able to abstract a proton from the carbon atom adjacent to the R group and place one on the terminal carbon. k_{cat} inhibitors can be most advantagously applied to a crude enzyme mixture where only the enzyme of choice can activate the analog (Rando, 1977).

C. Epoxide Affinity Labels

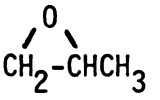
The use of the epoxide moiety as the reactive functional group attached to a substrate has yielded considerable success. Much of the earlier work with epoxide-containing affinity labels was summarized by Fee (1973). Rose and O'Connell (1969) designed glycidol phosphate as an affinity label for triose phosphate isomerase and enolase. Subsequent investigations determined that a glutamic acid residue was modified (Whaley *et al.*, 1970; Miller and Waley, 1971; Schray *et al.*, 1973). Glycidol phosphate, glycidol and propylene oxide were all found to be inhibitors of yeast glyceraldehyde-3-phosphate dehydrogenase (Mc Caul and Byers, 1976). This enzyme, being a tetramer, incorporated four moles of label per mole of enzyme, and a sulfhydryl group of cysteine was modified in each case.



glycidol phosphate



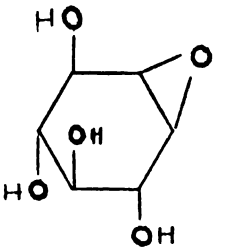
glycidol



propylene oxide

McCaul and Byers (1976), by using three structurally related epoxides, were able to shed some light on the relationship between subunit modification and the role of subunit interactions in the catalytic mechanism of the enzyme.

Following a report by Quaroni et al., (1974) and Quaroni and Semenza (1976) on the use of conduritol-B-epoxide as an affinity label for the sucrase-isomaltase complex from small intestine, Braun (1976) investigated its interaction with β-fructosidase (invertase).

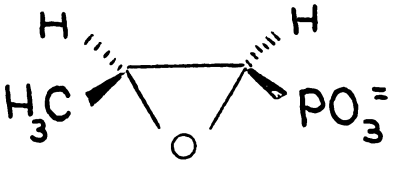


conduritol-B-epoxide

Braun (1976) determined kinetically that the inactivation is irreversible and first-order with respect to time and inhibitor concentration. Protection by the normal substrate was also shown, and it was suggested that a carboxylate group was modified based on a pH-dependence of the inactivation. In order to show that the inactivation of invertase by conduritol-B-epoxide was a function of the molecule's close similarity to the natural substrate, a series of

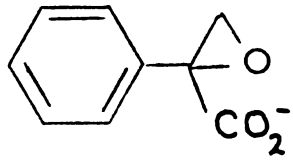
epoxides were tested. Epoxypropane 1,2-epoxybutane and 2,3-epoxy-1-propanol inhibited only very slightly or not at all and epoxycyclohexane was forty times slower than conduritol-B-epoxide.

Klinman (1975) showed that styrene oxide modified two cysteines per subunit of yeast alcohol dehydrogenase. Later investigations (Klinman et al., 1977) determined that cysteine residues 43 and 152 were alkylated by the epoxide. Phosphonomycin inactivates the enzyme pyruvyl transferase and it was also shown to modify a cysteine residue (Cassidy and Kahan, 1973; Hendlin et al., 1969; Christensen et al., 1969). Phosphonomycin is an example of an affinity label which has been found useful as an antibiotic. It prevents the biosynthesis of a key structural intermediate in the bacterial cell wall.

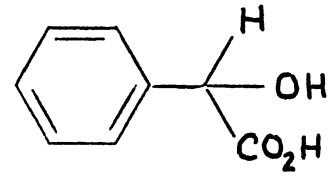


phosphonomycin

Fee et al. (1974) designed α-phenylglycidate as an affinity label for mandelate racemase. It kinetically conformed to being an affinity label, and evidence suggested that a carboxylate group may have been modified.



D,L-α-phenylglycidate



D,L-mandelic acid

Not all of the epoxide-containing substrate analogs have turned out to be affinity labels. Thus, neither cis- nor trans-epoxysuccinate inactivated fumarase (Bradshaw et al., 1969). 1L-1,2-anhydro-myo-inositol, which is a substrate analog for an α-glucoside, is a competitive inhibitor of yeast α-glucosidase (Barnett et al., 1971). Therefore, while some enzymes are rapidly inactivated by epoxide-containing substrate analogs, others can not only tolerate their presence but in some cases catalyze a reaction on them without inactivation (Albright and Schroepfer, 1971).

D. Reactions of Epoxides

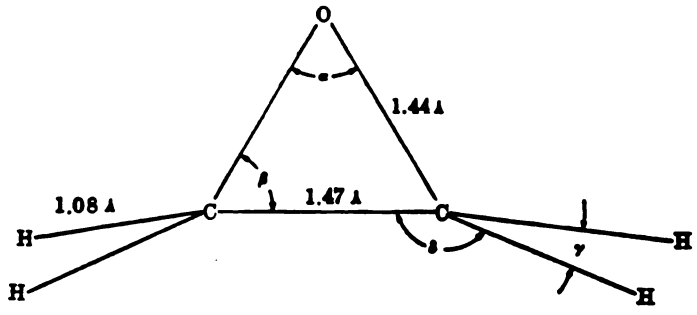
Oxiranes, which are the formal name given to the simplest oxygen-containing heterocyclic rings, are more commonly referred to as epoxides, 1,2-epoxides or ethylene oxides. The basic chemistry of epoxides reflects the fact that the strained three-membered ring system will undergo reactions in a facile manner which with non-strained cyclic ethers do not occur. Hence, alicyclic ethers will cleave only under the most vigorous conditions while the same reaction with epoxides proceeds easily under relatively mild conditions as illustrated below.

$$R\text{-}CH_2\text{-}O\text{-}CH_2\text{-}R \xrightarrow[\Delta]{HI} RCH_2\text{-}OH + RCH_2I$$

$$RCH_2\text{-}O\text{-}CH_2R \xrightarrow[\Delta]{HBr} RCH_2\text{-}OH + RCH_2BR \text{ (slow)}$$

$$\begin{array}{c} RCH\text{-}CHR \\ \backslash\ / \\ O \end{array} \xrightarrow[RT]{HX} \begin{array}{c} RCH\text{—}CHR \\ |\quad\ | \\ OH\ \ X \end{array}$$

Microwave spectroscopy and electron-diffraction studies have yielded a detailed structure of the epoxide ring and have been invaluable in predicting the unusual reactivity of it. The results from many laboratories are summarized in the following figure of the simplest epoxide, ethylene oxide (Rosowsky, 1964):

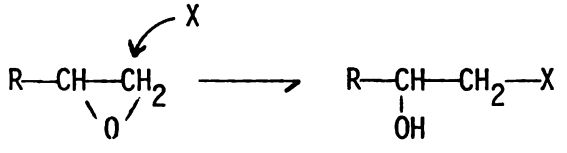


The C-C bond length is intermediate between a normal C-C bond (1.54 A) and that of a C=C bond (1.33 A), while the H-C-H bond angle is intermediate between the tetrahedral bond angle ($109^0\ 28'$) and the trigonal bond angle (120^0) Theoretical models that have been proposed attempt to reconcile the geometry of epoxides by forming hybridizations of carbon which are neither sp^3 nor sp^2 but some intermediate hybrid (Walsh, 1949; Campbell and Cromwell, 1957; Cromwell and Graff,

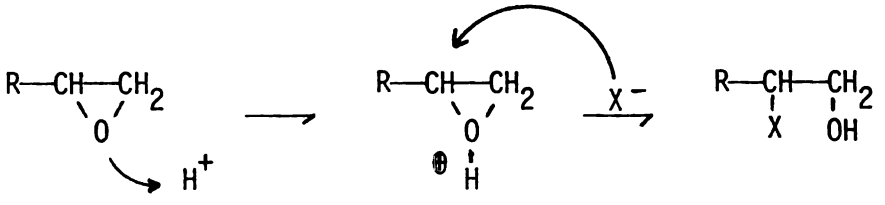
1952; Cromwell and Hudson, 1953; Cunningham et al., 1951). The generalized conclusion is that the reactivity of epoxides can be attributed to relief of ring strain. The electronegative oxygen atom in the ring polarizes the σ bonds, the result being that the oxygen is partially negatively charged and the carbon atoms partially positively charged.

The reactions which epoxides undergo in aqueous solution are most relevant to this discussion and are the only ones which will be treated here. The two most important reactions are: (1) the direct attack of a nucleophile on one of the partially electropositive carbon atoms and (2) acid-catalyzed ring opening, both of which are illustrated below:

(1)



(2)



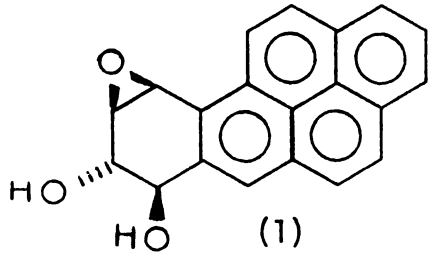
In the direct displacement reaction attack is indicated at the sterically less hindered position, and in the acid catalyzed reaction the ring is shown opening to give the more stable carbonium ion (R is considered to be aliphatic or electron-donating). The fact that the acid-catalyzed reaction proceeds at a much faster rate is supported by the following evidence (Ross, 1950).

Table II.1

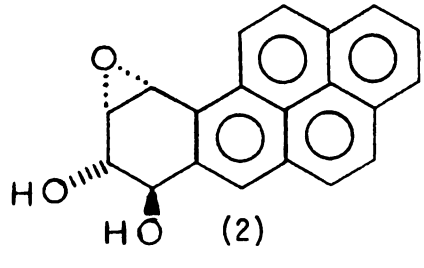
Compound	k_1	k_2
Propylene oxide	0.0091	445
Glycidol	0.0071	45
Epichlorohydrin	0.019	6.95
1,2-3,4-Diepoxybutane	0.0070	7.0
1,2-5,6-Diepoxyhexane	0.023	639
Di-(2,3-epoxypropyl)ether	0.0070	23.1

In this table, k_1 is the rate constant at pH = 7 of the reaction between the epoxide and water and k_2 is the rate constant for the acid-catalyzed addition of water both determined at 37^{o} C. The wide variation in the k_2 values indicates the influence of electronic factors on the protonation of the oxygen. Regardless of that variation the data show the overwhelming rate enhancement by the acid-catalyzed process.

Certain compounds which have been studied because of their mutagenic or carcinogenic or tumor growth inhibition properties contain epoxides, and their reaction mimics this acid-catalyzed process thereby producing a rate enhancement. Whalen et al. (1977) have studied the acid catalyzed and the solvolytic reaction of two benzo(a)pyrene derivatives shown below:

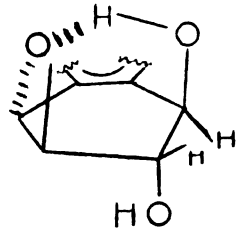


(1)

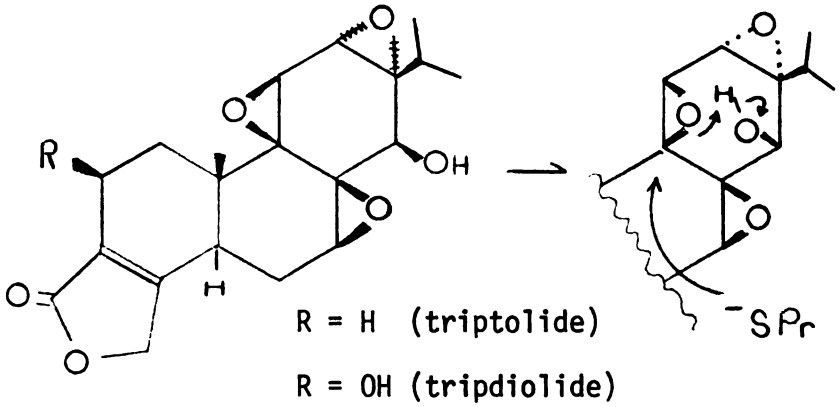


(2)

They found that at low pH (2) is slightly more than twice as reactive as (1) in the hydrolysis reaction. At higher pH (1) is 33 more times reactive than (2) where the reaction is the spontaneous hydrolysis of the compounds. The reasoning given for this enhanced reactivity in the spontaneous reaction is that compound (1) can assume the conformation shown below where the epoxide oxygen can hydrogen bond to the benzylic -OH at C-7. The derivative (2) cannot assume this conformation.



Another example of this enhanced reactivity comes from the work of Kupchan and Schubert (1974) while studying antileukemic plant natural products. Triptolide and tripdiolide contain a 9,11-epoxy-14β-hydroxy system which allows the C-14 -OH to hydrogen-bond to one of the epoxide moieties. The epoxide which alkylates propanethiol is the one which is capable of hydrogen bonding to the -OH. The compounds and proposed mechanism are shown below:



R = H (triptolide)

R = OH (tripdiolide)

Reactions of epoxides with proteins was studied by Frankel-Conrat (1944) in an effort to develop reagents which were capable of reacting with carboxylate residues. Reactions which were known at the time were considered harmful to the protein. The research was stimulated by the fact that ethylene oxide, 1,2-propylene oxide and epochlorohydrin were commercially available. A study of amino acid reactions with epoxides was initiated simultaneously. The protein studies used egg albumin and β-lactoglobulin. The results from these epoxide modifications of the proteins revealed that the treated protein contained fewer free carboxyl, phenolic, primary amino and sulfhydryl groups than the untreated protein. The proteins also showed a dramatic shift in their isoelectric points toward the alkaline side and a marked change in solubility.

E. Creatine Kinase: A Re-examination of the Physiological Role

Creatine kinase, as stated earlier, was thought to be concerned with maintaining the level of ATP constant despite the metabolic state of the cell. In other words, the enzyme serves the cell by providing a rapid reserve source of ATP when the ATP stores are being depleted by vigorous contraction. This concept of creatine kinase's function was developed by early studies on skeletal muscle and later by analogy the same function was applied to the enzyme in cardiac tissue. Recent evidence, taking advantage of both more in-depth studies plus advanced techniques, has suggested a different role for the enzyme. This new evidence will be sumamrized in this section and the controversy surrounding both the old and the new studies will be examined.

The first study, which shed some light on possibly a new function for creatine kinase, was published by Jacobs et al. (1964). In this study these workers discovered that there was high activity of creatine kinase in mitochondria where the tissue contained a large amount of the organelle. Such tissues were heart, pigeon breast and the mitochondria in skeletal muscle. Jacobs' striking result was that in rat hearts 48% of the total creatine kinase activity was located in the mitochondria. The large amount of the total activity in the mitochondria led the investigators to imply that this may represent an unknown pathway of phosphate metabolism. Jacobs and Klingenberg (unpublished results) observed on reactions of isolated mitochondria with external creatine and phosphocreatine that high energy phosphate is transferred by this way between intra- and extramitochondrial compartments. This important finding stimulated research in this area.

Investigators began to look for creatine kinase elsewhere and to check Jacobs' result, and this is where the major controversy arose. The literature in some cases suppported Jacobs' finding and in others did not. The conflict was helpful in that it stimulated a variety of groups to work on the problem and it pointed out difficulties in creatine kinase purification procedures. Kleine (1965) reported that human heart and skeletal muscle mitochondria contained very low values of creatine kinase acitivy. Ottoway (1967) provided evidence that in beef heart 50% of total creatine kinase activity was contained in the mitochondria, 22% was bound to myofi-

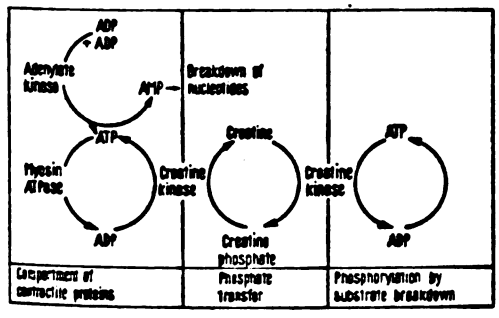
brillar structures and 28% was cytoplasmic. Sobel et al. (1972) determined that rabbit heart mitochondria consistently exhibited creatine kinase activity and that this enzyme was electrophoretically distinct from the other three known isoenzymes. They also determined that this mitochondrial form was more heat-labile, inhibited to a greater degree by urea, less susceptible to inactivation by 2,4-dinitrofluorobenzene and had a lower pH optimum. They noted the effect of external creatine and concluded that phosphorylation of creatine increases the local availability of ADP, which in turn stimulates oxidative phosphorylation. Sharov et al. (1977) devised a histochemical technique for localizing creatine kinase and found activity in mitochondria, on the membrane of the sarcoplasmic reticulum, on the myofibrils and the cytoplasm, on the plasma membranes and finally on the membrane of cell nuclei. In light of these data, namely that creatine kinase was found bound to all cellular structures of the heart where energy utilization takes place, the possibility emerges that creatine is a source of ATP for all energy-requiring processes in the cell.

Other investigators returned to skeletal muscle and found that the organization in that tissue was more complicated than first proposed. Sherwin et al. (1969), using an immunohistochemical technique, examined skeletal muscle and found creatine kinase completely soluble in the intermyofibrillar space. Evidence was also published which showed that while some techniques showed the enzyme to be soluble there might be some association with the contractile apparatus. Thus Turner et al. (1973) determined by enzymatic,

immunological and electrophoretic techniques that a protein which is bound specifically to the M-line of skeletal muscle is creatine kinase. They also proposed that the binding may alter the kinetic properties of creatine kinase to suit the metabolism of energy-utilization. Berson (1976), using the same techniques as Turner et al. (1973), showed that the "γ component" of skeletal troponin was creatine kinase. In the past this "γ component" was always thought to be a contaminent of troponin.

As the evidence for the organization of creatine kinase was mounting, investigators returned to the question of the function of the enzyme. Bessman and Fonyo (1966) designed experiments to determine the role that mitochondrial creatine kinase might play in regulation of respiration. They found that in the presence of creatine ATP was a powerful stimulator of respiration. They proposed that physiological changes in free creatine concentration resulting from muscle contraction may trigger a change in the respiration rate. They also determined that the steady state ADP concentration produced by mitochondrial creatine kinase was lower than optimal to stimulate respiration and that possibly the enzyme's role might be some sort of feedback regulation of respiration in response to muscular activity. A report by Gercken and Schlette (1968) on the poisoning of heart muscle with 2,4-dinitrofluorobenzene (FDNB) received much attention. The results were: (1) FDNB caused an energy deficiency which was not due to inhibition of ATP-utilization, (2) high levels of ATP were still present in the cell and that therefore ATP was

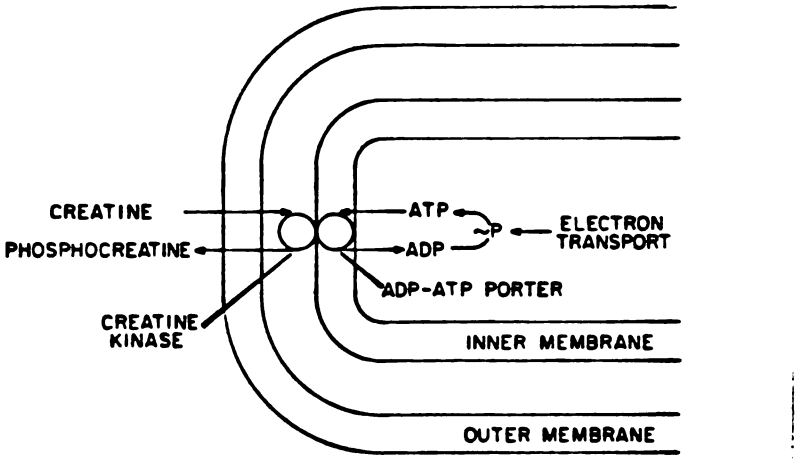
compartmentalized, and (3) that the hearts failed with normal glucose and glycogen content. They summarized their conclusions with the creatine kinase shuttle mechanism shown below:



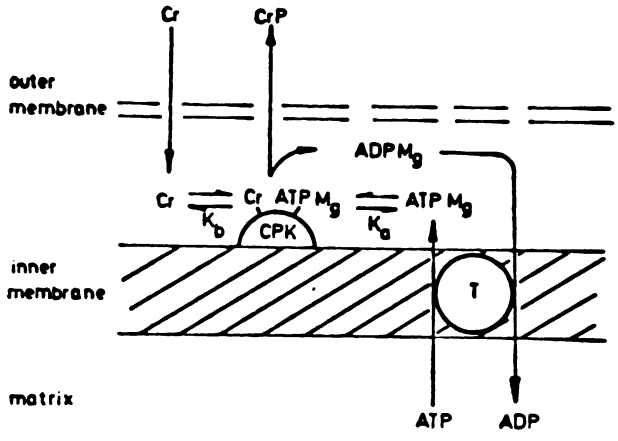
The drawback to this study is that the high level of non-specific reactivity of FDNB could have influenced the study in a way which the authors did not anticipate. Ideally, the FDNB would have inactivated only creatine kinase. Seraydarian and co-workers (1968) studied beating-heart cells in culture and concluded that their data supported the compartment picture of ATP. Gudbjarnason et al. (1970) studied the compartmentalization question by looking at normal and infarcted tissue. This data also supported the idea that in cardiac tissue there were non-interacting pools of ATP.

Jacobus (1972) determined that mitochondrial creatine kinase is strategically located so that creatine can serve as a phosphoryl acceptor and transport energy to the sarcoplasm. He also determined that the specific activity of mitochondrial creatine kinase was 24-fold greater than other nucleotide kinases in the mitochondria, suggesting a domination by creatine kinase. Jacobus and Lehninger (1973) found that creatine kinase was bound to the outside of the inner membrane

of the mitochondria. In conflict with Bessman and Fonyo (1966) they determined that enough creatine kinase was present to convert all ATP from oxidative phosphorylation to phosphocreatine. The diagram below from their work illustrates the intimate relationships of the electron-transport system and mitochondrial creatine kinase.



Saks and co-workers became interested in this problem and published a series of papers where they investigated both theoretically and kinetically this idea of energy shuttling by phosphocreatine. In one report (Saks et al., 1974) they present data to support the shuttle mechanism and involve an ADP-ATP translocase enzyme which would serve to deliver ATP from oxidative phosphorylation to the membrane-bound creatine kinase. This is illustrated below:



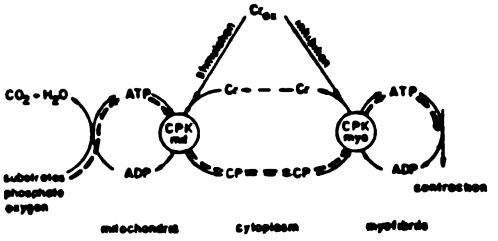
Support for the existence of the translocase enzyme resulted originally from the work of Heldt et al. (1965), where they studied a highly specific exchange reaction for adenine nucleotides in mitochondria. Isotonically-prepared, functionally intact mitochondria contain considerable amounts of endogenous adenine and pyridine nucleotides. These endogenous nucleotides are resistant to washing and therefore must be absorbed in a compartment of the mitochondria which is inaccessible to permeation. Heldt's work mentioned above determined that it was these nucleotides which participated in oxidative phosphorylation. Studies using C-14 labeled nucleotides showed that a rapid exchange process between endogenous and exogenous adenine nucleotides took place (Klingenberg et al. 1964; Pressman, 1958; Pfaff et al., 1965). Saks and co-workers (Saks, V.A., Lipina, N.V., Smirnov, V.N. and Chazov, E.I., 1976)

reported that mitochondrial creatine kinase reacts slowly with ATP in the medium, but reacts quickly when utilizing ATP from oxidative phosphorylation.

Yang et al. (1977) studied the formation of phosphocreatine in mitochondria using ^{32}P-ATP. They found that mitochondrial creatine kinase catalyzes the formation of larger amounts of phosphocreatine with mitochondrial synthesized ATP rather than with exogenous ATP. They concluded that this result supports the shuttle mechanism and also supports the translocase enzyme hypothesis. Saks et al. (1974) also hypothesized that the shuttle mechanism was more efficient because phosphocreatine would be able to diffuse more easily than ATP (of which there is some doubt) and more importantly ADP or ATP are not good regulators because of their ubiquitous biochemical reactions.

Saks et al. (1975) studied the kinetic properties of the mitochondrial enzyme and found properties very similar to those of the other isoenzymes of creatine kinase. They reported that the regulatory action of Mg^{2+} could have some physiological significance. In another report by Saks and co-workers (1976) a study was made concerning the effect of intracellular creatine content on the energetics and force of contraction of the frog heart. The results showed that (1) the force of contraction was dependent on the concentration of creatine and the effect can be both stimulatory and inhibitory and (2) the stimulatory effect was due to an increase in phosphocreatine synthesis by mitochondrial creatine kinase which in turn stimulated oxidative phosphorylation with the net effect being an increase of ATP at the

myofibrillar site. This presumably caused the increase in the force of contraction. The conclusion then involved not only Ca^{2+} in the force of contraction (the classical model) but also the high energy phosphate level. Saks' model for the shuttle mechanism is shown below:



In a series of reports (Saks, V.A., Lipina, N.V., Smirnov, V.N. and Chazov, E.I., 1976; Saks, V.A., Lipina, N.V., Lyulina, N.V., Chernousova, G.B., Fetter, R., Smirnov, V.N. and Chazov, E.I., 1976; Saks, V.A., Chernousova, G.B., Vetter, R., Smirnov, V.N. and Chazov, E.I., 1976), Saks and co-workers summarized and expanded upon results which had been obtained earlier.

In summary, then, at the present time there is sound evidence that in cardiac tissue much of the total creatine kinase activity is bound both at the energy production site and the energy utilization site and that phosphocreatine may serve as the high energy compound which shuttles the energy from one site to the other. Further, the mitochondrial enzyme may be part of the stimulation required for oxidative phosphorylation.

Another aspect of creatine kinases' physiological function which is currently under investigation is the effect of creatine concentration

on myofibrillar protein biosynthesis. Ingwall et al. (1972) and Ingwall et al. (1974) found that creatine was specific in the control of the biosynthesis of actin and myosin. Ingwall and co-workers (1975) also showed the same effect of creatine and of creatine analogs which had a high V_{max} relative to creatine. Analogs which had a low V_{max} relative to creatine suppressed the biosynthesis. It was also found that creatine and analogs which gave the positive effect stimulated the synthesis of the M·M isoenzyme of creatine kinase. Zil'ber et al. (1975) showed that creatine stimulated RNA synthesis in growing chick embryo myoblasts.

It is evident from the preceeding discussion that creatine kinase and the substrates involved in its function are intimately tied to many important processes in the cell and in time the research efforts of many laboratories will lend a clearer picture to their physiological importance.

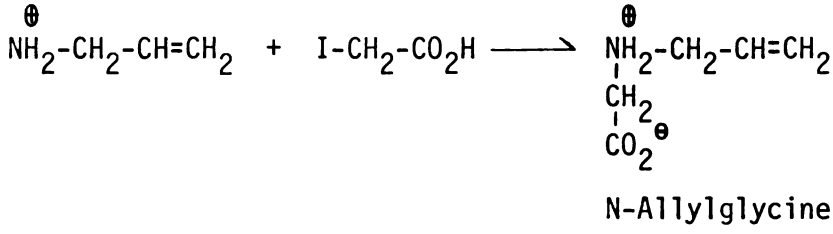
III. RESULTS AND DISCUSSION

A. Synthesis, Purification and Properties of Epoxycreatine

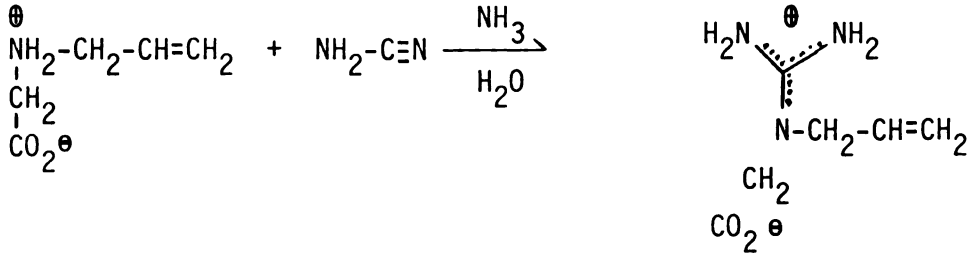
The synthesis of N-(2,3-epoxypropyl)-N-amidinoglycine, which for simplicity will be called epoxycreatine, was originally synthesized by the synthetic scheme below. It should be noted that a more correct name for the compound would be epoxyethylcreatine. Purification problems, which were related to the poor yield in the epoxidation reaction, stimulated attempts to synthesize the compound via a different route and eventually led to the changes made in the original scheme which gave a satisfactory yield of epoxycreatine. The problems involved in the synthesis and the purification will be discussed below.

1. Rationale for the Synthesis

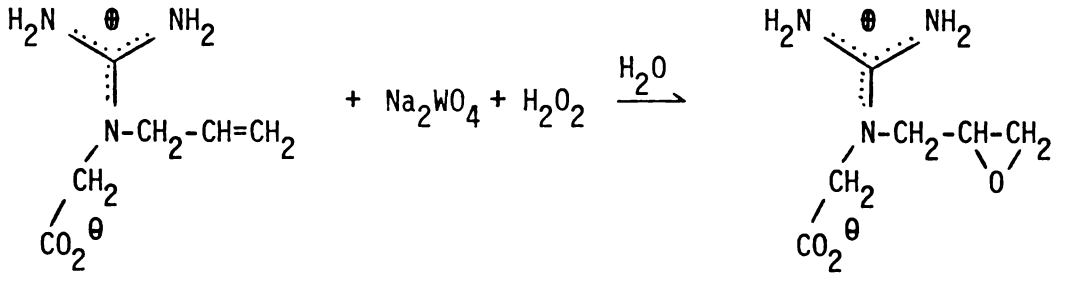
The synthesis involved three steps, all of which had precedents in the literature, and the originally proposed scheme is outlined below.



N-Allylglycine



Vinylcreatine



epoxycreatine

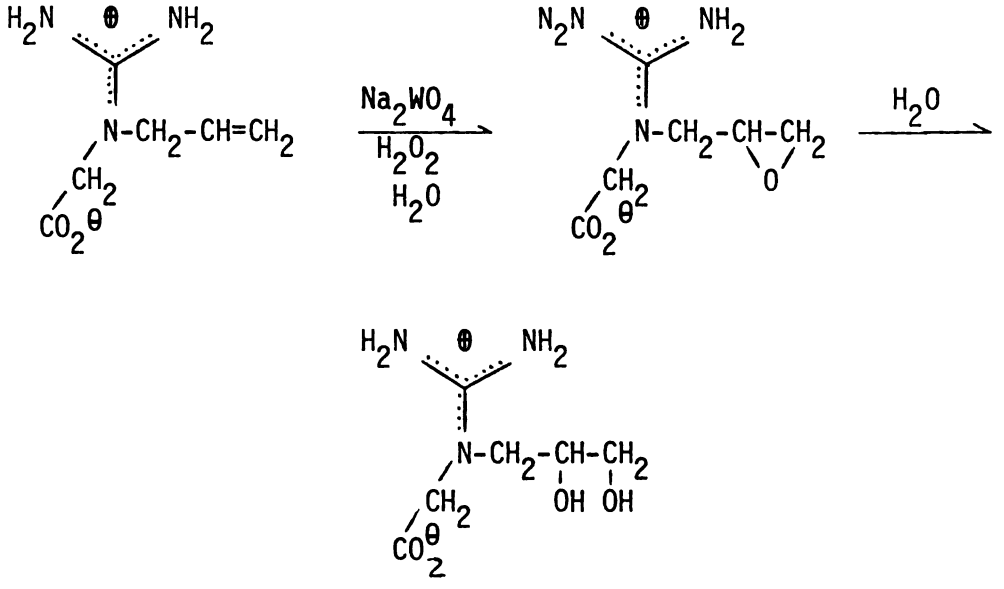
The first two steps of this synthesis, namely the generation of a N-substituted glycine derivative and then the reaction of it with cyanamide to obtain a creatine analog, had been utilized by Kenyon and co-workers previously (Rowley et al, 1971). They had worked out procedures for the first step of the synthesis which enabled the facile separation of the acetone-insoluble zwitterionic product from the acetone-soluble iodide salts which result from a large excess of the amine starting material. The procedure in the second step of the synthesis, the reaction with cyanamide to form the guanidine, was changed somewhat, which led to an increase in yield. The reaction is sensitive to the concentration of the reagents, and the addition of the reactants to the solvent influences their solubility. Thus, it was found that if cyanamide were dissolved first in the solvent it was much easier to dissolve the zwitterion in a small volume of water. This procedure, as stated in the experimental section, was followed for a series of analogs which were synthesized.

The choice of H_2O_2 peroxidation with Na_2WO_4 as a catalyst was made for two reasons: (1) it had been utilized with moderate to very good success when epoxidizing olefins which were electron deficient, i.e., conjugated to electron withdrawing groups (Payne and Williams,

1959), and (2) the reaction could be carried out in an aqueous medium. While the double bond of vinylcreatine is not directly conjugated to an electron-withdrawing substituent, it is allylic to the powerful withdrawing properties of the guanidine moiety. The inductive effect of this group may explain in part the difficulties in the epoxidation reaction. Perhaps more importantly, vinylcreatine is soluble only in water and therefore for the best results it seemed necessary to conduct the reaction in an aqueous solvent.

2. Purification of Epoxycreatine

In retrospect the synthesis of epoxycreatine itself was not a major problem; however, because of the poor yield ($\sim$ 15%) obtained in the epoxidation reaction and the apparent lability of the epoxide moiety the purification of the compound proved to be difficult. Originally the course of the reaction was followed by NMR spectroscopy by observing the disappearance of the olefinic protons and the appearance of upfield peaks characteristic of the epoxide ring. This method of analysis, however, was shown to be inadequate when the corresponding diol analog of epoxycreatine was synthesized and the NMR of it was analyzed. The 60 MHz spectrometer used for following the epoxidation reaction did not provide the resolution to estimate the amount of diol which could be present in the reaction mixture. The possible side reaction is shown below.

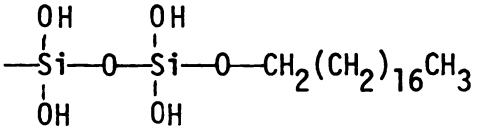


N-(2,3-dihydroxypropyl)-N-amidinoglycine

Formation of the diol was a distinct possibility because judging the yield of the reaction by the disappearance of the olefinic protons in the NMR spectrum it was necessary to run the reaction for at least 48 hours in order to see the absorption decrease to about 50% of the initial amount. The reactivity of epoxycreatine, of course, was not known at that time but by analogy to other epoxides that length of time in solution at room temperature was certain to generate some diol. For comparison purposes the diol was synthesized by an independent route. The conditions of the reaction were varied in order to investigate whether the length of time for the disappearance of the olefinic protons could be shortened. None of the modified conditions (pH, temperature, amount of Na_2WO_4) provided satisfactory results. All attempts to crystallize pure epoxycreatine failed. Many chromatographic techniques were attempted, and, while some separation of

vinylcreatine from the other reaction components was accomplished, the resulting reaction product consistently gave an unsatisfactory C, H and N analysis calculated for epoxycreatine.

High pressure liquid chromatography (HPLC) has recently come into regular use for organic synthesis purification. The sensitivity of the methods detection system (UV or refractive index) allowed it to be exploited by analytical and biochemists as well, and it was here that the purification of peptides was investigated with very satisfactory results. The column used for this type of purification was the newly developed reverse-phase column which is essentially a hydrophobic adsorbent, hence the name reverse phase, as it is the opposite of the normally polar column adsorbents, such as silica gel or alumina. The eluents normally used with a reverse phase column were buffered aqueous solutions. The reverse phase absorbent manufactured by Waters Associates, Inc. is called Bondapak C-18 and the chemical structure is shown below.



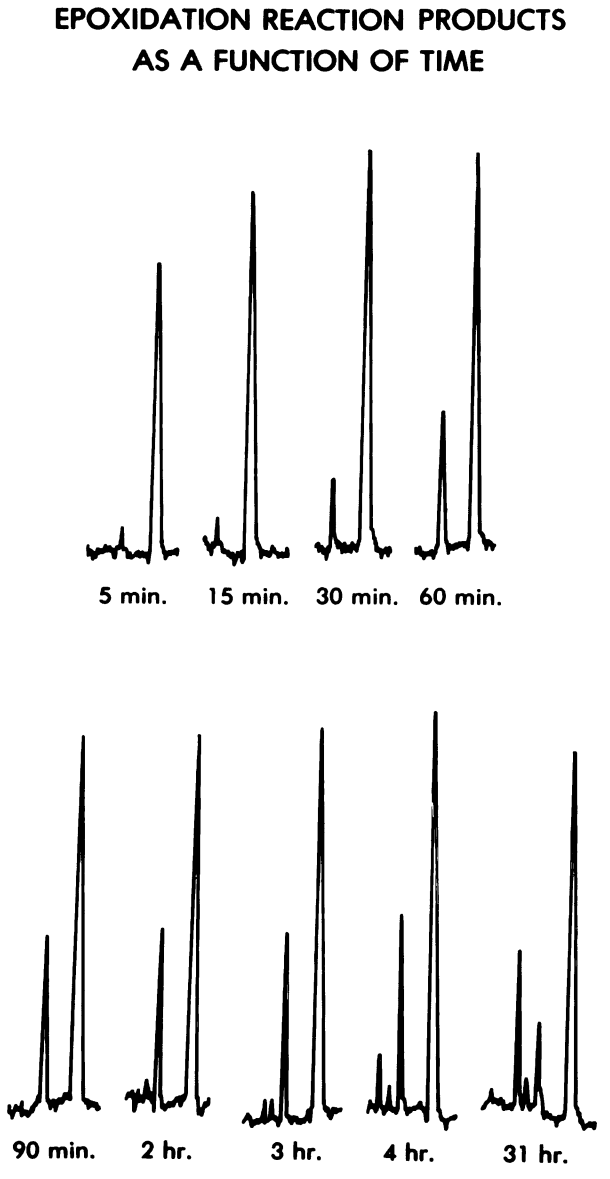
The microcrystalline grade contains particles 10 μ in size.

Since creatine and creatine analogs are derivatives of amino acids it was feasible that reverse phase HPLC could be used to purify epoxycreatine. Analytical samples of the diol and the starting material, vinylcreatine, were injected with distilled water as the eluent to determine if separation was possible with this system.

The retention times obtained using the refractometer detection system were 6 minutes for the diol and 11 minutes for vinylcreatine. It was reasonable to assume that epoxycreatine would have a retention time between these two compounds. When the epoxidation reaction mixture was injected, four peaks were observed, one corresponding to the diol at 6 minutes, one to vinylcreatine at 11 minutes, a very small peak at 7.2 minutes and a peak at 8.5 minutes. By visual inspection of the chromatogram it was determined that most of the reaction mixture consisted of vinylcreatine with a fair amount of diol present and a smaller amount of the peak at 8.5 minutes, which was assumed to be the epoxide.

The sample of the reaction mixture which had been injected was that which had been allowed to oxidize for 47 hours at room temperature and at neutral pH. HPLC provided a facile method to study the reaction products as a function of time. This was accomplished by simply withdrawing aliquots at indicated times, stopping the reaction by precipitation of the products with acetone, drying the samples and then analyzing them by HPLC. The results of this study are shown in the following figure.

Figure III.1

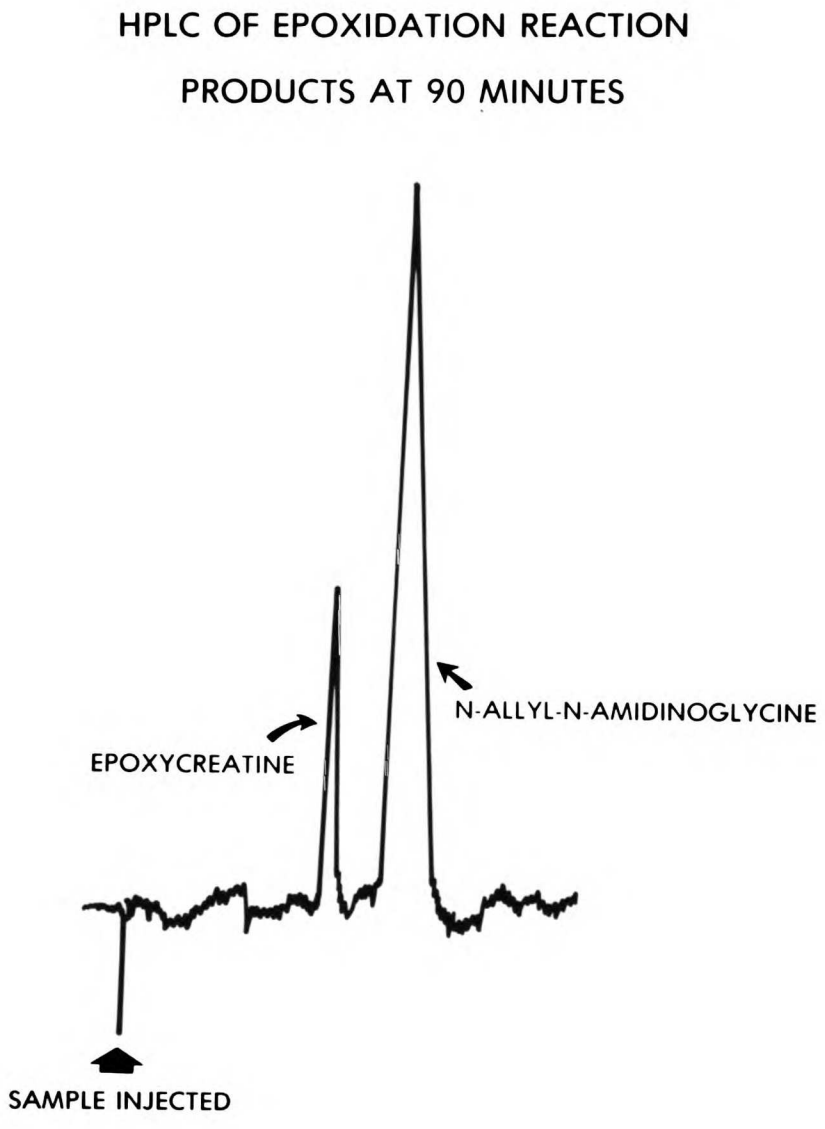

EPOXIDATION REACTION PRODUCTS
AS A FUNCTION OF TIME
5 min.
15 min.
30 min.
60 min.
90 min.
2 hr.
3 hr.
4 hr.
31 hr.

The first aliquot taken at 5 minutes indicates the beginning of the formation of the peak with the retention time of 8.5 minutes. This peak continues to increase up to 90 minutes of the reaction time. At the 2 hour time point the minor peak at 7.2 minutes appears and it increases very slowly. At 3 hours the diol appears and it continues to increase at the expense of the 8.5 minute peak, as is obvious by inspection of the 31 hour aliquot. It was now evident that by allowing the reaction to continue for 48 hours most of the epoxycreatine which was present had reacted with the solvent to form the diol. It was also determined from this study that the diol tended to tail on the column when it was overloaded. The loss in resolution severely limited the amount of the reaction mixture which could be applied to the column. The optimal reaction time therefore appeared to be 90 minutes. The reaction was repeated and this time allowed to continue for 90 minutes. Samples from this reaction were injected and the two peaks collected.

An advantage of using distilled water as the eluent was that it could be easily removed by lyophilization. The fact that the water was removed while frozen was also an advantage as it would certainly help to protect the epoxide during the isolation. The 8.5-minute peaks when collected were kept on ice while a series of injections were made in order to minimize any diol formation during this time. It was also determined that the maximum amount of the reaction mixture which could be injected each time was 20 mg. If more than 20 mg were injected the vinylcreatine peak began to spread out and decreased the resolution between it and the 8.5-minute peak.

The following figure shows a 20 mg injection of the reaction mixture:

Figure III.2



The areas of each peak indicated that vinylcreatine composed 85% of the product distribution and the other peak therefore 15%.

An important piece of evidence as to the identity of the 8.5-minute peak was also obtained using HPLC. A portion of the lyophilized material obtained from this peak was redissolved in water and reinjected into the HPLC and only one peak emerged at 8.5 minutes. Therefore no degradation of the suspected epoxycreatine peak had occurred during the isolation procedure. Another portion of the lyophilized material was redissolved in H_2O, placed in a boiling water bath for 5 minutes and reinjected into the HPLC. Only one peak emerged with a retention time of 6 minutes which corresponded to that of the diol. This will be discussed later in reference to the structure proof of epoxycreatine.

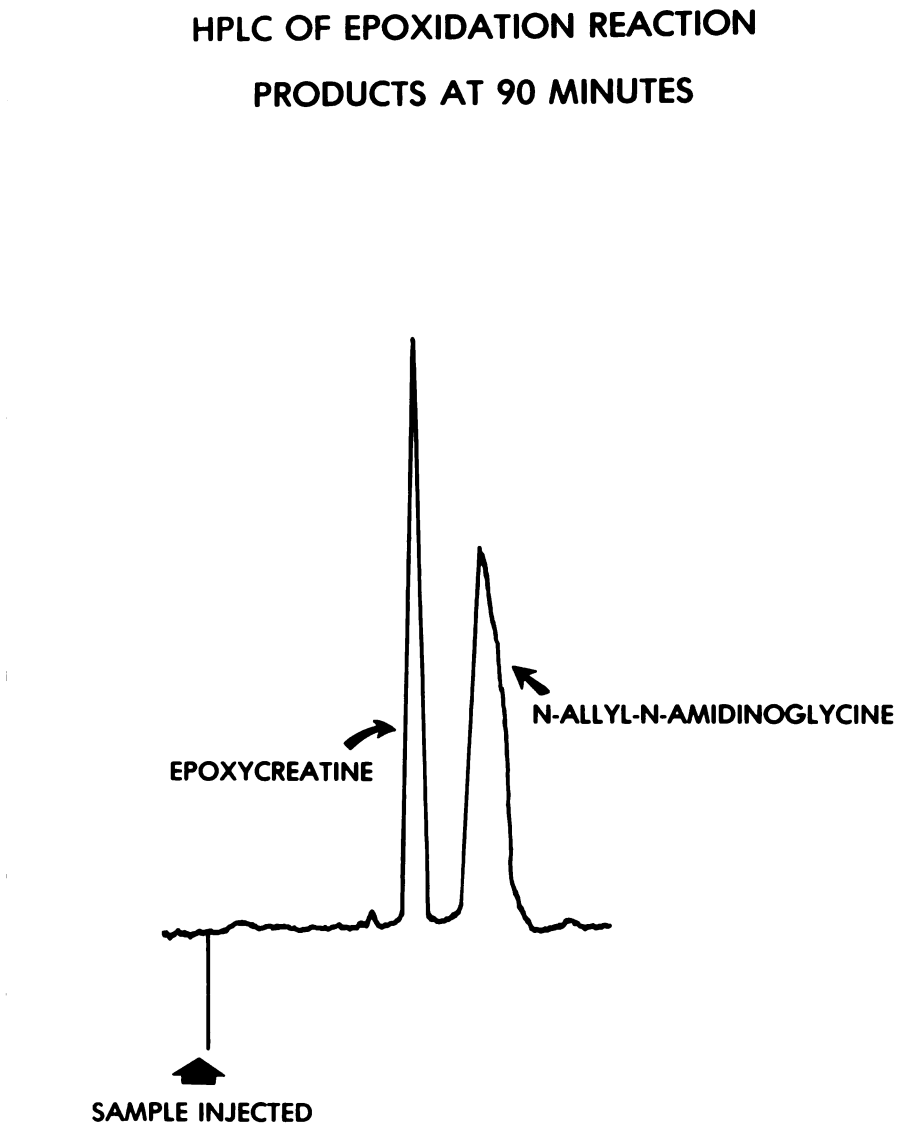
3. Improved Synthesis of Epoxycreatine

In an effort to obtain a higher yield of epoxycreatine the reaction procedure and the amount of 30% H_2O_2 was changed. Instead of using water as the solvent for the reaction , vinylcreatine

was dissolved directly in 30% H_2O_2 and solid Na_2WO_4 was added to initiate the reaction. The HPLC chromatogram indicated the yield of epoxycreatine was now about 25%. The reaction was repeated with the same modified procedure except that 50% H_2O_2 was used and the reaction was allowed to continue for 90 minutes. The chromatogram this time showed the yield of epoxycreatine to be 46%. Ironically the maximum amount which could be injected each time was still 20 mg.

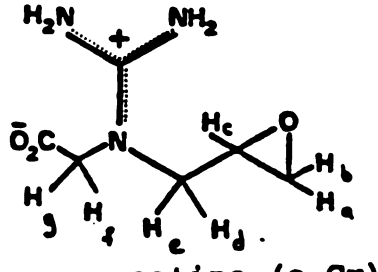
An injection larger than that caused decreased resolution because the epoxycreatine peak began to tail into the vinylcreatine peak. Attempts to increase the yeild of epoxycreatine further are currently under way. The following figure shows the chromatogram of the improved synthesis.

Figure III.3

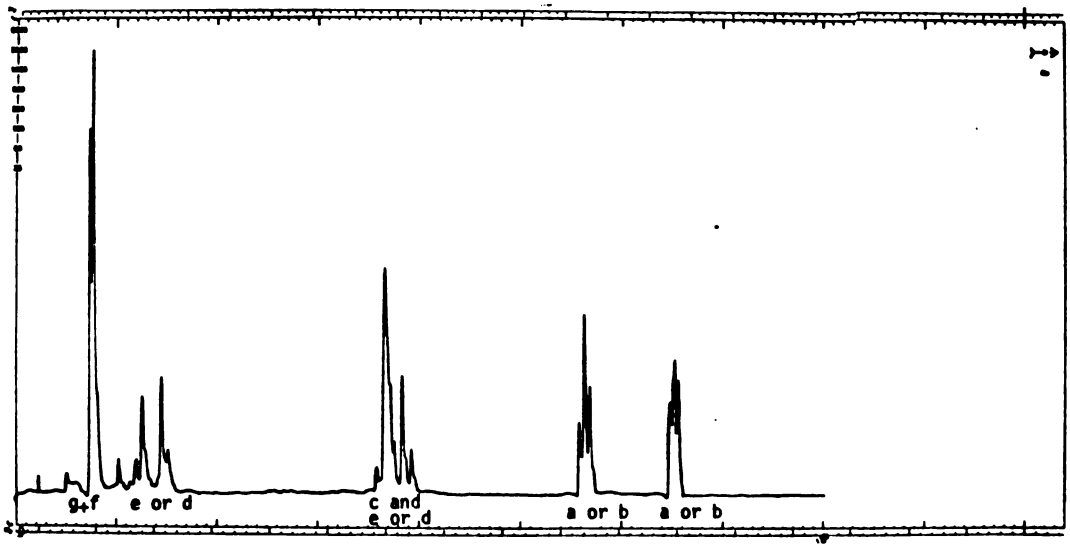


4. Properties of Epoxycreatine

The 100 MHz 'H-NMR spectrum of epoxycreatine revealed the characteristic upfield absorption of epoxide rings at 2.7 and 2.9 ppm. A corresponding spectrum of the diol was also taken for comparison purposes. Both spectra were complicated by the fact that the 100 MHz instrument did not provide the resolution necessary to interpret them. Consequently 360 MHz spectra were taken at the Stanford Magnetic Resonance Laboratory, Stanford University. The spectra taken on this instrument appear in Appendix A. The spectrum of epoxycreatine is shown below along with the probable assignments made from the decoupling experiments which were also performed.



epoxycreatine (e-Cr)



The infrared spectrum which was taken as a KBr pellet showed absorption characteristic of the epoxide ring at 1260 (weak), 900 (moderate) and 850 (strong) cm^{-1}. The guanidine moiety shows absorption at 1690 cm^{-1} and the carboxylate group at 1605 cm^{-1}. There is no evidence in the spectrum of ester formation (neither is there in the NMR spectrum). Ester formation could come about by the attack of the carboxylate group on the epoxide ring; however, it appears as if the zwitterionic attraction stabilizes the molecule and provides a barrier to the occurrence of this attack.

The long-term stability of epoxycreatine in aqueous solution is not known except that it is stable to hydrolysis at 0^{o} C for 12 hours when dissolved in distilled water. This was tested simply by analyzing the sample by HPLC.

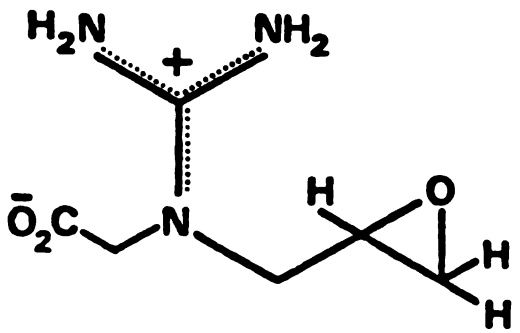
Epoxycreatine is soluble up to approximately 200 mM in a solution of 0.01 M HEPES, pH 7.45. The compound has never been crystallized but instead lyophilized to remove water, so it, as many lyophilized samples, contains residual amounts of water. The final analytical sample contained 0.25 mole H_2O/mole epoxycreatine; however, it is not known whether this represents a quarter hydrate or not. It is possible that a sample can be dried so that no water remains.

The structure proof relies on the C, H and N analysis, the NMR spectrum with the characteristic upfield absorption, the IR spectrum and the HPLC retention times. The epoxide would be predicted to elute between the diol and the olefin, and the hydroylsis of the epoxide to the diol with the corresponding change in retention times is an added proof.

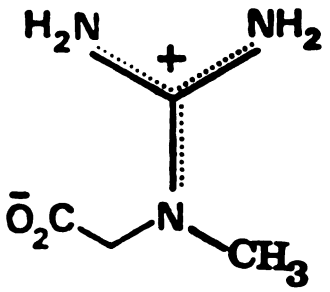
B. Interaction of Epoxycreatine with purified Creatine Kinase

1. Effect of Epoxycreatine on Enzymatic Activity

The structures of epoxycreatine and creatine are shown below.



epoxycreatine (e-Cr)



creatine (Cr)

The design of the creatine-based affinity label, epoxycreatine, resulted from detailed investigations of the bulk tolerances of the active-site of creatine kinase. Preliminary studies had shown that both glycocyamine (Tanzer and Gilvarg, 1959) and N-ethyl-glycocyamine (Ennor et al., 1955) could act as substrates. A more detailed and quantitative study (Rowley et al., 1971) found that the active-site could tolerate derivatives of creatine where the methyl group had been altered. Thus it was found that the ethyl and propyl homologs of creatine were substrates in the enzymatic reaction with an initial rate relative to creatine of 13 and 1%, respectively. The structure of epoxycreatine is, of course, very similar to the propyl derivative of creatine and therefore it was reasonable to assume that since they are sterically similar epoxycreatine would also be able to bind at the active-site and then possibly inactivate the enzyme.

Incubation of epoxycreatine with creatine kinase causes complete inhibition of enzymatic activity and the activity does not return after exhaustive dialysis. The initial experiments were performed at 0^{o} C, 0.01 M HEPES (pH 7.45), epoxycreatine concentration of 48 mM, and creatine kinase concentration of 1 mg/mL. Under these conditions the enzyme was completely inactivated in about 4.25 hours. The initial and later studies of the epoxycreatine-creatine kinase interactions were performed at 0^{o} C because it was known that epoxycreatine was stable to hydrolysis at that temperature for at least 12 hours and therefore the competing reaction of hydrolysis would not interfere with these studies.

2. Criteria for Active-Site Directed Irreversible Inhibition

Meloche (1967) in a paper on the inactivation of 2-keto-3-deoxy-6-phosphogluconic aldolase by bromopyruvic acid developed the kinetic derivations to which an active-site-directed reagent must conform and detailed the criteria which are necessary to prove that a compound is indeed reacting at the active site. These criteria are: (1) the inactivation must be complete and (2) irreversible. In addition, (3) the inactivation should obey saturation kinetics, (4) the normal substrate or a competitive inhibitor of the enzymatic reaction should protect against the inactivation in a competitive manner and (5) the irreversible binding of the inhibitor should be stoichiometric; i.e., one inhibitor molecule bound per active site.

The initial experiments with epoxycreatine described earlier showed that the interaction with the enzyme satisfied the first two points of the stated criteria. The inhibition was complete; in other words, enzymatic activity was completely abolished and exhaustive dialysis which would remove any small molecule either not covalently bound or tightly associated with the enzyme indicates that the inhibition is irreversible. The additional experiments which will be described will show that epoxycreatine satisfied points (3), (4) and (5) of the above-mentioned criteria.

3. Kinetics of Irreversible Inhibition

The kinetic derivations which follow are those derived by Meloche (1967) with some notation changes to simplify the equations.

The affinity label, if it is indeed reacting at the active site, should in the initial binding step act similarly to the normal substrate; that is, there should be a dissociable complex formed between the affinity label and the enzyme. This complex formation is represented by the following equation:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} E_{inact} \qquad \text{(Eqn. III.1)}$$

where E represents free enzyme, I the inhibitor (in this case epoxycreatine), EI the enzyme-inhibitor complex and E_{inact} the inactivated enzyme. Using the steady state approximation, i.e., dEI/dt = 0, and assuming (1)

$$E_t = E + EI + E_{inact} \qquad \text{(Eqn. III.2)}$$

where E_t represents the total enzyme present in all forms (2),

$$V'_{inact} = k_2 \; EI \qquad \text{(Eqn. III.3)}$$

and (3),

$$V_{inact} = k_2 (E_t - E_{inact}) \qquad \text{(Eqn. III.4)}$$

the following equation can be derived,

$$V'_{inact} = \frac{V_{inact}}{\frac{K_{inact}}{I} + 1} \qquad \text{(Eqn. III.5)}$$

where V'_{inact} represents the inactivation velocity at the inhibitor concentration I , V_{inact} the inactivation velocity at infinite

concentration I and K_{inact} is $\frac{k_2 + k_{-1}}{K_1}$ and represents that concentration of inhibitor which gives the half-maximal inactivation rate and presumably half-saturates the enzyme. This derivation is analogous to the Michaelis-Menten equation derived under steady-state conditions.

The initial inactivation rates were measured by determining the amount of time for a given inhibitor concentration to cause a 50% loss of enzymatic activity. This time is called the inactivation half-time ($t_{\frac{1}{2}}$). The inhibitor concentration is very high with respect to the enzyme; therefore the reaction should be pseudo first-order, and the following relationship applies:

$$t_{\frac{1}{2}} = \ln 2 / \text{first order rate constant}$$

It follows then that $t_{\frac{1}{2}} = \alpha\ 1/V'_{inact}$ and $T \alpha\ 1/V_{inact}$. Substituting these terms into Eqn. III.5 yields:

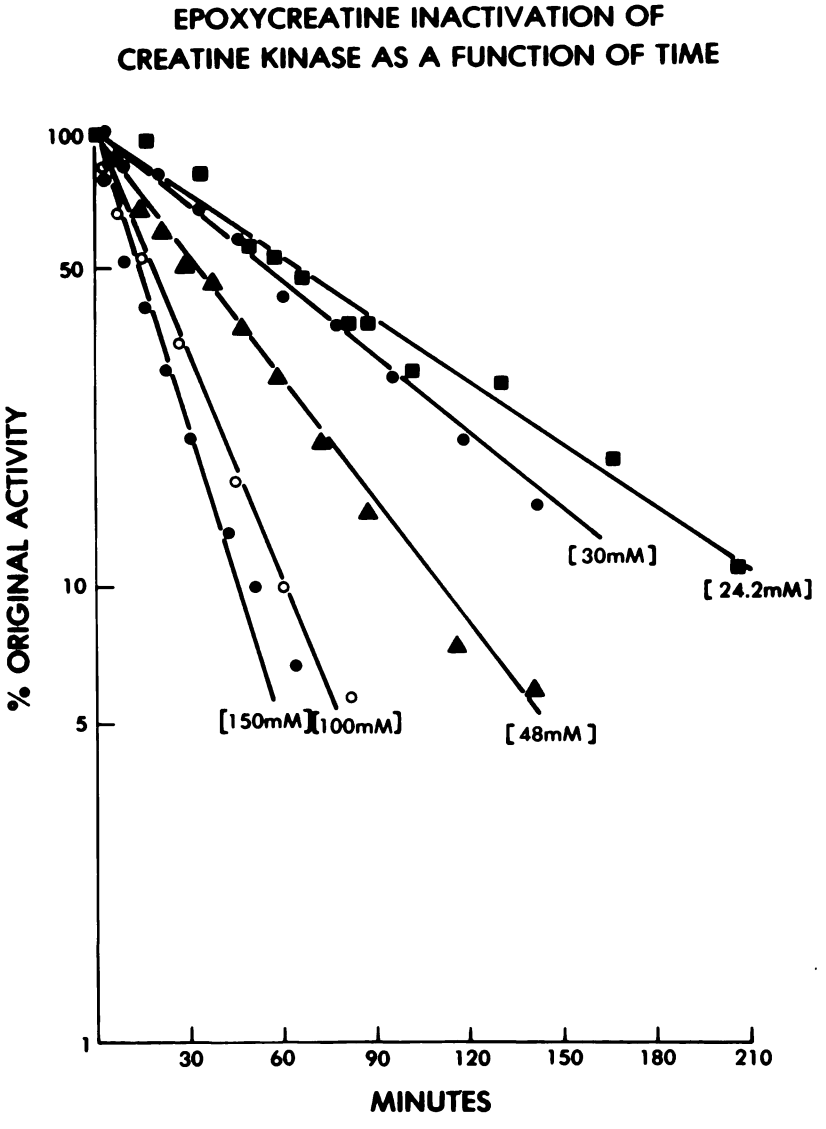
$$t_{\frac{1}{2}} = \frac{1}{[I]} \quad (T\ K_{inact}) + T \qquad \text{(Eqn. III.6)}$$

where T is the maximum inactivation half-time at infinite inhibitor concentration. All other parameters are the same as previously defined. This equation is the linear form of Eqn. III.5., and therefore a plot of $t_{\frac{1}{2}}$ vs. $1/[I]$ should give a straight line with an intercept T, if the interaction of the inhibitor and enzyme first forms a dissociable complex.

The inactivation half-times of creatine kinase were measured at several different concentrations of epoxycreatine at 0^{o} C, pH 7.45.

The figure below shows a portion of the data obtained when plotted as pseudo first-order reactions.

Figure III.4



Meloche (1967) and others have observed deviations from linearity which indicates the reaction is no longer pseudo first-order. Cooperativity between subunits or interconvertible forms of the enzymes have been given as reasons for this deviation. The deviation was not seen in this case; the inactivation remains pseudo first-order when the enzyme was 95% inactivated. The values of $t_{1/2}$ and the respective concentrations of epoxycreatine are shown in Table III. 1.

Table III. 1

Inactivation of Creatine Kinase with Epoxycreatine

epoxycreatine	$t_{1/2}$
24.2 mM	64 min.
30.0	55
33.3	46
36.0	39.6
48.0	32
100.0	19
150.0	14

The values of $t_{1/2}$ were plotted *vs.* 1/[epoxycreatine] according to equation III.6. The plot is shown in figure III.5. Based on the observation that the double reciprocal plot is linear and intersects the ordinate at a point greater than zero, epoxycreatine apparently

does form a dissociable complex prior to inactivation of the enzyme as described by eqn. III.1. The minimum inactivation half-time (T) is 3.44 min. This value corresponds to a first-order rate constant of

$$k = \frac{\ln 2}{3.44 \text{ min.}} = 3.36 \times 10^{-3} \text{ sec.}^{-1}$$

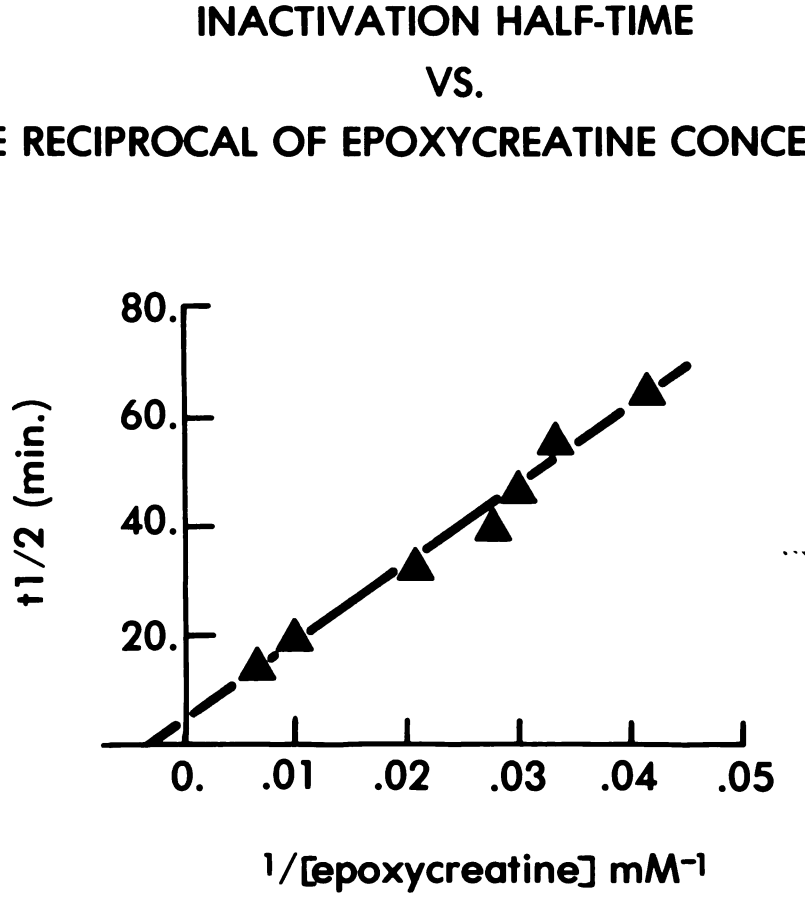
The value of K_{inact} can be obtained from the slope of the line or from the negative of the reciprocal of the intercept on the abscissa. Therefore for creatine kinase

$$K_{inact} = 417 \text{ mM}$$

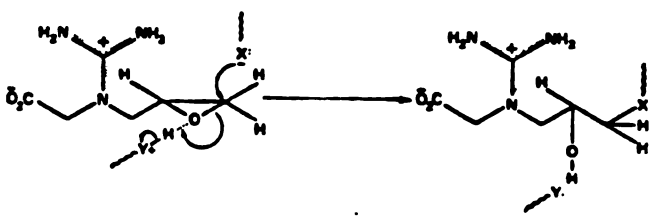
This value for K_{inact} seems reasonable when compared to other experimental data. Maggio and Kenyon (unpublished results) have found the K_s of the propyl analog of creatine to be 395 mM at 30° C with the beef skeletal muscle isoenzyme. While the K_s values vary somewhat from isoenzyme to isoenzyme the k_{inact} seems to be in general agreement with this K_s value. The fact that it is somewhat larger is not surprising because the epoxypropyl moiety is larger than the propyl moiety. Also the value was determined at 30° C and that with epoxycreatine at 0° C. McLaughlin et al. (1972) determined the K_m of creatine at 1° C and found it to be 5 mM as compared to that at 30° C which is 8.6 mM (Maggio and Kenyon, unpublished results). The K_s of creatine is 24.4 mM at 30° C; however, the value at 0° C has not been determined.

The first-order rate constant was stated earlier to be 3.36 x 10^{-3} sec.$^{-1}$ which can be converted to 12.1 hr.$^{-1}$ The value when converted to hr.$^{-1}$ can be compared to the data of Ross (1950)

Figure III.5


INACTIVATION HALF-TIME
VS.
THE RECIPROCAL OF EPOXYCREATINE CONCENTRATION
80.
60.
40.
20.
t1/2 (min.)
0.
.01
.02
.03
.04
.05
1/[epoxycreatine] mM-1

which was summarized earlier in Table II.1. The value of 12.1 $hr.^{-1}$ compares very favorably with values in that table for the acid-catalyzed addition of water to an epoxide. The data of Ross was determined at 37^{o} C and that for epoxycreatine at 0^{o} C. It is obvious that creatine kinase dramatically enhances the reactivity of the epoxide ring of epoxycreatine. The reasons for this enhanced reactivity could be that (1) the binding site for creatine or a creatine analog renders the epoxide more labile because of a particular environment created at the active site by the protein backbone or (2) the enzyme itself could hydrogen bond to the oxygen of the epoxide ring and therefore mimic the acid-catalyzed reaction. This is illustrated below:



where the group Y protonates the oxygen and the group X reacts with the epoxide ring itself. The attack of X is shown at the least sterically hindered position on the ring; however, bearing on the constraints at the active site, reaction at the other position cannot be ruled out. This observation of the reactivity of the epoxide ring may help in deducing the molecular mechanism of the creatine kinase reaction.

4. Substrate Protection of the Inhibition

If indeed epoxycreatine is inactivating creatine kinase by the irreversible reaction at the creatine binding site, then creatine present in solution should protect competitively against the inhibition. This can be derived in a similar fashion as before using the steady-state approximation. The reaction scheme is shown below:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} E_{inact} \qquad \text{(Eqn. III.7)}$$

$$+ \quad S \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ES$$

where S represents the normal substrate (creatine) and ES the enzyme-substrate complex. All other notations are the same as defined earlier. The sum of all forms of the enzyme is

$$E_t = E + EI + ES + E_{inact} . \qquad \text{(Eqn. III.8)}$$

The derived rate equation therefore is

$$V'_{inact} = \frac{V_{inact}}{\frac{K_{inact}}{[I]} + \frac{K_{inact}}{I}\frac{[S]}{K_s} + 1} \qquad \text{(Eqn. III.9)}$$

where $K_s = k_{-3}/k_3$ and represents the apparent dissociation constant of the enzyme-substrate complex. The linear expression for this equation is

$$t_{\frac{1}{2}} = \frac{1}{[I]} T \left(K_{inact} + \frac{K_{inact}[S]}{K_S}\right) + T \quad \text{(Eqn. III.10)}$$

This equation when plotted as $t_{\frac{1}{2}}$ vs $\frac{1}{[I]}$ at constant levels of [S] should result in straight lines whose slopes are determined by the substrate concentration and the lines should intercept the ordinate at the minimum inactivation time. In addition K_S can be determined from the slopes of the lines.

As before, the half-times of inactivation were determined graphically by plotting $t_{\frac{1}{2}}$ vs. $1/[I]$. this time, however, in the presence of either 20 or 40 mM creatine. The individual inactivation plots are contained in the Appendix B. The following tables show the results of the protection experiments.

Table III.2

20 mM creatine

epoxycreatine	$t_{\frac{1}{2}}$
24 mM	68 min.
26	67
30	53
33.3	52
36	48
48	37
100	19

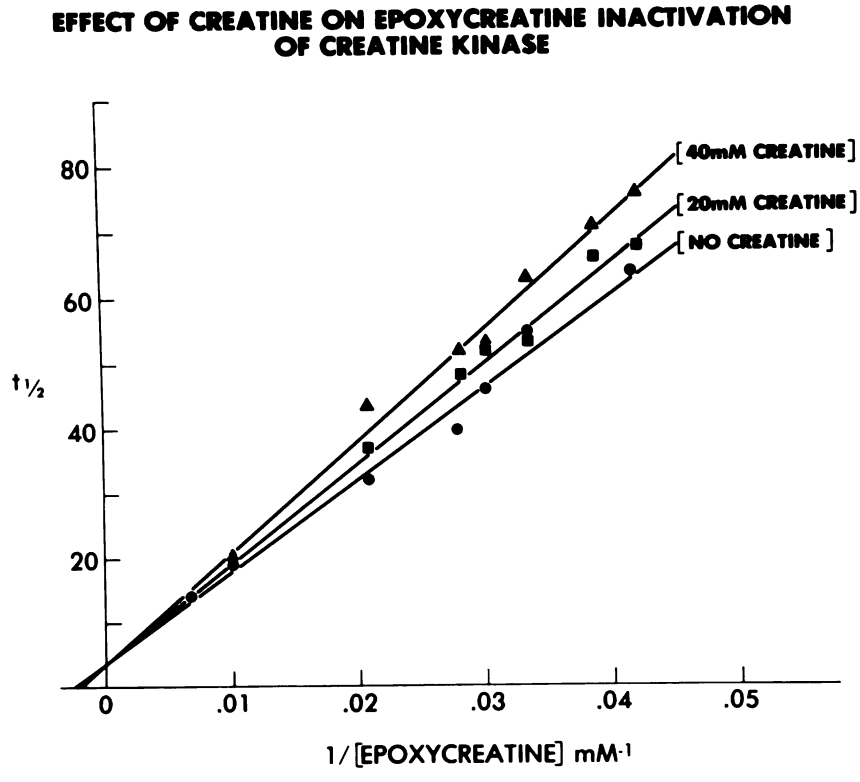
Table III.3

40 mM creatine

epoxycreatine	$t_{1/2}$
24 mM	76 min.
26	71
30	63
33.3	53
36	52
48	43.5
100	19

The following figure shows the results when the above data were plotted according to eqn. III.10. The result with no creatine present is included for comparison.

Figure III.6


EFFECT OF CREATINE ON EPOXYCREATINE INACTIVATION OF CREATINE KINASE
[40mM CREATINE]
[20mM CREATINE]
[NO CREATINE]
80
60
40
20
t½
0
.01
.02
.03
.04
.05
1/[EPOXYCREATINE] mM-1

From Figure III.6 it can be seen that creatine does protect the enzyme from inactivation in a competitive manner. This is evidenced by the fact that all 3 lines intersect the ordinate at the same value (T), which should be the case as this is the inactivation half-time at infinite epoxycreatine concentration. The following table summarizes the results.

Table III.4

creatine	slope	K_s
20 mM	1573 min. mM	207 mM
40 mM	1759 min. mM	177 mM

{The correlation coefficient (r) for each line was 0.994.}

From this data it becomes obvious that creatine is not protecting the enzyme from inactivation as well as it should. Although as stated earlier the K_s for creatine at 0^o C is not known, it would be very surprising if it were very different from the value of 24.4 mM determined at 30^o C. As can be seen from Table III.4 the values at 20 and 40 mM creatine are 207 and 177 mM, respectively. There are a number of possibilities which could account for these results. Evidence shown later will discount the possibility that epoxy-creatine is reacting at sites other than the active site and thereby causing the inactivation. Another possibility is that the site

which creatine binds and that which epoxycreatine binds are not exactly the same but overlap somewhat. This is certainly possible because the structural difference in the two molecules may cause epoxycreatine to bind in a somewhat different fashion, causing a distortion at the active site and allowing then an overlap of sites. Evidence will also be shown later to discount the possibility that a cooperative effect between subunits is the cause for the less than optimal protection by creatine. Actually, Figure III.2, which shows a linear pseudo first-order reaction for > 95% of the inactivation, tends to rule out such a cooperative effect.

The last possibility to consider is that creatine is able to bind at sites other than the active site. These sites may be specific (serving as some sort of regulatory mechanism) or non-specific (possibly due to the charged nature of the molecule). Equilibrium dialysis and gradient sedimentation studies by Kuby et al. (1962) determined the number of binding sites for the substrates of creatine kinase. They found this number equal to 2 for the nucleotide species; however, because of the weak association of creatine and phosphocreatine for the enzyme they found it difficult to determine the value and stated that the number of sites was at least 2 and probably greater than that. Therefore, if creatine is able to bind at more sites than the active site, the result would be a decreased effectiveness in protection against inactivation at the active site. Of course nothing is known about these other sites and certainly nothing is known about epoxy-

creatine's interaction (or lack of interaction) at these sites. Further investigation is needed to clarify this point.

5. Stoichiometry of the Epoxycreatine - Creatine Kinase Interaction

The stoichiometry of binding of epoxycreatine to creatine kinase was determined by the use of ^{14}C-labeled epoxycreatine. The synthesis of the radioactively labeled epoxycreatine, which was analogous to the unlabeled synthesis, is detailed in the experimental section (V.C.4.). The label was positioned at the carboxylate carbon of epoxycreatine, and introduced by using ^{14}C-labeled iodoacetic acid as a starting material. The labeled iodoacetic acid was diluted with unlabeled material at the start of the synthesis, with the final specific activity of epoxycreatine being 2.5×10^{-1} mCi/mmole. The protein concentration was determined spectrophotometrically as stated in the experimental section and was compared to the Lowry method (Lowry, 1951) of protein determination. Both methods yielded the same result and therefore the spectrophotometric determination was used because of its simplicity.

The particular experiment was carried out at an epoxycreatine concentration of 100 mM at 0^{o} C for 20 hours. The half-time at this concentration was 32 minutes and therefore the reaction was carried out for 37.5 half-times. The results are shown below.

(1) $cpm_{sample} = E \times dpm_{sample}$

(2) $cpm_{sample} + cpm_{std} = E \times dpm_{sample} + E \times dpm_{std}$

where E represents the efficiency

$$(2) - (1) = E \times dpm_{std}$$

$$E = \frac{(2) - (1)}{dpm_{std}}$$

(1) cpm_{sample} - background: 2102.5 cpm/mL

(2) $cpm_{sample} + cpm_{std}$ - background: 20521.4 cpm/mL

(2) - (1) = 18418.9 cpm/mL

$$(\text{Efficiency}) = \frac{18418.9}{41500} = .444$$

from eqn (1)

$$dpm_{sample} = \frac{cpm}{E}$$

$$dpm_{sample} = 4735.4$$

1 millicurie = 2.22×10^{9} dpm

2.13×10^{-6} mCi in sample

specific activity = 2.5×10^{-1} mCi/mmole

8.53×10^{-6} mmole lable present

protein concentration = 4.38×10^{-6} mmole/mL

(1.0 mL counted)

1.96 mmole epoxycreatine/mmole creatine kinase

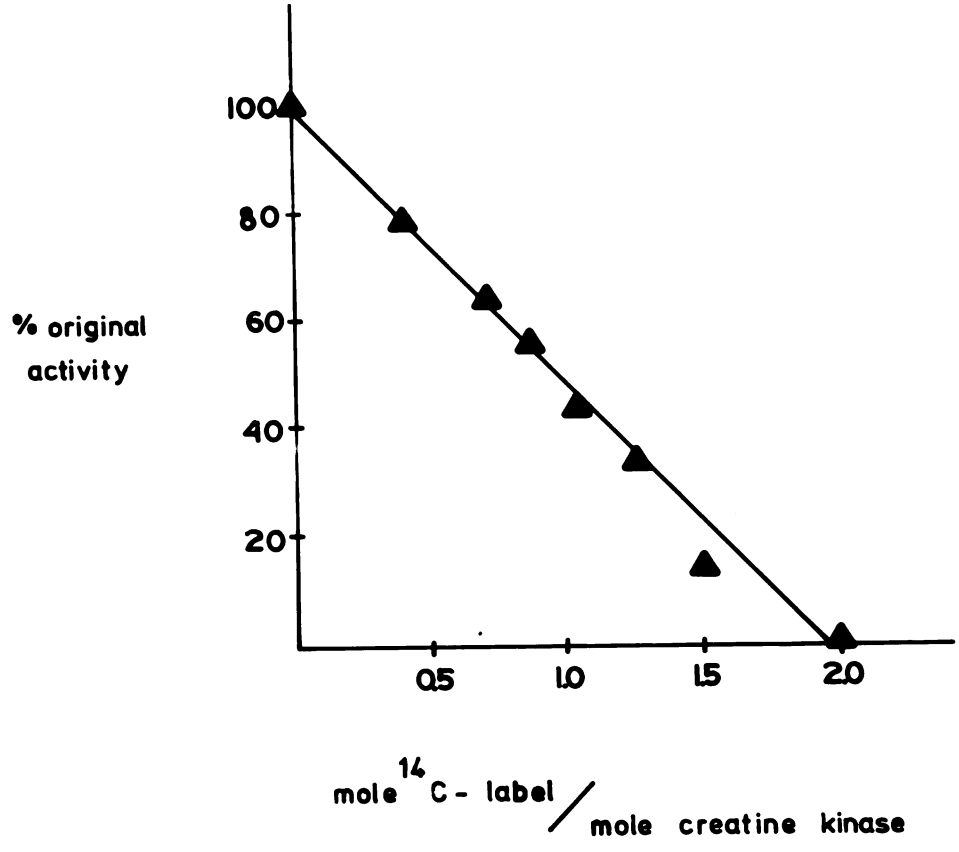
An aliquot of the above prepared protein solution was diluted and re-counted with the ratio being 2.02 mmole epoxycreatine/mmole creatine kinase. These ratios correspond to modification of one site at each subunit. As pointed out earlier the reaction was

allowed to proceed long after enzymatic activity had ceased and with a molar excess of epoxycreatine to creatine kinase of 480. The results indicate that only one site per subunit was modified and thus that the modification is highly specific.

Half-of-the-sites reactivity is a phenomenon observed in some enzymes which exist as dimers, tetramers and so on and where differential reactivity of the subunits is observed. In an experiment to check if the subunits of creatine kinase react at the same rate with epoxycreatine the enzyme was treated with the inhibitor, assayed for enzymatic activity and counted to determine the amount of epoxycreatine bound. The results are shown below. It should be noted that 5 points were taken at times after 2.0 mmole label/mmole enzyme, all of which indicated no further reaction, and the points were weighted accordingly. The results clearly indicate that both subunits react with epoxycreatine at the same rate and that incorporation of two moles of label/mole of enzyme is necessary to obliterate enzymatic activity.

Figure III.7

Enzyme Activity vs. Epoxycreatine Bound



6. Epoxycreatine as a Substrate for Creatine Kinase

The epoxide ring of epoxycreatine was placed on a portion of the molecule where it was known that the enzyme could tolerate some bulk. The actual molecule is close structurally to creatine and very close to that of the propyl analog which is 1% (V_{max} relative) as active as creatine (McLaughlin, et al., 1972). It seemed reasonable therefore that epoxycreatine might also be a substrate. The limited quantities of epoxycreatine which were available at the time prevented the use of the pH-stat assay and so the method of Rowley and Kenyon (1974) was used (see experimental V.D). The result showed that epoxycreatine had been phosphorylated and that there was an increase in the amount of ADP present at the expense of ATP based on a control. Obviously for epoxycreatine to be a substrate in the enzymatic reaction it must bind at the active site and therefore this experiment clearly shows that epoxycreatine is capable of interacting at the active site of the enzyme.

7. Effect of Other Substrates on the Inactivation Rate

All studies reported above involved epoxycreatine and creatine kinase. It was of interest to determine the inactivation rate in the presence of other substrates, being that there is synergism in substrate binding; i.e., at 30^{o} C K_s for creatine is 24.4 mM and the K_m is 8.6 mM. The enzyme preparation in this study was different from the earlier one and the specific activity of this later prepara-

tion was higher. The first preparation had a specific activity which was equal to the highest reported and the fact that this later enzyme was higher yet is unexplained. The small amount of precipitate in the vial may have been a minor contaminant, which, by falling out, increased the activity.

The concentration of epoxycreatine used in these experiments was 48 mM, and the inactivation half-time was 21 minutes. The earlier reported half-time at this concentration was 32 minutes. The difference was reproducible and has been ascribed to the difference in enzyme activity. The results of this study are summarized below.

Table III.5

epoxycreatine	creatine	MgATP	MgADP	NO_3	$t_{1/2}$
48 mM	—	—	—	—	21
48	—	4	—	—	29
48	—	—	4	—	24
48	40	8	8	8	366

(All concentrations are in mM and $t_{1/2}$ in min.)

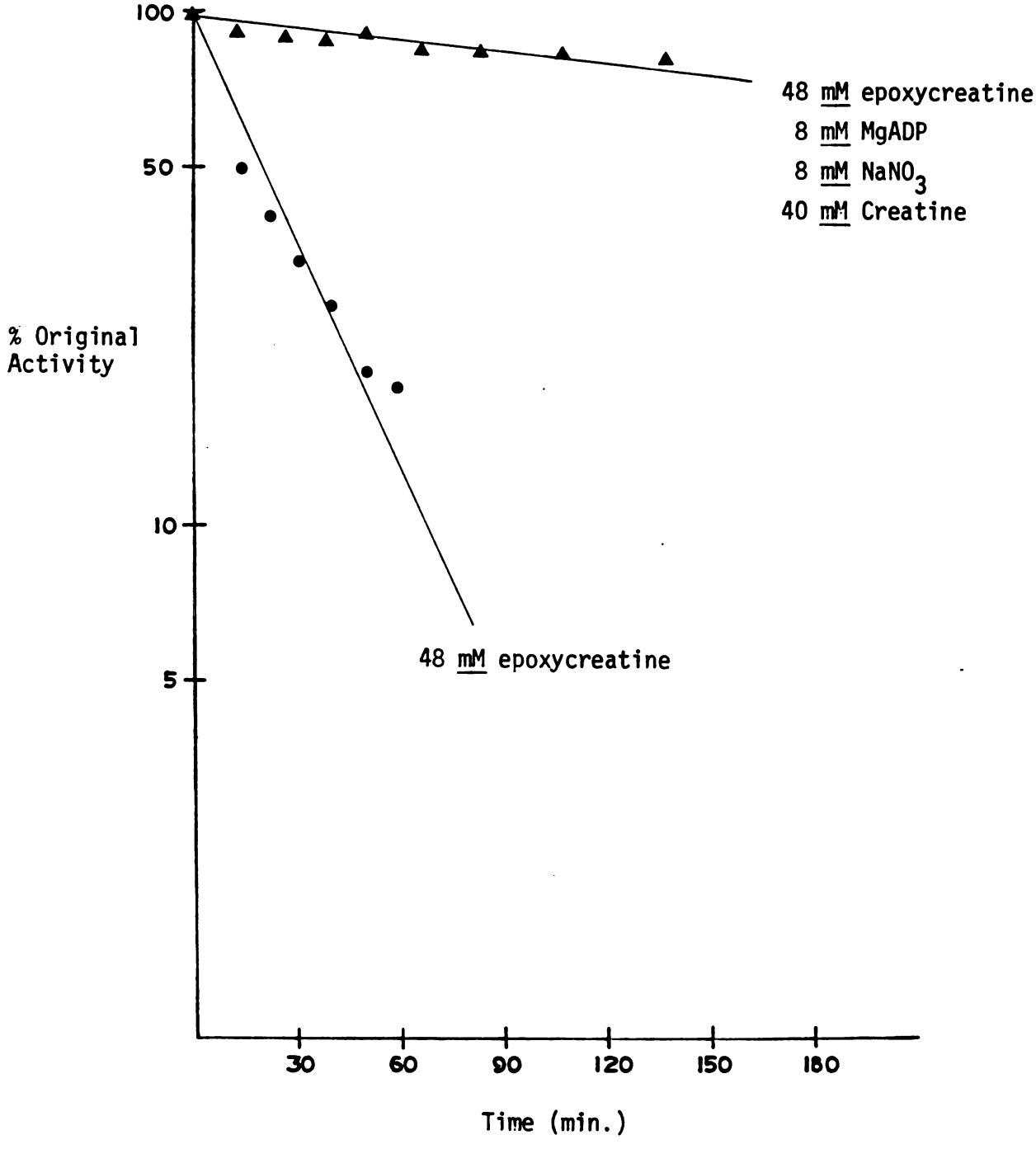
The following interpretation is given for these data. The half-times increase in the following fashion: control < MgADP < MgATP. One might expect that the half-times would have decreased because of the synergistic effect of the nucleotides on creatine (and

presumably epoxycreatine) binding. However, based on the efforts of Struve and Kenyon (1973), Phillips and Quiocho (unpublished results cited in Annesley and Walker, 1977) and Struve et al. (1977) it has been suggested by use of a creatine analog that the nitrogen trans to the carboxyl group of the creatine structure is the one which is phosphorylated. The nitrogen which is presumably phosphorylated is thus on the same side of the molecule as the epoxypropyl group of epoxycreatine. A simple interpretation of these results would be that the epoxypropyl group overlaps partially in the nucleotide binding site and the increase in inactivation half-time is a steric phenomenon. It should be also pointed out that the experiment with epoxycreatine and MgATP has the additional variable of enzyme turnover, and the effect of phosphorylated epoxycreatine on creatine kinase is not known.

Milner-White and Watts (1971) first postulated that the abortive ternary complex of creatine-MgADP-NO_3^-, when bound to creatine kinase, forms an extraordinarily tight complex because the planar NO_3^- mimics the transferred phosphoryl group and is therefore a transition-state complex (Wolfenden, 1972). A number of research groups have investigated this idea since then and have supported it for the most part. As can be seen from the table above this complex protects the enzyme from inactivation by epoxycreatine to a very large degree. The figure below shows the transition-state complex rate of inactivation and the control inactivation rate is included for comparison.

Figure III.8

Inactivation rate in the presence of the transition state complex.



This result ($t_{\frac{1}{2}}$ = 366 min.) shows that in this tight complex epoxycreatine is presumably prevented from access to the active site and therefore the rate of inactivation is very slow, indicating the inactivation process is taking place solely at the active site. This result should be thought of in terms of the protection experiments with creatine where creatine did not protect as well as it should have. As mentioned earlier, creatine kinase possesses synergism in the binding of substrates. The transition-state complex exemplified this effect with the net result being very effective protection against the active site reaction of epoxycreatine.

8. Removal of the Affinity Label from Creatine Kinase

In studies mentioned in the Historical Background of this dissertation it was found for many epoxide-containing affinity labels that a carboxylate group of either aspartic or glutamic acid residue was modified. This reaction with the carboxylate group would create an ester functional group on the enzymic surface. In these cases the investigators usually treated the inactivated enzyme with dilute alkaline solutions and studied the effect of such base-treatment on the enzyme-affinity adduct. In cases when the label was labile in alkaline solutions, the next step usually was the effect on this label's removal rate by hydroxylamine. If an ester hydrolysis had occurred, then hydroxylamine should increase this rate.

a. Treatment with NaOH

Creatine kinase which had been treated with ^{14}C-epoxycreatine for another experiment was utilized, and it contained 1.5 mmole ^{14}C-label/mmole of enzyme. The protein was treated for 12 hours at 25° C with 0.1 N NaOH. The protein sample was counted to determine the amount of radioactivity present and the ratio was 0.2 mmole ^{14}C-label/mmole enzyme; therefore 87% of the radioactive label was lost. The ease and mild conditions which caused the label's removal implied that perhaps a carboxylate group of creatine kinase had been modified.

b. Comparison of the effect of NaOH and NH_2OH

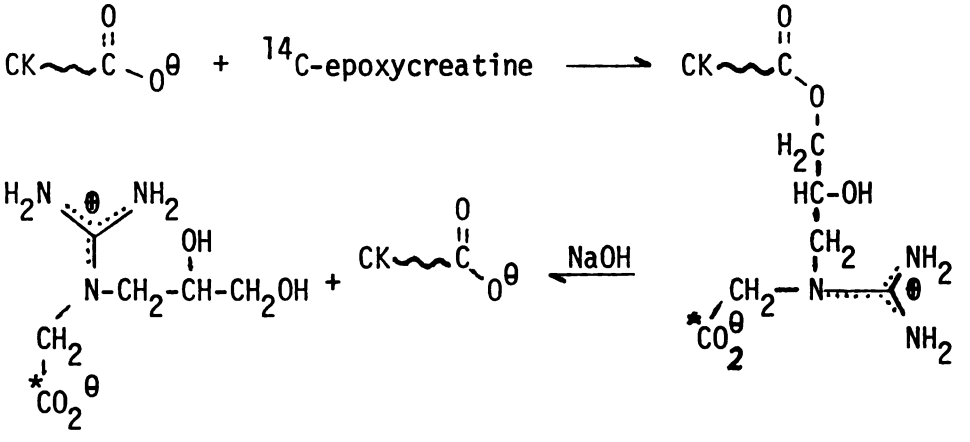
Two samples of ^{14}C-epoxycreatine-treated creatine kinase were treated analogously except one was in an aqueous solution, pH 9.0, and the other in 2.0 M NH_2OH, pH 9.0. The samples were treated for 5 hours and then counted. The results showed that the base treated sample lost 18% of the label while the NH_2OH sample lost 70% of the label. The results clearly indicate that NH_2OH accelerates the rate of label removal and implicates further a carboxylate residue of the enzyme.

9. Attempts to Determine the Modified Residue

The amino acid residue which was modified by epoxycreatine is still not known; however, the rationale of the attempts to determine this will be summarized here.

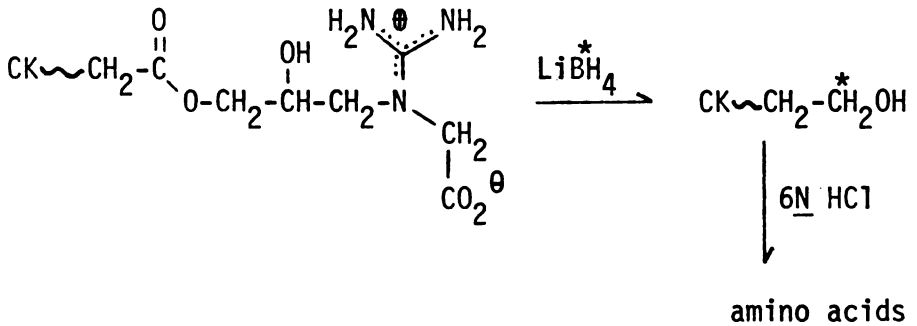
The solution to this problem was approached from two perspectives. First, if an ester were formed, then basic hydrolysis would yield the diol analog of the epoxide. This diol had been synthe-

sized earlier by an independent route and therefore its properties of it were known. Second, the ester adduct should be reducible by $LiBH_4$ (Chibnall and Rees, 1958). After hydrolysis of the protein, the amino acid analog generated should be observed using an amino acid analyzer. The experiments are diagrammed below.

(1) 

After separation of the expected diol from the protein by gel filtration the diol could presumably be analyzed by Fourier Transform NMR, retention time in the HPLC and finally counted to determine if it was radioactive. The results of this experiment are inconclusive because the sample obtained was not clean. It contained some component which interfered in the NMR and which by HPLC was shown to absorb at 280 nm. No attempts were made to see if it was protein in nature; however, the NMR spectrum did not resemble dibutyl phthalate, a common substance in dialysis membranes.

The second experiment was a modification of the method of Chibnall and Rees (1958). The modification was that labeled $LiBH_4$* (tritium)was used instead of unlabeled $LiBH_4$. The reason for this will become clear in the experiment outlined below (beginning with the proposed ester).



If the modified residue was an aspartic acid group, then tritium-labeled homoserine will be found in the amino acid analysis. If the residue was glutamic acid then the tritium labeled next higher homolog of homoserine will be found. This compound has been called either bis-homoserine or pentahomoserine. Due to a series of instrument problems no results are available from this experiment. Homoserine and bis-homoserine are commercially available and therefore their retention times could be easily determined.

C. Interaction of Epoxycreatine with the Perfused Cat Heart

1. Preliminary Experiments with a Rat Heart Homogenate

The newly emerging evidence for a more involved physiological role for creatine kinase in heart muscle was stated earlier. One of the more important experiments detailed there was the FDNB-treatment of heart muscle (Gercken and Schlette, 1968). The non-specific reactivity of this compound was mentioned as the drawback to this experiment. Epoxycreatine, as has been demonstrated, is highly and specifically reactive toward creatine kinase and would seem to be therefore an ideal tool to use to investigate the physiological function of the enzyme. By inactivating the enzyme in its physiological environment and monitoring resulting function, it should be possible to deduce the importance of the enzyme. Indeed, this is one of the most important uses of active-site directed reagents.

The problems and complexity associated with the purified enzyme and its interaction with epoxycreatine are minor when approaching the problem on the physiological level. There are many questions which remain unanswered, and these results are preliminary, but nevertheless point to some interesting conclusions.

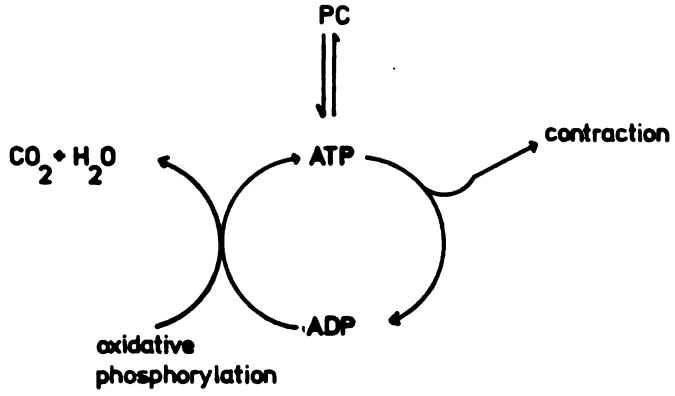
Substrate analogs react with the skeletal and heart muscle isoenzymes of creatine kinase with different rates. Epoxycreatine's effect on the heart muscle isoenzyme had never been tested; therefore, a preliminary experiment was carried out to determine

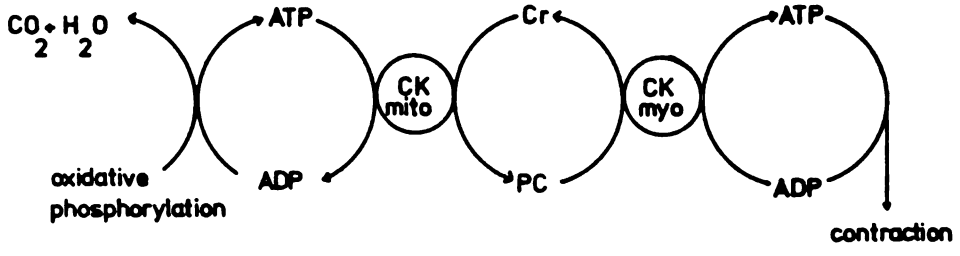
if the compound could inactivate this isoenzyme. A rat heart homogenate was used for this experiment. The results indicated that 67% of the total creatine kinase was inactivated. At the concentration of epoxycreatine used (58 mM) and the total amount of activity in the rat heart sample, the results show that epoxycreatine is capable of inactivating the heart muscle isoenzyme.

2. Results with Perfused Cat Hearts

The physiological function of creatine kinase, as stated earlier, is currently under investigation again. Research concerning the function of the enzyme has been stimulated by the findings presented in the Historical Background (II.E). The enzymes' role as a rapid source of ATP is well-documented. The newly emerging evidence, however, implies a more direct role for creatine kinase in energy transfer. The experimentation with epoxycreatine is planned to investigate if either or both of the models shown below are physiologically relevant.

two possible schemes of cardiac muscle energetics





These experiments were conducted in collaboration with Dr. William Jacobus, Dept. of Cardiology, Johns Hopkins University. The experimental protocol was decided upon because of the vast amount of research carried out at Hopkins on perfused cat hearts. The preliminary work aimed at better ways to carry out open heart surgery indicated how long a time period these perfused hearts were stable to manipulation. It was found that a heart once it was

stabilized on the perfusion apparatus (approximately 15 min. after removal from the animal) could be arrested with KCl and kept in the arrested state for about 1 hour. After that time it could be re-perfused and regain 100% of its function monitored by the developed pressure (systolic-diastolic pressure). The experiment therefore consisted of a control which when arrested had an 80 mM solution of creatine injected into the heart cavity and the experimental heart which had an 80 mM solution of epoxycreatine injected into the cavity. The reasoning was that the epoxycreatine would diffuse into the myocardium and inactivate the creatine kinase. The results are summarized below:

preliminary results of perfusion experiments

	control	epoxycreatine treated
% developed pressure returned	97	50
ATP(u moles/gm.)	1.95	1.24
PC (u moles gm.)	3.11	3.77
creatine kinase (IU/gm.)	275	170

The results clearly indicate that inactivating creatine kinase in the perfused heart alters function. These results again are preliminary but some speculation as to what occurred is possible. It seems unreasonable if the rapid, reserve mechanism were operable that any effect should be observed. The heart was not stressed in any way and therefore even if creatine kinase were inactivated the heart should be able to continue functioning at its original level. The result is striking because the correlation of function lost to creatine kinase inactivation is approximately 1:1. This result implies that creatine kinase is responsible for energy transfer and interruption of that transfer pathway will affect the function. The fact that the ATP and phosphocreatine levels are not statistically different from the control values indicates some sort of metabolic regulation of cardiac function. From these preliminary results, though, it appears as if the mechanism first proposed by Gercken and Schlette (1968) is possible in cardiac muscle. Clearly there are many questions unanswered. An important question is the relative reactivities of the creatine kinase bound at the myofibrils and that inside the mitochondria. A number of experiments are planned and with the use of epoxycreatine will help explain the physiological role of creatine kinase.

D. Specificity of Epoxycreatine

1. Interaction with Actin and Myosin

In an effort to determine if epoxycreatine is specific in its association with creatine kinase in an *in vivo* situation its effect on actin and myosin were investigated. These studies were carried out with the help of Dr. Roger Cooke, Dept. of Biochemistry and Biophysics, University of California, San Francisco. The actin (Spudich and Watt, 1971) and myosin (Tonomura *et al.*, 1966) were purified from rabbit skeletal muscle. Essentially the experiment consisted of incubating actin and myosin separately with epoxycreatine and assaying the myosin ATPase enzyme by means of a pH-stat. The myosin ATPase can be activated by either Ca^{2+} or actin, and both activators were used. The results indicate that epoxycreatine does not affect the activity of the myosin ATPase either by its interaction with myosin or actin. The results are summarized below.

Table III.6

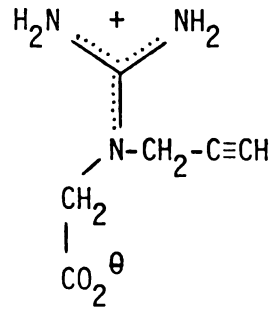
Sample	Ca^{2+} activated (μmole/g.s.)	actin activated (μmole/g.s.)
myosin + creatine	9.0	4.7
myosin + epoxycreatine	9.0	4.2
actin + epoxycreatine		4.5

The differences seen here are within the limit of experimental error of this assay method and are average values for three experiments. The results indicate that epoxycreatine does not inhibit or interfere with the contractile apparatus.

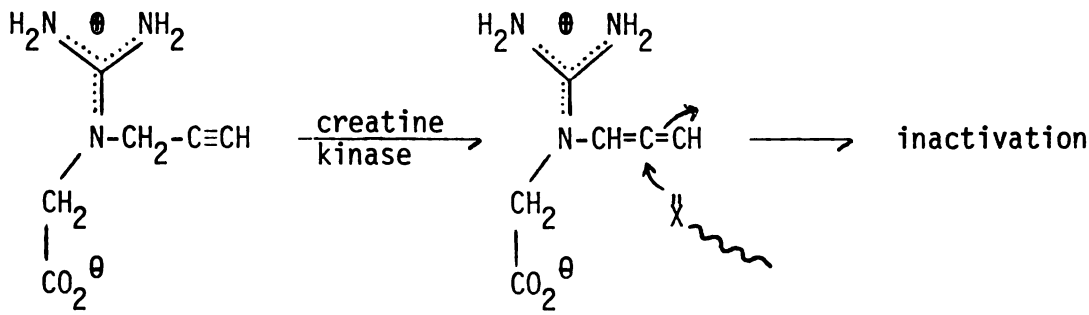
E. Other Studies

1. N-(2-propynyl)-N-amidinoglycine and Creatine Kinase

The structure of this analog is shown below:



The compound was synthesized in the hope that it would be a mechanism-based irreversible inhibitor (Rando, 1977). The possible mechanism of inactivation was envisioned as proceeding by the pathway shown below:



an allene

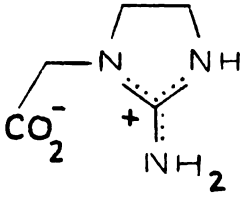
Acetylenic analogs have been used as inactivators, as mentioned earlier, and the proposed mechanism is the generation by the enzyme at the active site of an allene which is very reactive toward nucleophiles. Creatine kinase must, during the course of the

phosphorylation, add or remove a proton, and therefore inactivation by the acetylene was possible.

Incubation of creatine kinase with the analog produced no inactivation. It is possible that in order to activate the analog the enzyme would be turning over. No further studies were carried out with the other substrates except to study the acetylene as a substrate. The results of this study are found in Appendix C.

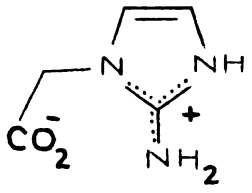
2. 1-Carboxymethyl-2-iminoimidazole and Creatine Kinase

The Kenyon laboratory has been very active in the synthesis of new creatine analogs and their interaction with the enzyme. Rowley et al. (1971) produced the most active (relative to creatine) analog to date. 1-Carboxymethyl-2-iminoimidazoline has an initial rate which is 31% that of creatine.



1-carboxymethyl-2-iminoimidazolidine

With this in mind 1-carboxymethyl-2-iminoimidazole was synthesized.



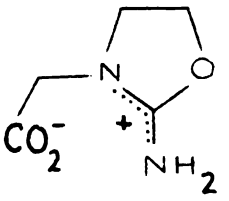
1-carboxymethyl-2-iminoimidazole

This compound was interesting from a number of viewpoints. First, was it a substrate? And second, if not, was it a competitive inhibitor? The answer to both questions is no. The reaction was assayed by the spectrophotometric method because this analog is a buffer at ∿ pH 7 and prevents the use of the pH-stat. This, in fact, may explain its lack of interaction with the enzyme in that it does not appear to act like a guanidine but rather a substituted imidazole. When it was tested as a substrate, the concentration was the same as creatine normally is, namely 40 mM. When the analog (30 mM) was added to the assay system with 40 mM creatine, it did not inhibit the initial rate.

The compound also was of interest because if it could be epoxidized it could prove to be a very effective affinity label owing to the characteristics of its precursor. Such a study must await further research.

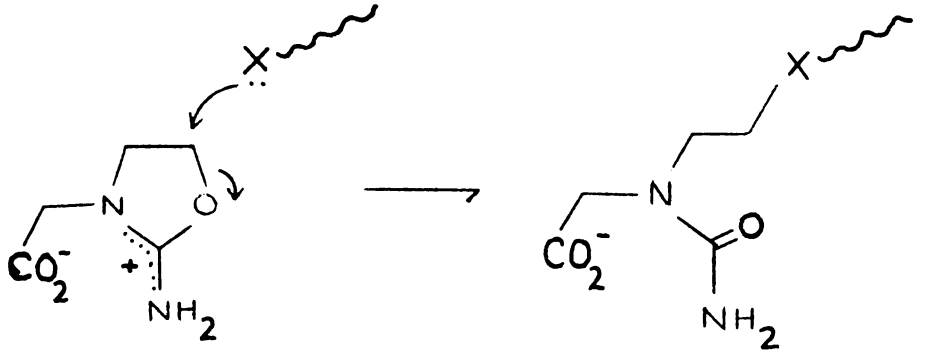
3. 3-Carboxymethyloxazolidine and Creatine Kinase

The structure of this analog is shown below:



3-carboxymethyloxazolidine

This compound was synthesized because it is an O-alkylurea derivative and therefore due to the inherent reactivity of such a group may be an affinity label. The possible mechanism of inactivation was envisioned to proceed as illustrated below:



where X is a nucleophile at the active site. It is possible that a full complement of substrates is needed for the inactivation to occur because a partial reaction with the gamma phosphoryl group of ATP would begin to place a positive charge on the oxygen atom thereby labilizing the carbon-oxygen bond.

The reaction was carried out at room temperature with MgATP and the analog present in the same concentrations they would be

in the normal assay for activity. The samples (control and experimental) were assayed periodically and after 4 days the analog-treated enzyme was 60% inactivated. No further experimentation was done with this compound.

IV. CONCLUSIONS

This section will summarize in point form the conclusions which may be reached from the experiments which have been detailed in this report.

1. Epoxycreatine completely and irreversibly inhibits creatine kinase.

2. The inhibition follows saturation kinetics which it must if it is to be a true enzyme-substrate analog interaction.

3. At 0^{o} C (pH 7.45) the K_{inact} = 417 mM where K_{inact} is defined as the concentration of epoxycreatine which causes the half-maximal inactivation rate and presumably the concentration which half-saturates the enzyme.

4. The mimimum inactivation half-time is 3.44 minutes and the pseudo first-order rate constant = 3.36×10^{-3} $sec.^{-1}$

5. Creatine competitively protects against the inactivation, mgATP and MgADP slow down the inactivation rate slightly, and the so-called tranistion state complex of MgADP, NO_3^- and creatine provides remarkable protection for the enzyme.

6. Epoxycreatine is a substrate in the enzymatic phosphorylation reaction.

7. The stoichiometry of the epoxycreatine-creatine kinase reaction is 2 mole affinity label/mole of enzyme, i.e. one label/subunit, suggesting a very limited and specific reaction.

8. Basic hydrolysis cleaves the label from the enzyme by a mechanism which is not known at this time. The rate of this hydrolysis is hastened by hydroxylamine. These results suggest that a carboxylate group may be part of the amino acid residue modified by epoxycreatine.

9. Epoxycreatine does not interfere with the myosin ATPase activity nor does it appear to inhibit actin and its association with myosin.

10. Preliminary experimentation into creatine kinase's physiological function indicate that epoxycreatine will be helpful in delineating that role.

V. EXPERIMENTAL

A. General Methods and Procedures

1. Melting Points, Microanalyses, Spectra and Scintillation Counting

All melting points or decomposition points were determined on a Thomas capilliary melting point apparatus and are uncorrected. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. ^{1}H NMR spectra were measured on a Varian A-60A spectrometer and unless otherwise indicated the solvent was D_2O and the reference was 2,2-Dimethyl-2-silapentane-5-sulfonate (DSS). Infrared spectra were run as KBr pellets on a Perkin Elmer 457 spectrophotometer. All radioactive samples were counted in a Beckman LS-100 scintillation counter, and either Beckman Ready-Solv GP or Aquasol were used as cocktails.

2. Purification of Cyanamide

Cyanamide was routinely stored at -20^o C and prior to purification was brought to room temperature. An amount was weighted (usually 50% over what was needed), dissolved in anhydrous ether and filtered to remove the ether-insoluble dicyandiamide (1-cyanoguanidine) and/or urea. The ether was then removed *in vacuo* with the temperature on the flask kept constant with the aid of a water bath and kept under this vacuum until dry. It was immediately weighed and dissolved in the amount of water specified by each reaction procedure.

3. Materials and Assay Methods

Materials used were obtained from the following sources: cyanamide (J. T. Baker or Eastman), allylamine (Aldrich or Eastman), iodoacetic acid (MCB or Aldrich), sodium tungstate dihydrate (Alfa), propargylamine, aminoacetaldehyde diethylacetal, aminoacetaldehyde dimethylacetal, 3-amino-1,2-propanediol, N-bromosuccinimide, ethanolamine, and cyanogen bromide (Aldrich), 30% H_2O_2 (MCB), 50% H_2O_2 (DuPont), bovine serum albumin (Armour), ATP (Sigma), magnesium acetate (J. T. Baker), creatine (Eastman), N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) (Calbiochem), all solvents, 47% hydroiodic acid (HI), and 48% aq. NH_3 (Mallinckrodt), lactate dehydrogenase, pyruvate kinase, phosphoenol-pyruvate (PEP) and NADH (Sigma). Water for buffer solution was distilled and deionized, and water used as the solvent for HPLC was distilled by Corning water distillation apparatus model AG-2.

Creatine kinase assays were carried out on a Radiometer TTT2 pH-stat at 30^{o} C, Mahowald et al. (1962), and the protein concentrations were determined from the following relationship:
$A_{280\,nm} = 7.1 \times 10^{4}\ M^{-1}\ cm^{-1}$ (C) (1), Noda et al. (1954), where $A_{280\,nm}$ was the absorbance at 280 nm, (C) was the concentration and (1) the cell path length. Spectrophotometric assays were performed on a Gilford 2220 spectrophotometer equipped with a Beckman DU monochrometer Model 2400 and a Gilford 6040 recorder. The cell compartment was equipped with thermo-spacers and thermostatted at 30^{o} C.

B. Creatine Kinase

1. Source and Isolation

Creatine kinase was isolated from fresh rabbit skeletal muscle as described by Kuby et al. (1954) in their procedure B. The enzyme obtained from this purification had a specific activity of 135 μ equiv./mg protein/min. as assayed by the pH-stat method in the forward direction. When this source lost some activity, the purification procedure B was performed a number of times and the high specific activity could not be obtained; therefore a crystallization of the enzyme according to the method of Keutel et al. (1972) was attempted which also failed. The original purified enzyme which was in solution with 20% ethanol was dialyzed against 0.01 M HEPES, pH 7.4, at 4^{o} C and it regained all of the activity which it had lost due to a cold room failure. This sample was used for all continuing studies.

2. Assay Procedures for Creatine Kinase

a. pH-stat method

A thorough account of the operation of the Radiometer TTT2 pH-stat has already been written by Smith (1974), and no changes were made in that procedure; however, some changes were made in the components of the assay system. It was determined that SO_4^{2-} anion inhibits the creatine kinase reaction, presumably by binding in an anionic site where the transferred phosphoryl group binds, (Nihei et al.,1961) Therefore, instead of using $MgSO_4$, $Mg(OAc)_2$ was used. In previous studies the ATP solution was brought to pH 9.0-9.5, and in this study all ATP solutions were adjusted to pH 8.5. Therefore a typical assay contained the following:

solution	volume (mL)	final concentration
0.080 M creatine	2.0	0.040 M
0.040 M Na_4ATP (pH 8.5)	0.4	0.004 M
0.040 M $Mg(OAc)_2$	0.4	0.004 M
1% (w/v) BSA	0.4	0.1 %
titrant and H_2O	0.8	
	4.0	

b. spectrophotometric coupled enzyme assay

The procedure followed was that of McLaughlin et al. (1973) and consisted of the following:

solution	volume	final concentration
80 mM creatine	0.375 mL	30 mM
40 mM $Mg(OAc)_2$	0.15 mL	6 mM
0.27 M glycine buffer (pH 9.0)	0.370 mL	0.1 M
40 mM ATP (pH 8.5)	25 λ	1.0 mM
10 mg/mL phosphoenolpyruvate	20 λ	0.2 mg/mL
10 mg/mL NADPH	10 λ	0.1 mg/mL
10 mg/mL pyruvate kinase	2.5 λ	0.025 mg/mL
10 mg/mL lactate dehydrogenase	2.5 λ	0.025 mg/mL
	1.0 mL total volume	

Typically approximately 1.2×10^{-3} mg of creatine kinase was added to the cuvette which had been equilibrated at 30^{o} C for 5 min.

C. Chemical Syntheses

1. General Method for the synthesis of N-substituted glycine derivatives

To an ice cold, rapidly stirred amine (0.68 mole) was added dropwise a solution of iodoacetic acid (14.9 g, 0.08 mole) in 40 mL of water. The reaction was allowed to stand for 20 h after which time the water and excess amine were removed under reduced pressure leaving a viscous, amber-colored oil. The oil was dissolved in 40 mL of absolute ethanol and added to a stirred solution of 50 mL absolute ethanol and 470 mL dry acetone. The solution was allowed to stand at 4^{o} C for 12 h. During this time a white precipitate generally formed. The white powder was filtered, washed with dry acetone and dried. The product was recrystallized, dried and then characterized.

The specifics of each synthesis including recrystallization solvent and physical characterization data are listed in the procedure for each individual compound.

2. General method for the synthesis of guanidines

Cyanamide was purified by extraction from ether by the method stated earlier. Typically the cyanamide (0.04 mole) was dissolved in approximately 2 mL of H_2O and 4 drops of an aqueous ammonia solution. The N-substituted glycine derivative (0.032 mole) was added in small aliquots and swirled after each addition until a clear solution was obtained. When the addition was complete the flask was equipped with a greased ground glass stopper and allowed to stand for 3 to 5 days depending on when crystallization appeared to have ceased. At that point the crystals and

residual solvent were ground with the aid of a mortar and pestle, filtered on a sintered glass funnel until dry, washed with 50 mL of acetone and finally dried again. The product was recrystallized, dried and then characterized.

The specifics of each synthesis including recrystallization solvent and physical characterization data are listed in the procedure for each individual compound.

3. Specific syntheses

a) N-(2-Propenyl)glycine was prepared from a solution of iodoacetic acid (14.9 g, 0.08 mole) in 40 mL of water which was added dropwise to ice-cold, rapidly stirred allylamine (38.8 g, 0.68 mole). The reaction mixture was allowed to stand for 20 h after which time the water and excess allylamine were both removed in vacuo, leaving a viscous, amber-colored oil. The oil was dissolved in 40 mL of absolute ethanol and added slowly to a stirred solution of 50 mL absolute ethanol and 470 mL acetone. The solution was allowed to stand at 4^{o} C for 12 h during which time a white precipitate formed. The white powder (4.8 g) was filtered, dried and recrystallized by dissolving it in boiling absolute ethanol and adding acetone to turbidity. The white crystals which formed were filtered and dried,giving 4.6 g (50% yield), mp 165-166^{o} C (dec.)

Anal. Calcd. for $C_5H_9NO_2$: C, 52.15; H, 7.89; N, 12.17.
Found: C, 51.93; H, 7.77; N, 11.97.

^{1}H NMR δ 3.65 (2H, s), 3.72 (2H, d, J = 7 Hz), 5.30-5.60 (3H, m).

b) N-amidino-N-(2-propenyl)glycine (vinylcreatine) was prepared by dissolving freshly ether-extracted cyanamide (1.73 g, 0.041 mole) in a solution of 2 mL of water and 4 drops of an aq. NH_3 solution (58%). N-(2-Propenyl)glycine (3.8 g, 0.033 mole) was then added in small aliquots and swirled after each addition until a clear solution was obtained. When all of the N-(2-propenyl)glycine was in solution, the flask was sealed with a greased ground glass stopper and the reaction solution was allowed to stand for 5 days. At this concentration crystallization began to occur after approximately 24 hours. After 5 days the crystals and residual solvent were ground in a mortar, filtered on a sintered glass funnel until dry, washed with 50 mL of acetone on the funnel, and finally dried again. The white powder (4.6 g) was recrystallized suspending it in boiling 95% ethanol and adding water dropwise until a clear solution was obtained. The white crystals which formed were filtered, washed with cold absolute ethanol and dried giving 4.13 g of product (80% yield), mp 244-245° C (dec.).

Anal. Calcd. for $C_6H_{11}N_3O_2$: C, 45.84; H, 7.08; N, 26.74. Found: C, 45.79; H, 7.05; N, 26.46.

^{1}H NMR δ 3.95 (2H, s), 4.03 (2H, d, J $\sim$ 4 Hz), 5.3-6.2 (3H, m).

c) N-Amidino-N-(2,3-epoxypropyl)glycine (epoxycreatine) was prepared by dissolving N-amidino-N-(2-propenyl)glycine (1.5 g, 9.5 mmoles) in 10 mL of 50% H_2O_2. To initiate the reaction sodium tungstate dihydrate (0.13 g, 0.4 mmole) was added and the reaction solution was stirred at room temperature for 90 min. When the sodium tungstate was added the clear solution immediately turned pale yellow and remained so during the course of the reaction. After that period of time 100 mL of acetone was

added and the white powdery precipitate (1.5 g) was filtered, washed with acetone and dried. The product was purified by high pressure liquid chromatography using a reverse phase Waters Assoc. Inc. microcrystalline Bondapak C-18 preparative column* with water as the eluent. The crude product was dissolved in a minimum amount of water, passed through a millipore filter to remove dust and other insoluble material, reprecipitated with acetone, filtered on a fine sintered glass funnel and dried. The crude product was then weighed out in 20 mg lots. Each 20 mg sample was dissolved in 150 μL of water just prior to injection. The flow rate was 2.0 mL per min and a refractive index detector was used. The epoxide product had a retention time of 8.5 min and the starting material eluted at 11 min. The product-containing solution once collected was kept on ice until all injections were made, and then it was frozen, lyophilized and stored at room temperature under vacuum in a desiccator. The refractive index detector indicated a 46% yield in the reaction and based on that 95% of the theoretical amount of epoxycreatine was obtained after lyophilization.

Anal. Calcd. for $C_6H_{11}N_3O_3 \cdot 0.25\ H_2O$: C, 40.55; H, 6.25; N, 23.65. Found: C, 40.77; H, 6.24; N, 23.70.

^1H NMR (360 MHz) δ 2.82 (1H, t, J = 3 Hz), 3.00 (1H, t, J = 3 Hz), 3.35-3.44 (2H, m), 3.85 -3.94 (1H, m), 3.99-4.03 (2H, d, J = 2.5 Hz).

* The column dimensions were 0.7× 30 cm.

d) N-(2,3-Dihydroxypropyl)glycine was prepared from a solution of iodoacetic acid (11.16 g, 0.06 moles) in 35 mL of water which was added dropwise to an ice-cold solution of 3-amino-1,2-propanediol (42.5 g, 0.47 mole) in 20 mL of water. The reaction mixture was allowed to stand for 30 h after which time the water and excess 3-amino-1,2-propanediol were both removed in vacuo leaving a viscous, pale yellow oil. Normal attempts to isolate the zwitterion failed. Solvent systems containing acetone, ethanol and chloroform also failed to effect crystallization. The oil, now dark brown in color, was heated on a steam bath in 20 mL of absolute ethanol and added slowly to a warm solution of 50 mL absolute ethanol and 100 mL acetone. This solution was allowed to stand at 4^{o} C for 72 h after which time a brown precipitate formed. The brown powder was filtered, dried and recrystallized from 95% ethanol. The white crystals which formed were filtered and dried giving 2.54 g of the product (28% yield), mp 152-153^{o} C (dec.).

Anal. Calcd. for $C_5H_{11}NO_4$: C, 40.26; H, 7.45; N, 9.39. Found: C, 40.42; H, 7.41; N, 9.31

^{1}H NMR δ 3.20 (2H, t, J = 4.5 Hz), 3.7 (2H, s), 3.66 (2H, d, J = 4.5 Hz), 4.1 (1H, s, J = 4.5 Hz).

e) N-Amidino-N-(2,3-dihydroxypropyl)glycine was prepared by dissolving freshly ether-extracted cyanamide (1.05 g, 0.025 mole) in a solution of 2 mL of water, and 4 drops of an aq. NH_3 solution (58%). N-(2,3-Dihydroxypropyl)glycine (3.0 g, 0.020 mole) was then added in small aliquots and swirled after each addition until a clear solution

was obtained. When the addition of N-(2,3-dihydroxypropyl)glycine was complete, the flask was sealed with a greased ground glass stopper and swirled again until the solution was clear. The reaction was then allowed to stand for 5 days. Crystals began to form after 20 h. After 5 days the crystals and residual solvent were ground in a mortar, filtered on a sintered glass funnel until dry, washed on the funnel with 50 mL of acetone and finally dried again. The white powder (2.5 g) was recrystallized by suspending it in boiling 95% ethanol and by dropwise addition of water until clear. The white crystals were filtered, washed with cold absolute ethanol and dried giving 1.43 g (37% yield), mp 185-187° C (dec.).

Anal. Calcd. for $C_6H_{13}N_3O_4$: C, 37.68; H, 6.87; N, 21.98. Found: C, 37.54; H, 6.83; N, 21.71.

^1H NMR δ 3.33-3.72 (5H, m), 4.02 (2H, s).

(f) N-(2-Propynyl)glycine was prepared from a solution of iodoacetic acid (4.1 g, 0.022 mole) in 10 mL of water which was added dropwise to ice-cold, rapidly stirred propargylamine (2.05 g, 0.011 mole). The reaction mixture was allowed to stand for 20 h after which time the excess propargylamine and water were both removed in vacuo leaving a viscous brown oil. The oil was dissolved in 10 mL of absolute ethanol and added slowly to a stirred solution of 14 mL absolute ethanol and 130 mL of acetone. The solution was allowed to stand at 4° C for 12 h during which time a light brown precipitate formed. The brown powder (2.2 g) was filtered, dried, and recrystallized by suspending it in boiling 95% ethanol and adding water dropwise until the powder had all dissolved. The white crystals which formed were

filtered and dried giving 2.1 g (85% yield), mp 210-212° C (dec.).

Anal. Calcd. for $C_5H_7NO_2$: C, 53.08; H, 6.25; N, 12.38. Found: C, 53.00; H, 6.28; N, 12.34.

^1H NMR δ 3.02 (1H, t, J = 2.6 Hz), 3.72 (2H, s), 3.98 (2H, d, J = 2.6 Hz).

(g) N-Amidino-N-(2-propynyl)glycine was prepared by dissolving freshly ether-extracted cyanamide (0.53 g, 12.7 mmoles) in a solution of 2 mL of water and 4 drops of aq. NH_3 solution (58%). N-(2-Propynyl)-glycine (1.15 g, 10.2 mmoles) was then added in small aliquots and swirled after each addition until a clear solution was obtained. When addition of N-(2-propynyl)glycine was complete, the flask was sealed with a greased ground glass stopper and swirled again until the solution was clear. The reaction was then allowed to stand for 6 days. Crystals began to form after approximately 3 days. After 6 days the crystals and residual solvent were ground in a mortar, filtered on a sintered glass funnel until dry, washed on the funnel with 50 mL of acetone and finally dried again. The light brown powder (0.81 g) was taken up in 25% ethanol, filtered while hot, and evaporated slightly to effect crystallization. The very slightly brown crystals which formed were filtered, washed with cold, absolute ethanol and dried giving 0.64 g (40% yield), mp 204-205° C (dec.).

Anal. Calcd. for $C_6H_9N_3O_2$: C, 46.44; H, 5.86; N, 27.08. Found: C, 46.16; H, 5.87; N, 26.83.

^1H NMR δ 2.84 (1H, t, J - 2.5 Hz), 4.05 (2H, s), 4.23 (2H, d, J = 2.5 Hz).

h) N-(2,2-Diethoxyethyl)glycine was prepared from a solution of iodoacetic acid (14.88 g, 0.08 mole) in 40 mL of water which was added dropwise to ice-cold, rapidly stirred aminoacetaldehyde diethylacetal (1-amino-2,2-diethoxyethane) (90.58 g, 0.68 moles). The reaction mixture was allowed to stand for 20 h after which time the water and excess aminoacetaldehyde diethylacetal were both removed in vacuo leaving a viscous yellow oil. The oil was dissolved in 40 mL of absolute ethanol and added slowly to a stirred solution of 50 mL absolute ethanol and 470 mL acetone. The solution was allowed to stand at 4^{o} C for 12 h during which time a white precipitate formed. The white powder (12.6 g) was filtered, dried, and recrystallized from absolute ethanol. The white crystals which formed were filtered and dried giving 12.4 g (81% yield), mp 160-162^{o} C (dec.).

Anal. Calcd. for $C_8H_{17}NO_4$: C, 50.24; H, 8.98; N, 7.33. Found: C, 49.98; H, 8.75; N, 7.18.

^{1}H NMR δ 1.23 (6H, t, H = 7 Hz), 3.24 (2H, d, J = 5 Hz), 3.48-4.03 (6H, m), 4.93 (1H, t, J = 5 Hz).

i) N-Amidino-N-(2,2-diethoxyethyl)glycine was prepared by dissolving freshly ether-extracted cyanamide (1.14 g, 0.027 mole) in a solution of 2.5 mL of water and 4 drops of an aq. NH_3 solution (58%). N-(2,2-Diethoxyethyl)glycine (4.13 g, 0.022 mole) was then added in small aliquots and swirled after each addition until a clear solution was obtained. When the addition of N-(2,2-diethoxyethyl)glycine was complete, the flask was sealed with a greased ground glass stopper and swirled again until the solution was clear. The reaction was then

allowed to stand for 5 days. Crystals began to form after approximately 3 days. After 5 days the crystals and residual solvent were ground in a mortar, filtered on a sintered glass funnel until dry, washed on the funnel with 50 ml of acetone and finally dried again.

'H NMR δ 1.05-1.40 (6H, t, J = 3 Hz), 3.35-4.2 (8H, m), 4.6-4.93 (1H, t, J = 5 Hz).

(No C, H and N analysis was obtained for this compound and it was not used in any synthesis beyond this point.)

j) N-(2,2-Dimethoxyethyl)glycine was prepared from a solution of iodoacetic acid (18.6 g, 0.1 mole) in 50 mL of water which was added dropwise to ice-cold, rapidly stirred aminoacetaldehyde dimethylacetal (1-amino-2,2-dimethoxyethane, 98.0 g, 0.93 mole). The reaction mixture was allowed to stand for 20 h after which time the water and excess aminoacetaldehyde dimethylacetal were both removed in vacuo leaving a viscous yellow oil. The oil was dissolved in 40 mL of absolute ethanol and was added slowly to a stirred solution of 50 mL absolute ethanol and 470 mL acetone. The solution was allowed to stand at 4^{o} C for 12 h during which time a white precipitate formed. The white powder (12.8 g) was filtered, dried, and recrystallized from absolute ethanol. The white crystals which formed were filtered and dried giving 11.76 g (72% yield), mp 178-179^{o} C (dec.).

Anal. Calcd. for $C_6H_{13}NO_4$: C, 44.15; H, 8.05; N, 8.58. Found: C, 44.20; H, 8.14; N, 8.40.

^{1}H NMR δ 3.26 (2H, d, J = 5 Hz), 3.50 (6H, s), 3.75 (2H, s), 4.82 {1H, t (partially hidden by HOD peak), J = 5 Hz}.

k) N-Amidino-N-(2,2-dimethoxyethyl)glycine was prepared by dissolving freshly ether-extracted cyanamide (1.26 g, 0.03 mole) in a solution of 2 mL of water and 4 drops of an aq. NH_3 solution (58%). N-(2,2-Dimethoxyethyl)glycine (4.08 g, 0.025 mole) was then added in small aliquots and swirled after each addition until a clear solution was obtained. When addition of N-(2,2-dimethoxyethyl)glycine was complete, the flask was sealed with a greased ground glass stopper and swirled again until the solution was clear. The reaction was then allowed to stand for 5 days. Crystals began to form after approximately 2 days. After 5 days the crystals and residual solvent were ground in a mortar, filtered on a sintered glass funnel until dry, washed on the funnel with 50 mL of acetone and finally dried again. The white powder (2.7 g) was recrystallized from 95% ethanol, filtered and dried giving 2.58 g (50% yield), mp 214-216^{o} C (dec.).

Anal. Calcd. for $C_7H_{15}N_3O_4$: C, 40.96; H, 7.38; N, 20.48. Found: C, 40.93; H, 7.34; N, 20.28.

^{1}H NMR δ 3.53 (6H, s), 4.02 (2H, s), other absorption hidden by HOD peak at 4.7.

l) 1-Carboxymethyl-2-aminoimidazole was prepared using a procedure modified from that of Lancini and Lazzari (1966). N-(2,2-Dimethoxyethyl)glycine (1.0 g, 4.9 mmoles) was dissolved in 2 mL of water with the aid of a steam bath. Hydroiodic acid (1.0 mL, 47%) was added, and the reaction solution was heated on the steam bath for 20 min. When cool the pH of the solution was adjusted to 6.5 with NaOH, and then 60 mL of acetone was added. The brown powder which was obtained was filtered, washed repeatedly with acetone, dried, and recrystallized

from water to give 0.69 g (100% yield) of off-white crystals, mp > 300° C (dec.).

Anal. Calcd. for $C_5H_7N_3O_2$: C, 42.54; H, 5.01; N, 29.78. Found: C, 42.20; H, 5.29; N, 29.48.

^1H NMR δ 4.42 (2H, s), 6.77 (2H, m).

UV λ_{max} (H_2O) 210 mm, ε = 10,395.

m) N-Amidino-N-(2-hydroxy-3-bromopropyl)glycine and N-Amidino-N-(2-bromo-3-hydroxypropyl)glycine were both prepared using a slightly modified version of the method of Guss and Rosenthal (1955), by dissolving N-amidino-N-(2-propenyl)glycine (2.0 g, 0.013 mole) in 15 mL of water and stirred vigorously at room temperature until the solution was clear. Recrystallized N-bromosuccinimide (Dauben and McCoy, 1959) (2.7 g, 0.015 mole) was added all at once, still continuing to stir the reaction. The reaction, two phases both solid and liquid, was stirred for an additional 12 h. After that time the reaction mixture was filtered and the precipitate was washed twice with 20 mL portions of acetone and twice with 20 mL portions of absolute ethanol. The white powder obtained was dried giving 1.3 g (34% yield), mp 176-179° C (dec.).

Anal. Calcd. for $C_6H_{12}BrN_3O_3$: C, 28.36; H, 4.77; N, 16.54; Br, 31.45. Found: C, 28.56; H, 4.64; N, 16.54; Br, 31.34.

n) N-(2-Hydroxyethyl)glycine was prepared from a solution of iodoacetic acid (10.0 g, 0.054 mole) in 25 mL of water which was added dropwise to ice-cold, rapidly stirred ethanolamine (26.39 g, 0.43 mole). The reaction mixture was allowed to stand for 20 h after which time the water and excess ethanolamine were both removed *in vacuo* leaving a viscous yellow oil. The oil was dissolved in 25 mL of absolute ethanol and added slowly to a stirred solution of 30 mL absolute ethanol and 310 mL acetone. The solution was allowed to stand at 4^{o} C for 12 h during which time a white precipitate formed. The white powder (5.6 g) was filtered, washed with acetone, dried and used without further purification (87.5% yield), mp 170-172^{o} C (dec.); lit. Stewart, (1962) 174-175^{o} C.

^{1}H NMR δ 3.22 (2H,unsymmetrical quartet), 3.68 (2H, singlet), 3.87 (2H, unsymmetrical quartet).

o) 2-Amino-3-carboxymethyloxazolidine was prepared using a modification of the method of Newhall *et al*. (1964). N-(2-hydroxyethyl) glycine (2.0 g, 17 mmoles) was dissolved in a solution of 1 eq. of NaOH and 5 mL of water. To the resulting solution, BrCN (1.8 g, 17 mmoles) dissolved in 5 mL of methanol was added dropwise over a 2 h period. The reaction solution was stirred overnight, then 20 mL of acetone was added and the flask placed at 4^{o} C for 12 h. The oil which was obtained after acetone addition solidified, was filtered and dried yielding 0.7 g. The crystals were recrystallized by dissolving in a minimum amount of water, adding absolute ethanol until turbid,and keeping the mixture at 4^{o} C. The crystals were filtered and dried, giving 0.31 g

(13% yield), mp 184-185° C (dec.).

Anal. Calcd. for $C_5H_8N_2O_3$: C, 41.66; H, 5.61; N, 19.44. Found: C, 41.59; H, 5.60; N, 19.37.

^1H NMR δ 3.77-4.15 (4H, m), 4.74-5.00 (2H, m).

p) N,N-bis(2-Hydroxyethyl)glycine (Bicine) was prepared from a solution of iodoacetic acid (14.88 g, 0.08 mole) in 40 mL of water which was added dropwise to N,N-(2-hydroxyethyl)amine (diethanolamine) (52.3 g, 0.50 mole). The diethanolamine was rapidly stirred and kept at room temperature by use of a water bath. The reaction solution was stirred for 2 h after the addition of the iodoacetic acid was complete and then added to a stirred solution of 300 mL absolute ethanol and 500 mL acetone. The white powder obtained (12.16 g) was filtered, washed with acetone, dried and recrystallized by suspending the powder in boiling 95% ethanol and adding water until solution was attained. The white crystals which formed were filtered, washed and dried giving 10.7 g (82% yield), mp 192-193° C (dec.); lit. (Kiprianov, 1926), 193-195° C.

4. Radioactive synthesis of epoxycreatine

The synthesis is exactly the same as the one used to obtain unlabeled epoxycreatine except that the quantities and experimental set-up differed. These differences will be described in detail here.

Iodoacetic-1-C^{14} acid (1.5 mCi, 13.8 mCi/mmole) was purchased from New England Nuclear and diluted with cold iodoacetic acid so that the specific activity was approximately 2.8 x 10^{-1} mCi/mmole.

[^{14}C]-N-(2-Propenyl)glycine was prepared by the dropwise addition, through a syringe, ofa solution of iodoacetic acid (1.02 g, 5.48 mmoles) in 1.1 mL of water to ice-cold, rapidly stirred allylamine

(3.04 g, 53.3 mmoles) contained in a small round-bottom flask equipped with a stirring bar and rubber septum. The reaction mixture was allowed to stand for 20 h after which time the water and excess allylamine were both removed in vacuo leaving a viscous, amber-colored oil. The oil was dissolved in 3 mL of absolute ethanol and added slowly to a solution of 2.5 mL absolute ethanol and 30 mL acetone. The solution was allowed to stand at 4^{o} C for 12 h during which time a white precipitate formed. The white powder was filtered, washed with acetone and dried, giving 0.35 g (56% yield), mp 165^{o} (dec.). The product was used without further purification. [^{14}C]-N-Amidino-N-(2-propenyl)glycine (vinylcreatine) was prepared by dissolving freshly ether-extracted cyanamide (0.16 g, 3.8 mmoles) in a solution of 3 drops of water and 2 drops of an aq. NH_3 solution (58%). [^{14}C]-N-(2-Propenyl) glycine (0.35 g, 3.94 mmoles) prepared above was then added and swirled until a clear solution was obtained. The small round-bottom flask was equipped with a greased ground glass stopper, and the reaction solution was allowed to stand for 5 days during which time crystallization took place. After 5 days the residual solvent and crystals were ground in a mortar, filtered on a sintered glass funnel, and finally dried again. The white powder (0.381 g) was recrystallized by suspending it in boiling 95% ethanol and adding water dropwise until a clear solution was obtained. The white crystals which formed were filtered, washed with cold absolute ethanol and dried, giving 0.317 g (66% yield). This material was used to prepare [^{14}C]-N-Amidino-N-(2,3-epoxypropyl)glycine (epoxycreatine) by dissolving the olefin

(0.317 g, 2.02 mmoles) and sodium tungstate dihydrate (0.028 g, 0.085 mmoles) in 2.1 mL of water. 30% H_2O_2 (2.0 mL) was added and after 90 min. the product was precipitated with 30 mL of acetone.

The white precipitate was filtered, washed with acetone and dried giving 0.330 g of crude reaction mixture. This crude mixture was purified using HPLC as described earlier giving 49 mg of epoxycreatine. The labeled starting material was also recovered. The specific activity was determined to be 2.5×10^{-1} mCi/mmole.

D. Interaction of Epoxycreatine with Creatine Kinase

1. Kinetics of irreversible inhibition

a. Measurement of the inhibition

All of the inactivation kinetic experiments were performed on creatine kinase purified from fresh rabbit muscle. The specific activity of the enzyme was 135 μequiv/mg protein/min. The enzyme was assayed by the pH-stat method outlined earlier.

The inactivation experiments were carried out in small (0.3 mL) conical vials (Reacti-vial, Pierce Chemical Co.) at 0^{o} C and in 0.01 M HEPES buffer, pH 7.45. Creatine kinase concentration in all experiments was 1 mg/mL (12 μM). A typical inactivation experiment involved adding 0.05 mL of 2 mg/mL creatine kinase solution in 0.01 M HEPES (pH 7.45) to a vial. A calculated amount of epoxycreatine was weighed on a Cahn Electrobalance and added to a separate vial. The epoxycreatine was dissolved in 0.06 mL of HEPES buffer which was cooled to 0^{o} C.

At time 0, 0.05 mL of the epoxycreatine solution was added to the enzyme solution, mixed quickly and placed in an ice bath at 0^{o} C. Aliquots (usually 5 λ) were withdrawn every 10 or 15 minutes (depending on the epoxycreatine concentration) and assayed normally. A control which consisted of the stock enzyme solution diluted in half was assayed at both the start and conclusion of the experiment.

The results of the assays were converted to per cent activity remaining and plotted as first order reactions, that is, % activity remaining vs. time on semi-logarithmic graph paper. The activity at time 0 corresponding to the unmodified enzyme's activity varied slightly from one experiment to the next, probably due to pipetting errors. Therefore when all kinetic runs were completed all of the 100% original activity points were averaged together and the % inactivation for each inactivation experiment was determined by comparison to this average value. This practice gave reproducible values for the inactivation half-times at a given inhibitor concentration. The inactivation half-times were read from the graph as the time necessary for the enzyme to become 50% inactivated and therefore reflected an initial rate. The lines were linear throughout the experiment and therefore the half-times of inactivation could have been determined from any part of the line.

b. Substrate protection of the inhibition

These experiments were carried out under the exact conditions stated above; however, the epoxycreatine was dissolved in 0.06 mL HEPES buffer, pH 7.45, which contained a calculated amount of creatine.

Therefore, when the experiment was begun the creatine and epoxycreatine were mixed with the enzyme at the same time. The creatine-HEPES solutions were made up fresh daily. The data was plotted and inactivation half-times determined according to the methods stated earlier.

2. Exhaustive dialysis of inactivated enzyme

The general procedure for treating creatine kinase with epoxycreatine as outlined earlier was followed. A sample of creatine kinase was treated with epoxycreatine and a control diluted with buffer was treated in an analogous fashion. When the epoxycreatine-treated enzyme had become totally inactive, a 0.08 mL aliquot of it was dialyzed against 0.01 M HEPES (200 mL) buffer for 96 h and was assayed periodically during this time. No activity returned in the epoxycreatine-treated sample, whereas the control retained all of its activity.

3. Epoxycreatine as a substrate in the enzymatic reaction

Using the method of Rowley and Kenyon (1974) it was determined that epoxycreatine is a substrate in the enzymatic reaction. The polyethylenimine-cellulose thin layer chromatography plates were prepared as stated in the above reference. The reaction was carried out at 0^{o} C and the reaction mixture consisted of the following:

solution	volume	final concentration
0.04 M Na_4ATP (pH 8.5)	50 λ	0.004 M
0.04 M $Mg(OAc)_2$	50 λ	0.004 M
0.1 M glycine buffer (pH 9.0)	0.35 mL	0.07 M
creatine kinase (26.1 mg/mL)	50 λ	2.6 mg/mL
epoxycreatine	5 mg	58 mM

All components of the reaction mixture were combined except for the enzyme. A control reaction was set up which consisted of the same components except that creatine (5 mg) was substituted for epoxycreatine. At time 0 the enzyme was added, and the reaction was allowed to proceed for 60 min. The reaction was quenched by the addition of a solution of 2,4-dinitrofluorobenzene (50 λ, 0.1 M) in 2-propanol. A sample (5 λ) of the epoxycreatine and creatine reaction were spotted and developed in a 1.2 M NaCl solution. The Rowley and Kenyon (1974) visualization technique was used, and this indicated that epoxycreatine had been phosphorylated in the reaction. Further evidence for this phosphorylation came from the observation that ADP was being formed at the expense of ATP concomitantly with formation of the putative phosphorylated epoxycreatine. At the same time, no inorganic phosphate was being generated.

4. Stoichiometry of the creatine kinase-epoxycreatine reaction

To a vial containing ^{14}C-labeled epoxycreatine (1.73 mg, 100 mM final concentration) was added 100 μL of a solution of creatine kinase (17.1 mg/mL, 209 μM) in 0.01 M HEPES, pH 7.4, at 0^{o} C. The incubation

was allowed to continue for 20 h at which time the solution was dialyzed against three changes of 0.01 M HEPES, pH 7.4, at 4° C (1000 mL each for 3 h). The protein concentration was determined spectrophotometrically and the amount of radioactivity bound was determined by scintillation counting. (Protein concentrations were also determined by the Lowry method and found not to differ significantly from the spectrophotometric technique providing the absorbance was below 0.20).

5. % Residual activity vs. epoxycreatine binding.

To 1.0 mL of a creatine kinase solution (17.1 mg/mL), 209 μM) at 0° C in 0.01 M HEPES, pH 7.4, was added at time 0 [^{14}C]-labeled epoxycreatine (6.7 mg, 38.6 mM). Aliquots (100 μL) were withdrawn at specified times and immediately passed through a column (7 mm x 17 cm) containing Sephadex G-25 medium grade which had been equilibrated with 0.01 M HEPES, pH 7.4, and creatine (80 mM). The eluent was monitored by observing the absorption at 280 nm. The protein-containing eluent was collected and dialyzed against three changes of 0.01 M HEPES (1000 mL each), assayed for creatine kinase specific activity and then the amount of radioactivity bound was determined by scintillation counting. The entire procedure following the initial incubation was carried out at 4° C.

6. Removal of the Label from Creatine Kinase

a. NaOH treatment: A sample of creatine kinase which had been previously treated with epoxycreatine was used for this study. To a sample of the blocked protein (0.6 mg/mL, 200 μL) was added 0.2 N NaOH (200 μL). Immediately the solution was mixed and then left to stand for 12 h at room temperature. After that time the solution was dialyzed against 1000 mL of deionized, distilled water (3 changes for 5 h each). The protein concentration was determined spectrophotometrically, and then the sample was counted.

b. Comparison of the rate of label removal by NaOH and NH_2OH: The same protein sample was used in this experiment as was used above. To one protein sample (0.6 mg/mL, 400 μL) was added 2 M NH_2OH, pH 9.0 (400 μL), and to the other protein sample (0.6 mg/mL, 400 μL) was added water, pH 9.0 (400 μL). (The water at pH 9.0 was part of a 0.10 glycine buffer solution.) The samples stood for 4 h after which time they were dialyzed against deionized, distilled water (3 changes for 5 h each). Protein concentration was measured spectrophotometrically and the samples counted.

7. Attempted Determination of the Modified Residue at the Active Site.

a. Isolation and identification of the basic hydrolysis product of the inactivated enzyme: A sample of creatine kinase was pressure-concentrated until the concentration was 45 mg/mL. A portion of this enzyme (7 mL) was treated with [^{14}C]-epoxycreatine

(2 mg, 3.3 mM) for 2.7 days. After this time the enzyme was assayed and found to be inactive. The protein sample was dialyzed against 1000 mL distilled, deionized water, (3 changes, 5 h each), the sample volume measured, and an equal volume of 0.2 N NaOH was added slowly. The basic hydrolysis continued for 18 h. The protein solution was passed through a column (27 mm inside diameter) containing 150 mL of Sephadex 6-25 (medium grade). The elution volume of the diol on this colmn had previously been determined. The A_{280} of the column effluent was followed. After the protein eluted, the fraction where the presumed diol would be was collected, partially lyophilized, neutralized with dilute HI and finally lyophilized to dryness. The white powder was then washed 3 times with dry acetone and then dried at room temperature under vacuum. The sample was lyophilized 3 times from 100% D_2O and analyzed by NMR. A thin layer chromatogram (cellulose adsorbent, n-butanol-pyridine-water, 1:1:1) indicated radioactivity was present from the origin up to an R_f of 0.6. HPLC analysis showed sample absorbed at 280 nm and consisted of many peaks. A control sample of protein treated in analogous fashion exhibited the same properties.

b. Reduction of proposed ester from epoxycreatine-creatine kinase reaction: Creatine kinase (45 mg/mL, 1.0 mL) was treated with epoxycreatine (10 mg, 58 mM) for 4 h. A control sample was treated with creatine. Both samples, after that time, were dialyzed against 2000 mL of distilled, deionized water for 18 h, and then lyophilized. Diglyme was dried by suspending CaH_2 in it overnight, adding $LiAlH_4$ slowly and distilling it from the mixture.

The diglyme was stored, tightly sealed, and in the dark. $NaB^{*}H_4$ (tritium labeled, sp.act. = 25 mCi/mmole) was taken up in the dried diglyme (final conc. 10 mg/mL). To the $NaB^{*}H_4$ solution (1.0 mL) was added dry LiBr (23 mg) and stirred for 30 m at room temperature with N_2 gas atmosphere (Brown et al., 1955). After that time either the epoxycreatine-treated creatine kinase or the control, creatine-treated sample, was added (50 mg). The reaction mixture was then heated in an oil bath to 70^{o} C for 6 h. Dilute HCl was added to destroy the excess hydride and the sample was stirred for 1 hr at room temperature. The sample was diluted with water, dialyzed against 1000 mL distilled, deionized water (3 changes, 4 h each) and lyophilized. This procedure was a modification of that of Chibnall and Rees (1958). The samples were hydrolyzed, under vacuum, in 6N HCl for 22 h, and lyophilized.

8. Effect of Other Substrates on the Inactivation Rate.

A sample of creatine kinase in 0.01 M HEPES, pH 7.45 (2.0 mg/mL, 50 λ) was added to a solution of epoxycreatine (48 mM) and the other substrate or substrates also in 0.01 M HEPES pH 7.45. As in the typical inactivation experiments, aliquots (5 λ) were withdrawn at noted times and assayed on the pH-stat.

E. Epoxycreatine Interaction with the Perfused Cat Heart

1. Preliminary experiments with rat heart homogenate

A freshly excised rat heart was cut into small peices and homogenized in a solution of sucrose (0.25 M), Tris (5 mM) and EDTA (0.01 mM), pH 7.5, at 4° C. An aliquot of the homogenate (100 µL) was withdrawn, epoxycreatine (1 mg, 58 mM) was added and the resulting solution was incubated at 0° C. An additional aliquot (100 µL) was also withdrawn which served as the control. The control was then assayed for creatine kinase activity using the pH-stat method outlined earlier. After an incubation period of 5.5 h the epoxycreatine-treated homogenate was found to be 62% inactivated relative to the control. The same comparison after 22.5 h incubation resulted in 67% inactivation.

2. Treatment of perfused cat hearts with epoxycreatine

a. preparation of the perfusing fluid

The Krebs-Ringers-Bicarbonate (KRB) perfusing solution contained the following:

NaCl	120 mM
KCl	4.8 mM
KH_2PO_4	1.2 mM
$MgSO_4$	0.4 mM
$NaHCO_3$	25 mM
$CaCl_2$	1.3 mM
Dextran 70	1 %
glucose	16.7 mM

All components of the KRB perfusate were made up from stock solutions and were combined the night before except for the bicarbonate solution and the glucose. Just prior to the experiment the glucose was added to the solution not containing the bicarbonate, and the two flasks, one containing the bicarbonate solution and the other containing all other components of the perfusate, were brought to 37^{o} C in a temperature bath. When the two solutions were equilibrated with the bath, they were saturated with 95% O_2 - 5% CO_2 gas by bubbling for 20 m. The two solutions were then combined and saturated with 95% O_2 - 5% CO_2 for another 20 m after which time the perfusate was ready for the experiment. The gas saturation and temperature control were continued during the entire course of the experiment.

b. protocol of a perfusion experiment

A cat ($\sim$ 2.7 kg) was anesthetized with pentobarbital (5.0 mL, 65 mg/mL). When the anesthetic had taken effect the heart was removed from the animal and immediately plunged into perfusate which had been chilled in ice. The heart was then tied to the perfusate column by the aorta and perfusion was begun. A small catheter was then inserted in the lower end of the left ventrical which served as a vent for perfusate. The left atrial appendage was removed and a balloon was inserted for isovolumic pressure measurements. Electrodes were attached and the heart was paced at 120-150 beats per minute. The heart was allowed to function until a stable developed pressure was obtained, which usually was about 10 minutes. (Developed pressure is the systolic minus the diastolic pressure and hearts with a

developed pressure of less than 80 were deemed not acceptable.) The heart was closed off to the persuate by means of a three-way stopcock and arrested with Gay-Ebers* solution. Through the same stopcock a solution of either creatine or epoxycreatine (80 mM, 5 mL) was injected. The incubation was continued for 1 h with the heart cooled to 27^{o} C by means of a water bath. The balloon was deflated during the incubation. After the incubation time perfusion with normal perfusate was begun. If the heart was not beating regularly it was defibrillated. The balloon was reinflated after 15 m of reperfusion. After 45 m of reperfusion the developed pressure was noted and the experiment terminated. The apex of the heart was frozen with liquid N_2 freeze clamps and analyzed later for ATP and phosphocreatine content. A sample was also trimmed, homogenized and analyzed for creatine kinase activity by the spectrophotometric assay which was outlined earlier.

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* 90 mM NaCl

37 mM KCl

23 mM $NaHCO_2$

pH adjusted to 7.4

F. Specificity of Epoxycreatine

1. Interaction of Epoxycreatine with Actin and Myosin.

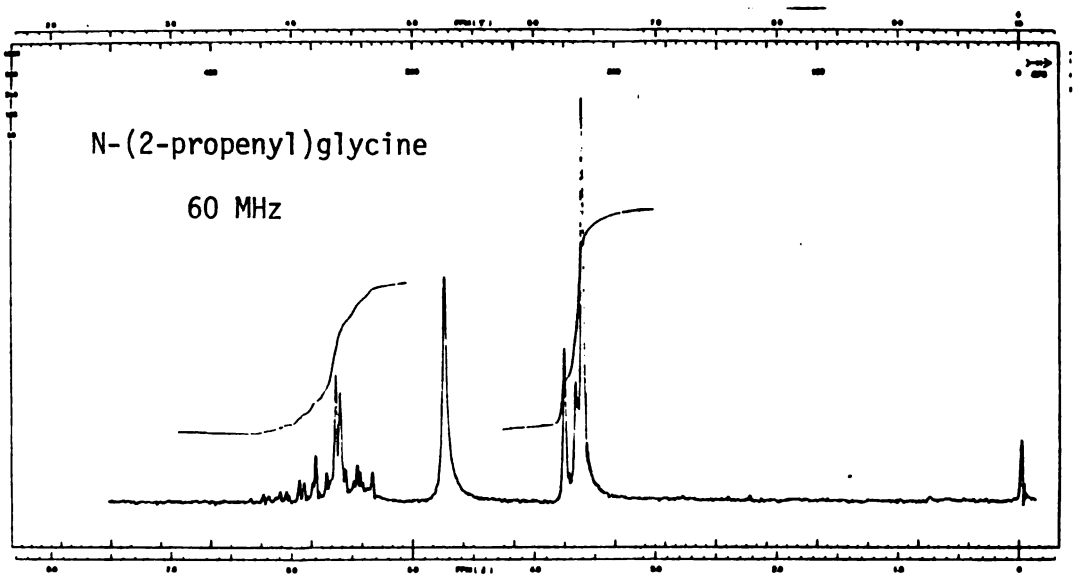
Epoxycreatine or creatine (both at 48 mM) were incubated with both myosin and actin for 1 h. The myosin ATPase activity was then determined in the case of Ca^{2+} activation and actin activation by use of a pH-stat. Accordingly, a myosin control sample was used to assay the epoxycreatine-treated actin. The Ca^{2+} activated assay contained 0.6 M KCl, 4 mM Ca^{2+}, 100 mM ATP (25λ) and 0.28 mg myosin at pH 8.0. The actin activated assay involved 50 mM KCl, 4 mM Mg^{2+}, 100 mM ATP (25 λ), actin (25 λ, 3.2 mg/mL) and myosin (0.28 mg) at pH 8.0. (Cooke, personal communication).

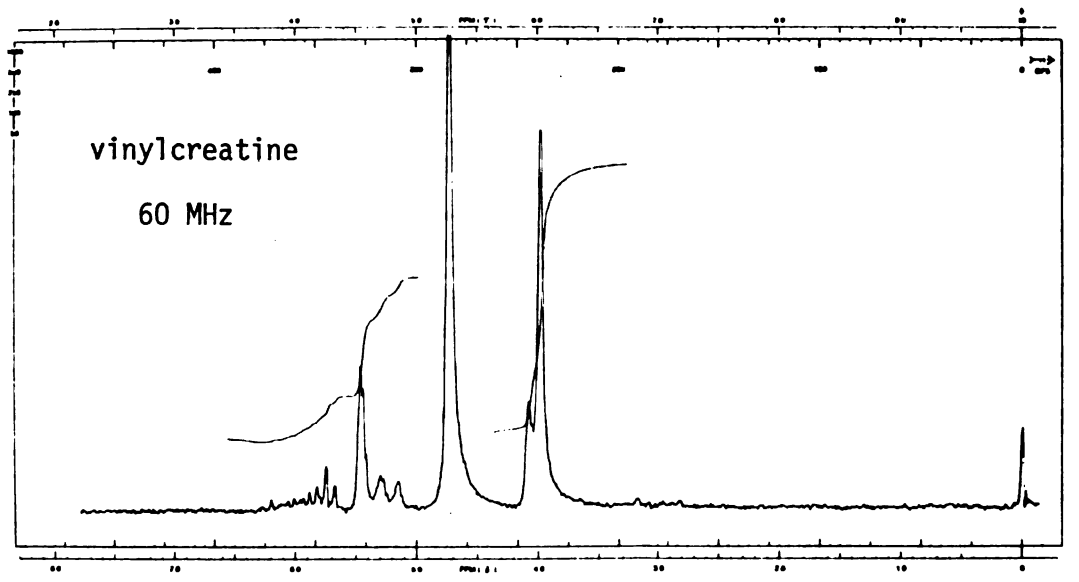
APPENDIX A

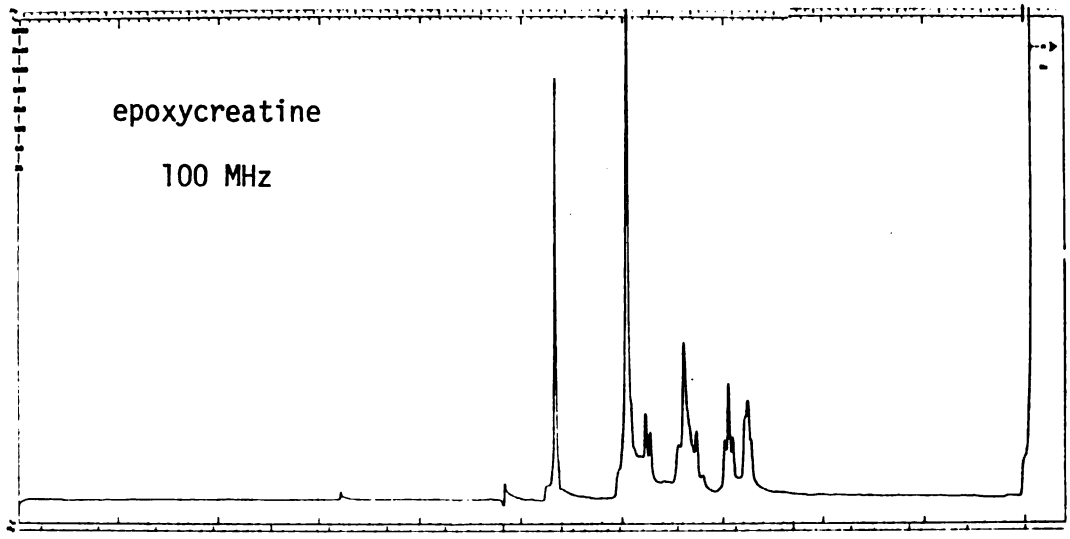
NMR and IR Spectra

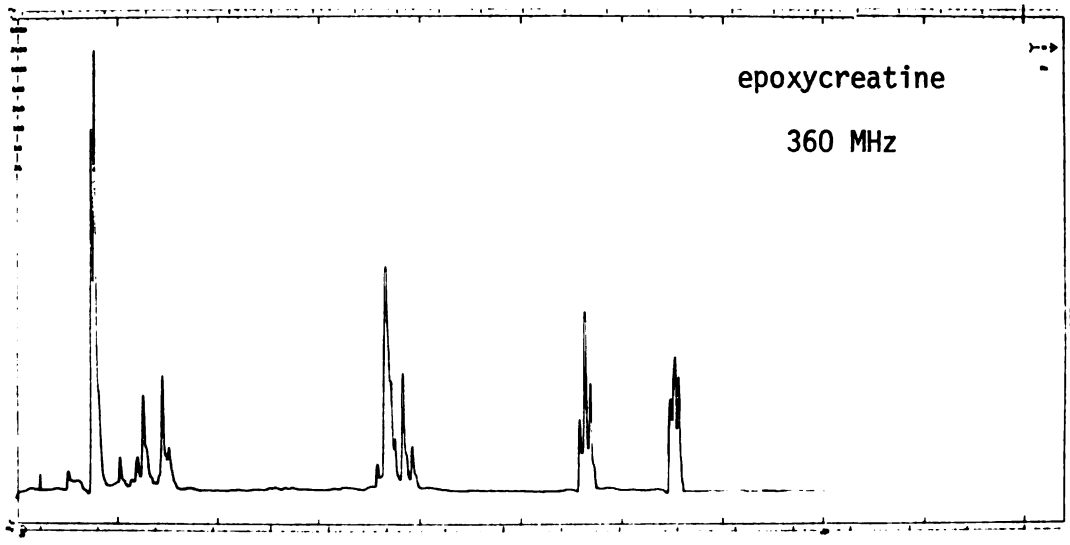
The NMR spectra shown on the following pages have chemical shifts stated in the Experimental Section. All spectra were analyzed in D_2O with either DSS (2,2-dimethyl-2-silapentane-5-sulfonate) or TSP {3(trimethylsilyl)tetradeutero Sodium propionate} as the reference. The decoupling experiments with epoxycreatine carried out at 360 MHz have the second irradiating frequency applied where the arrow indicates.

The IR spectra were taken as KBr pellets.

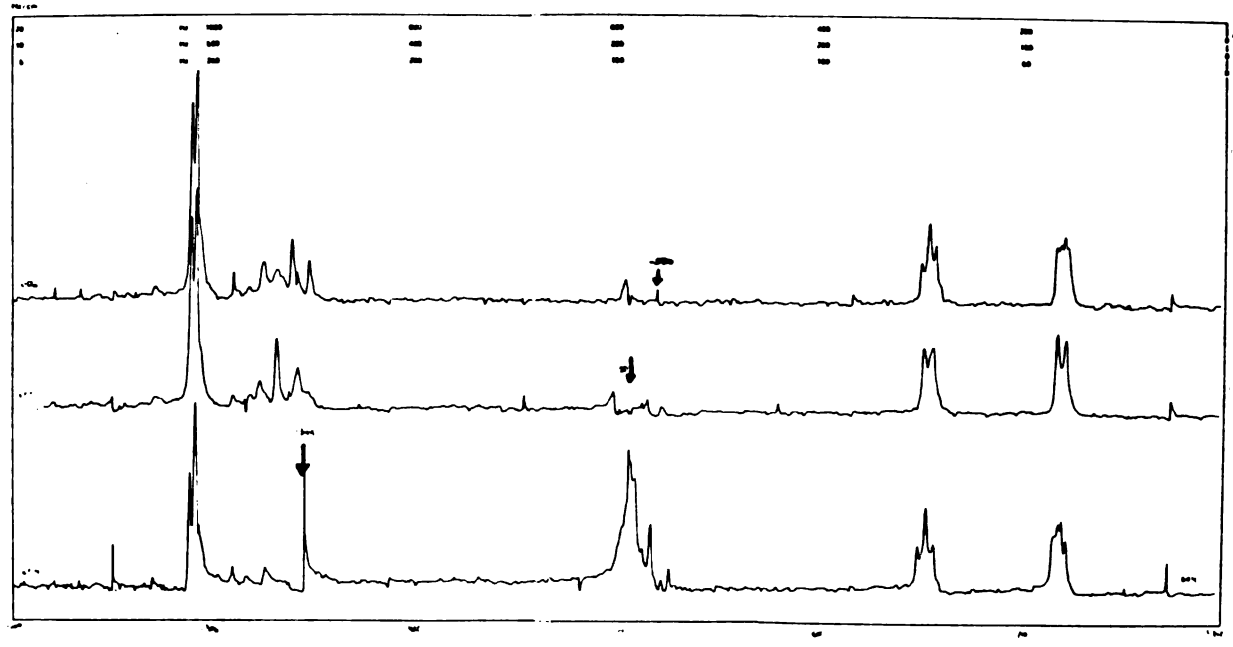

N-(2-propenyl)glycine
60 MHz

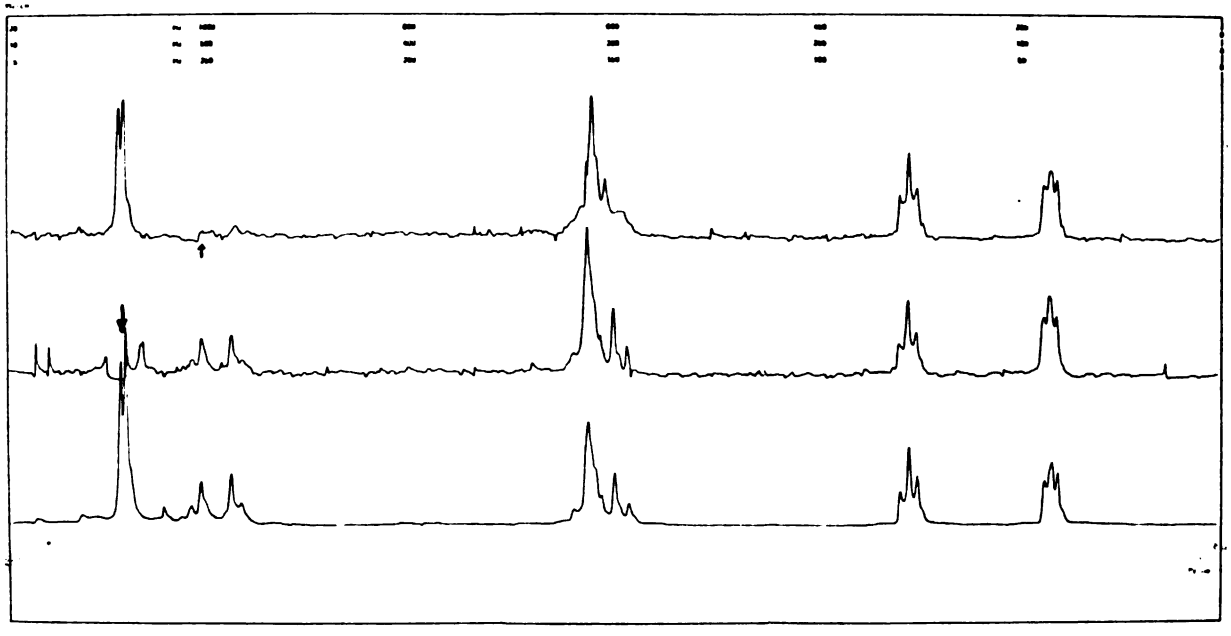

vinylcreatine
60 MHz

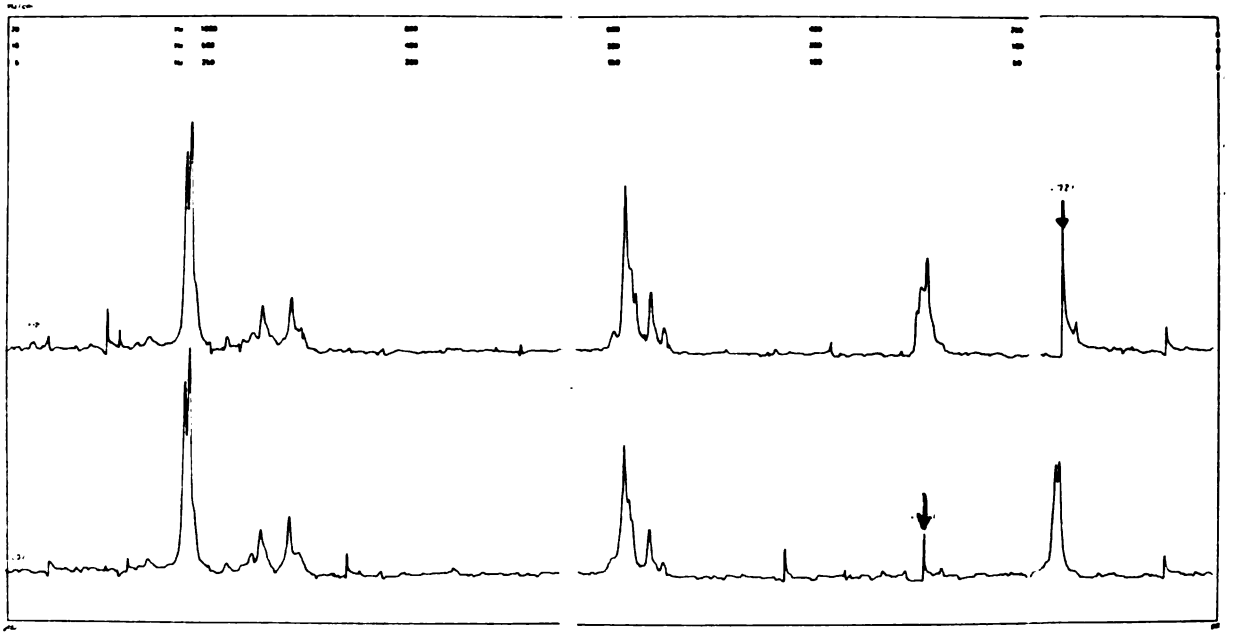

epoxycreatine
100 MHz

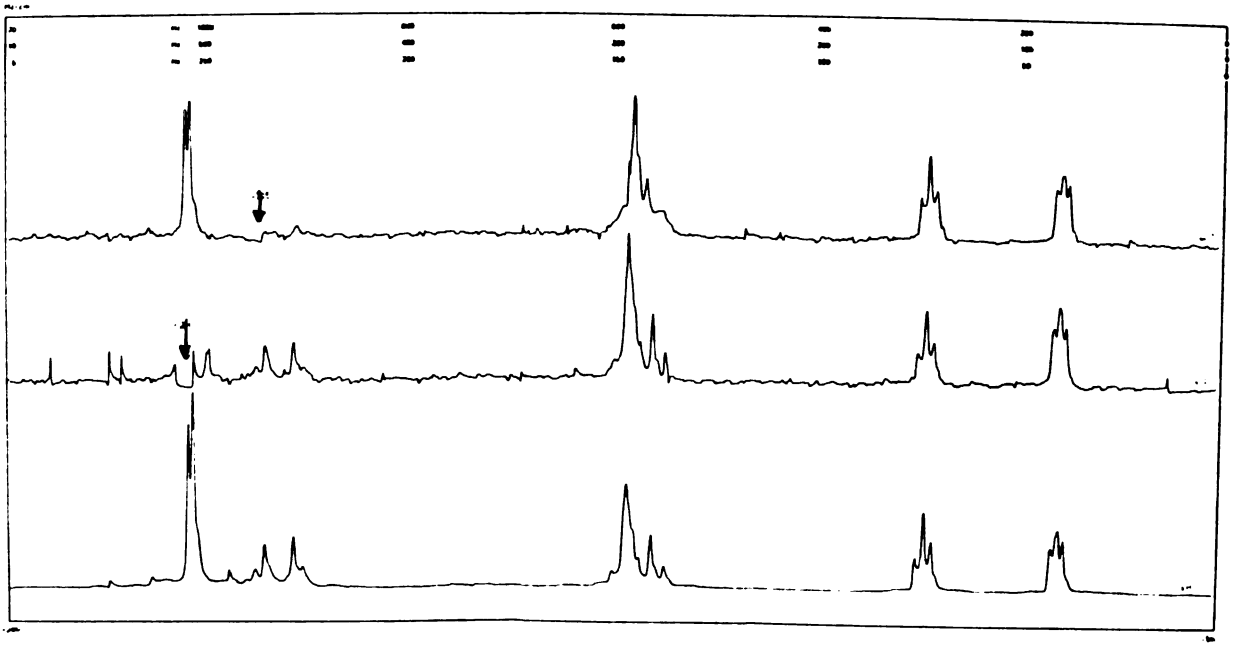

epoxycreatine
360 MHz

Epoxycreatine decoupling experiments (360 MHz)

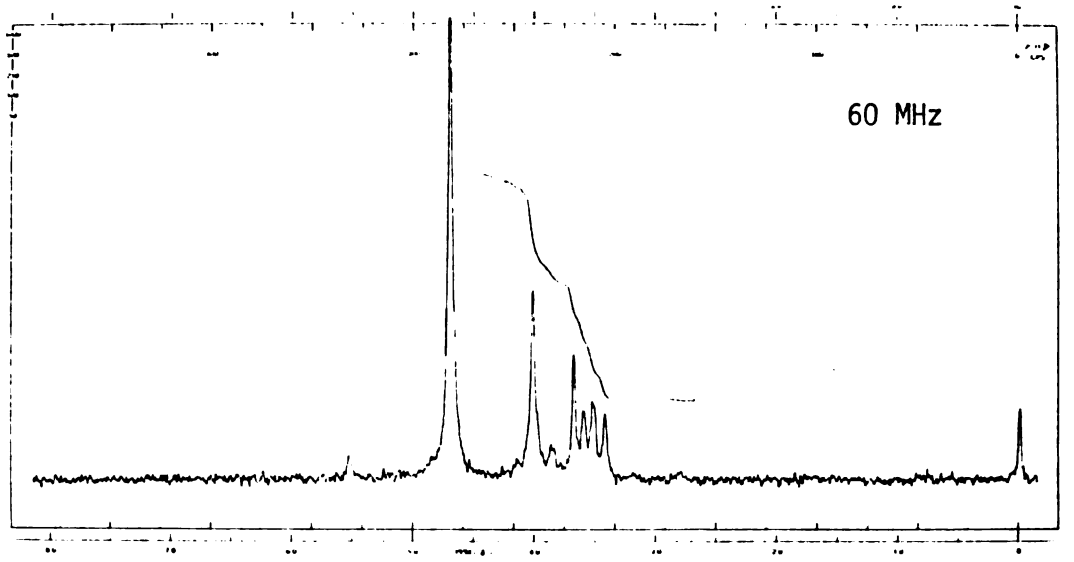


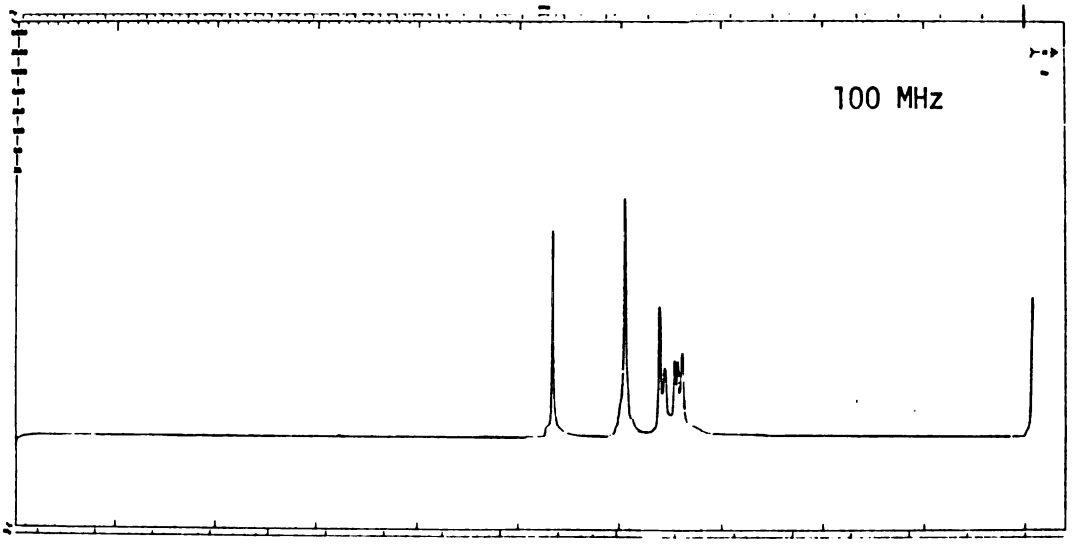


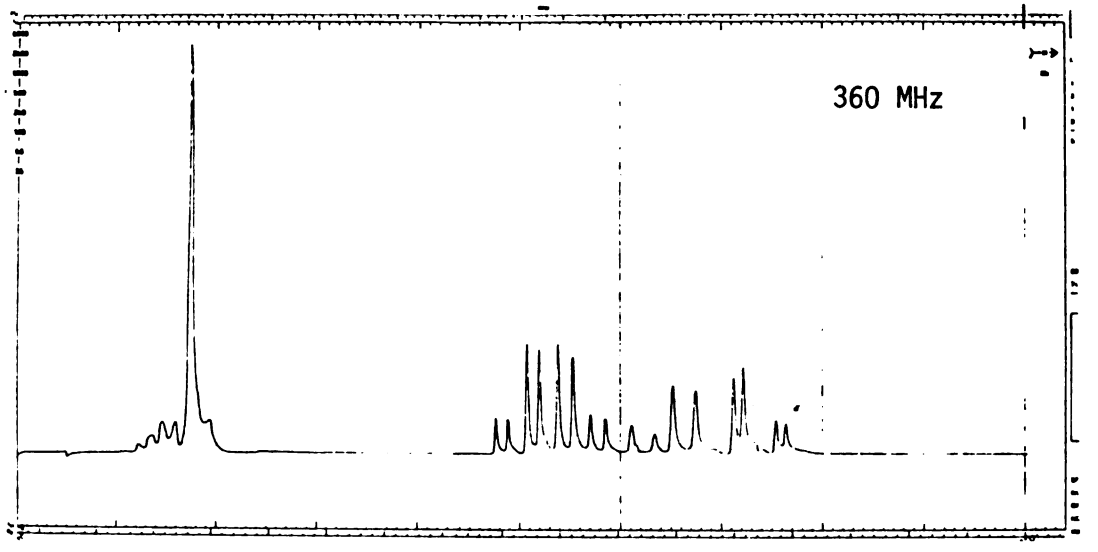


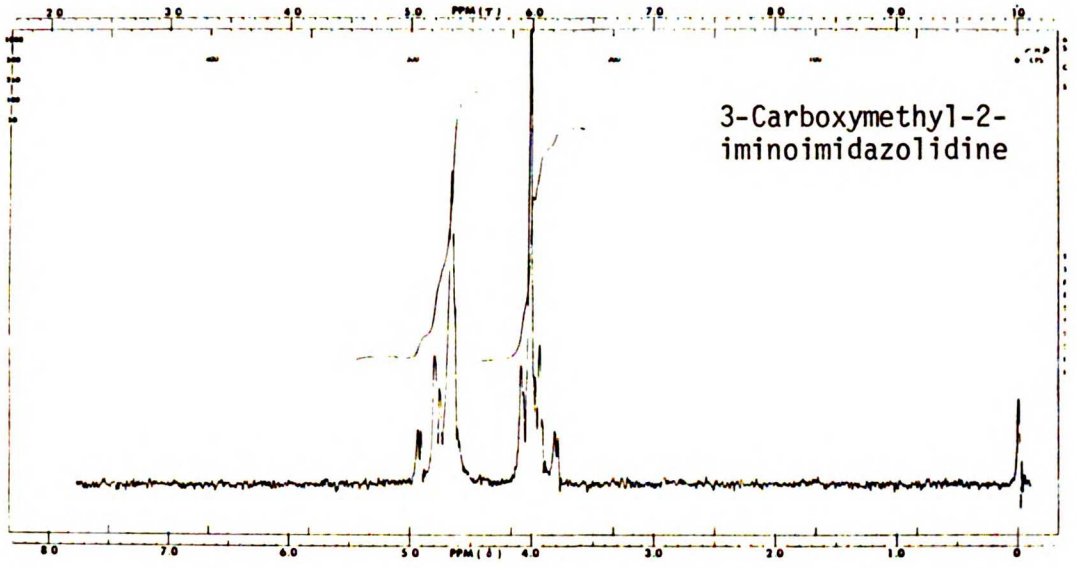


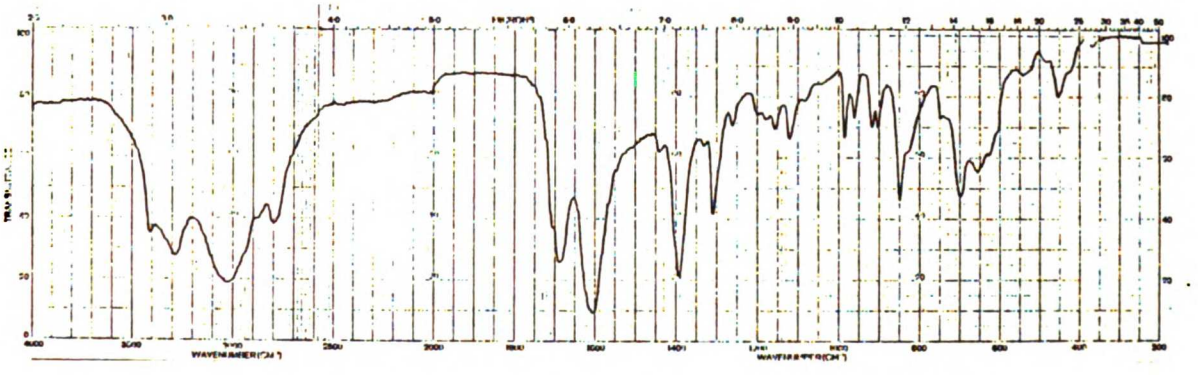
N-Amidino-N-(2,3-dihydroxypropyl)glycine



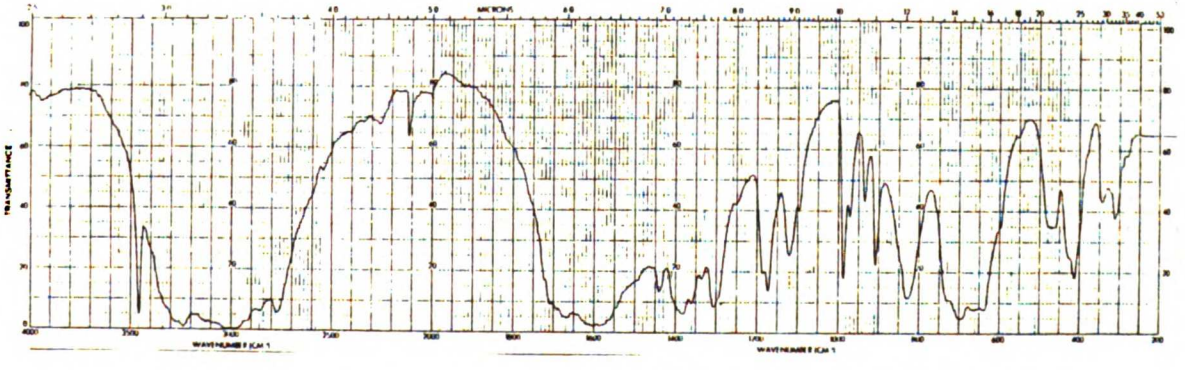








epoxycreatine

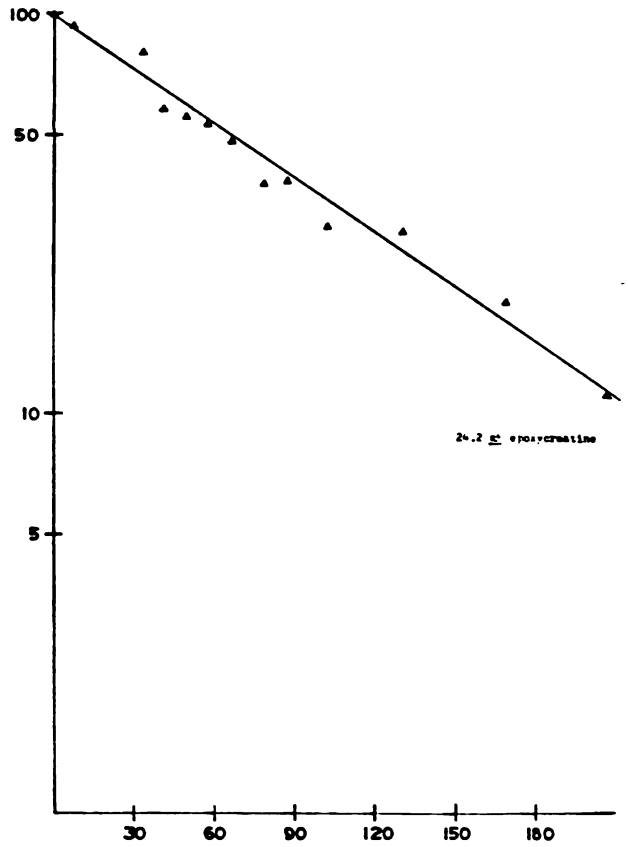


N-Amidino-N-(2-propynyl)glycine

APPENDIX B

Kinetics of Irreversible Inhibition

The pseudo first-order plots shown on the following pages are all plotted as % Original Activity *vs*. Time (min.). All concentrations (epoxycreatine or epoxycreatine and creatine) are given in *m*M.


100
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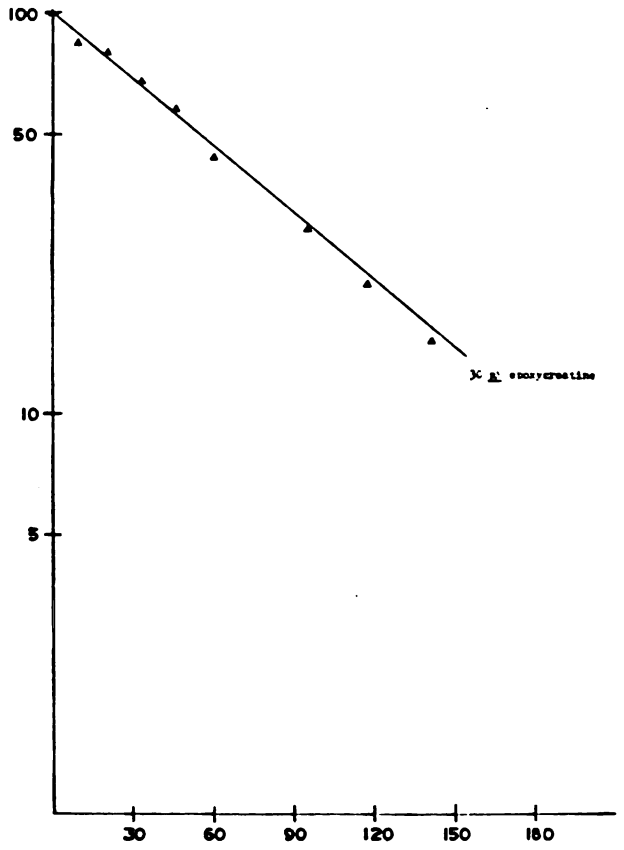
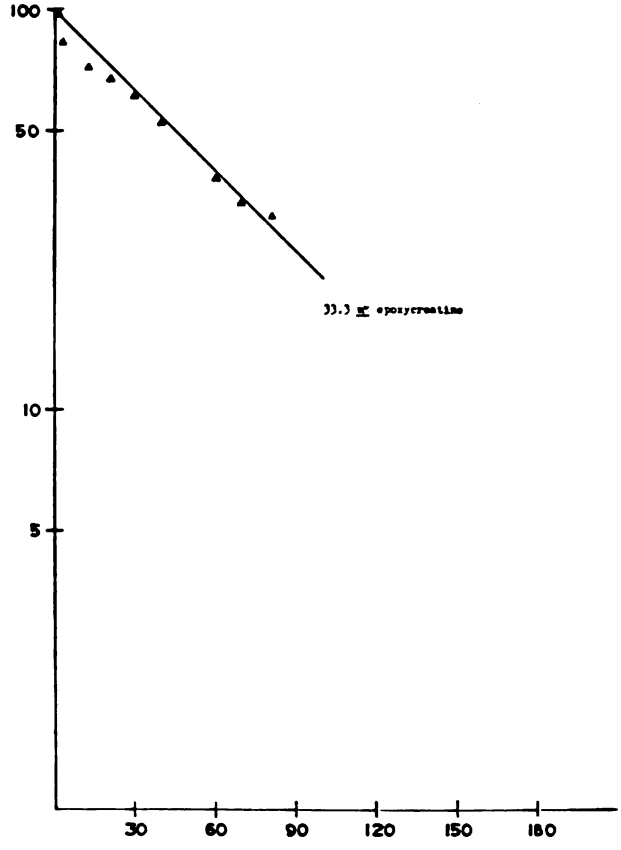
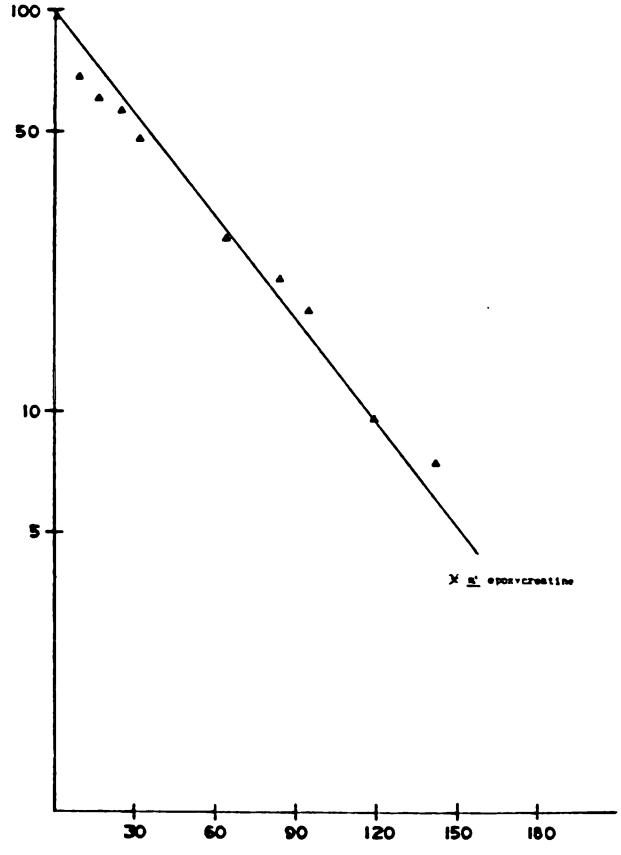
10
5
30
60
90
120
150
180

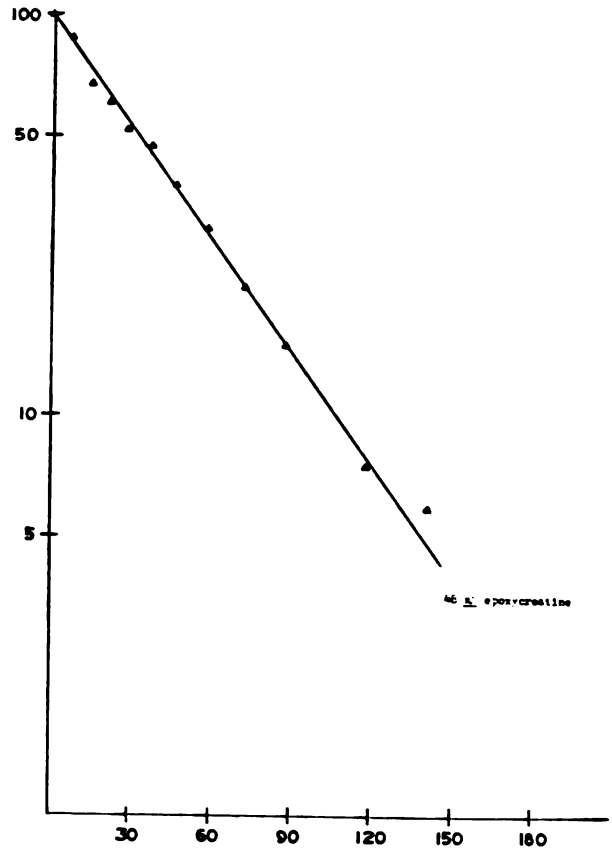

100
50
10
5
30
60
90
120
150
180



33.3 M epoxycreatine



X M epoxycreatine


100
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10
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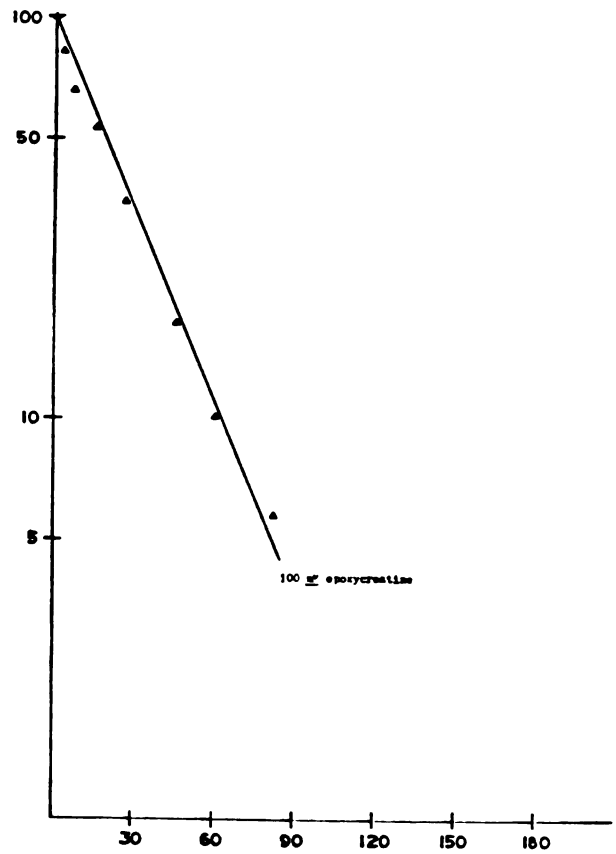
30
60
90
120
150
180


100
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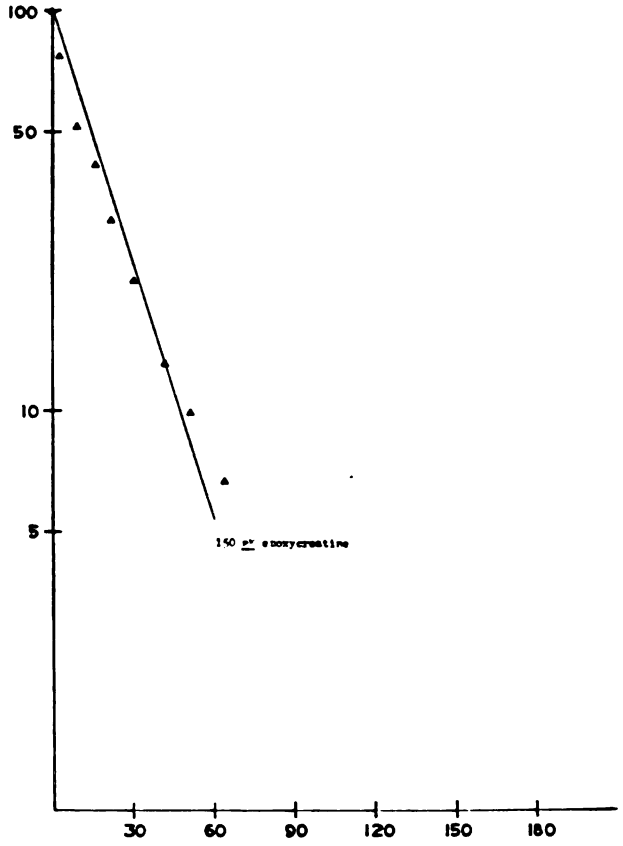
60
90
120
150
180


100
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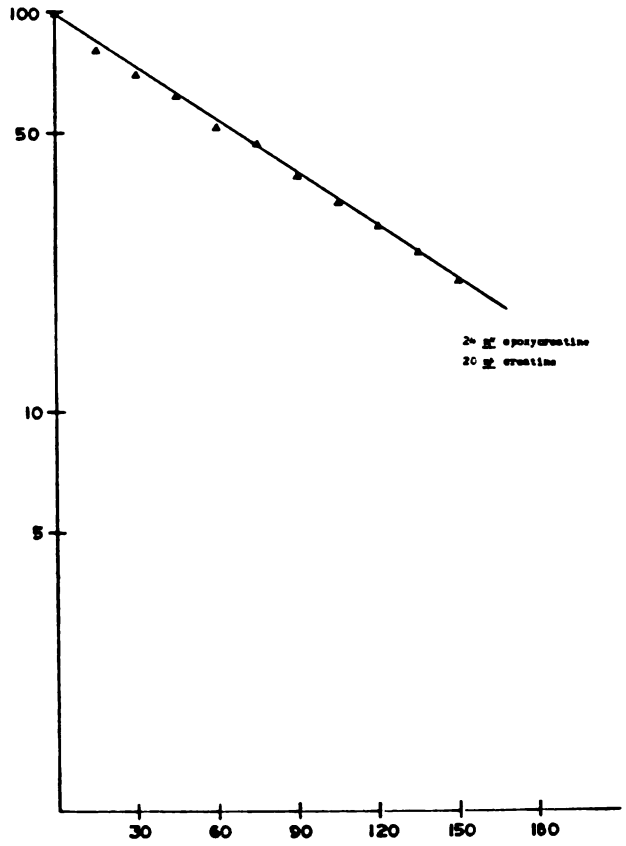
100
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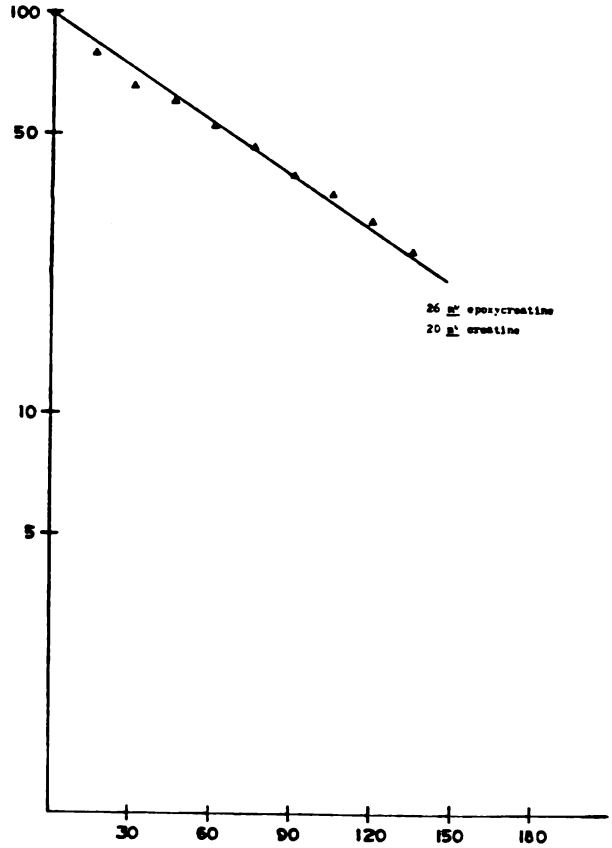
90
120
150
180


100
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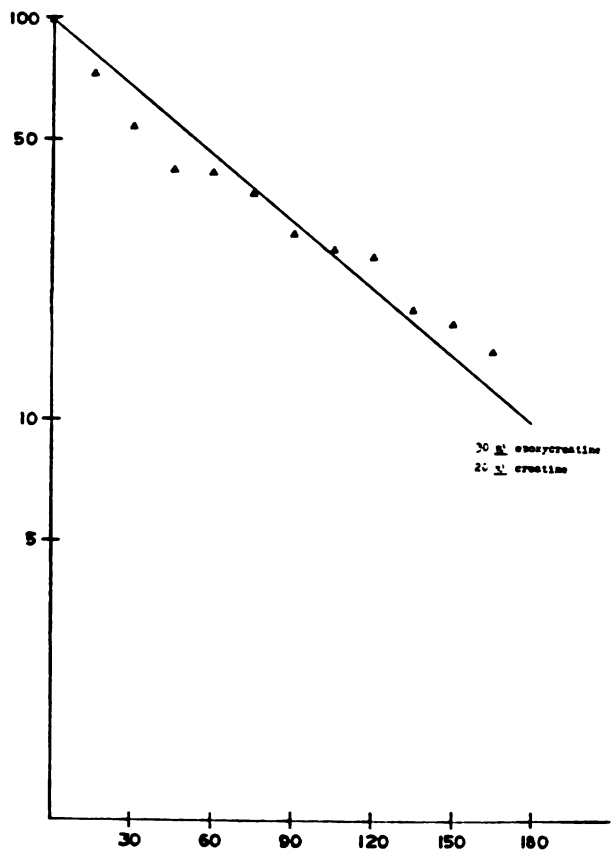
5
30
60
90
120
150
180


100
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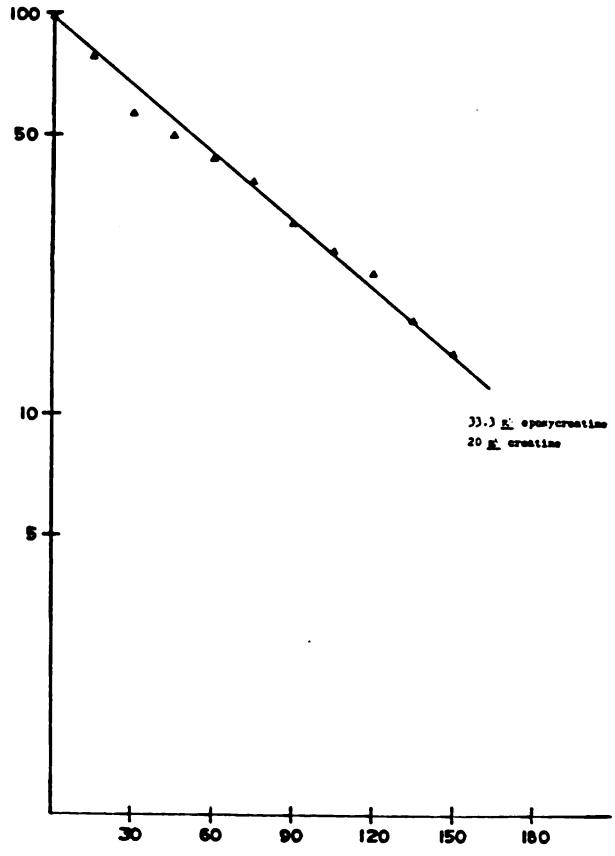
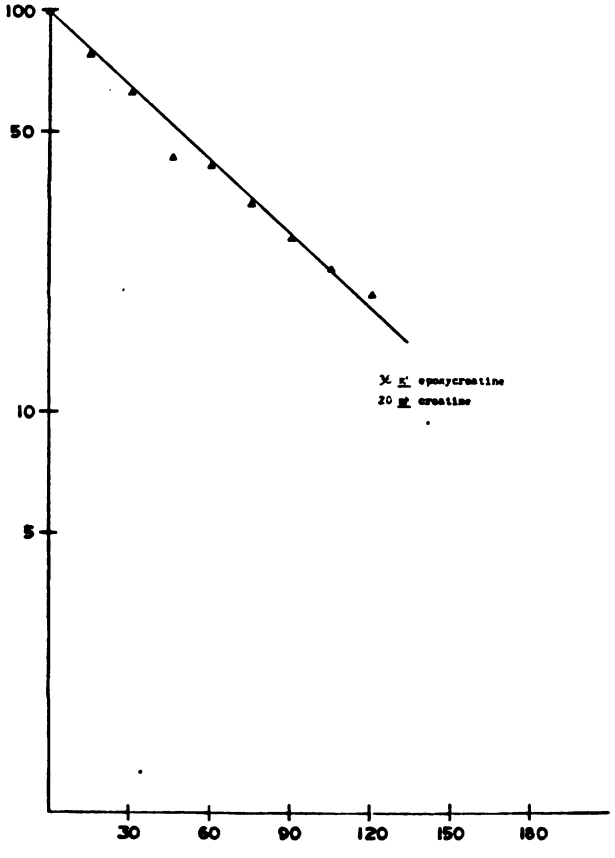
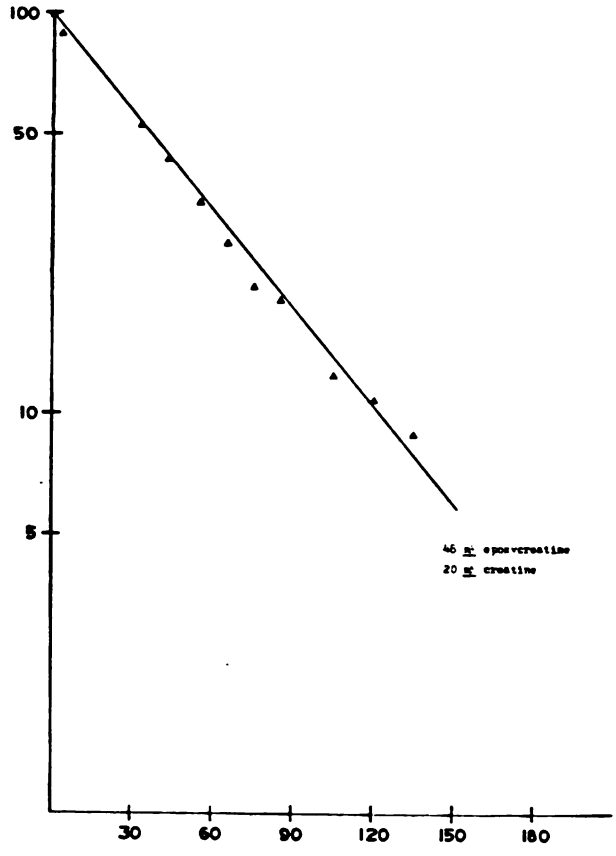
120
150
180

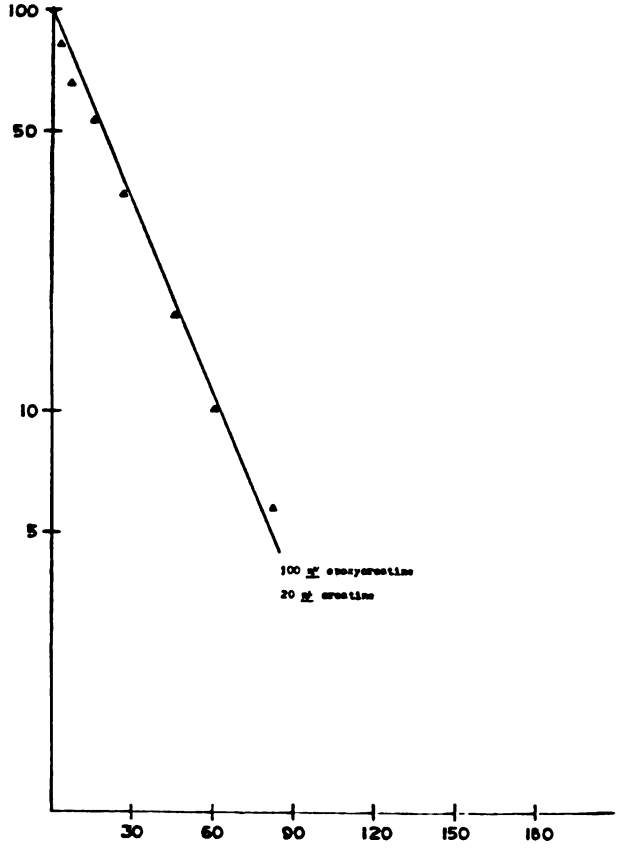


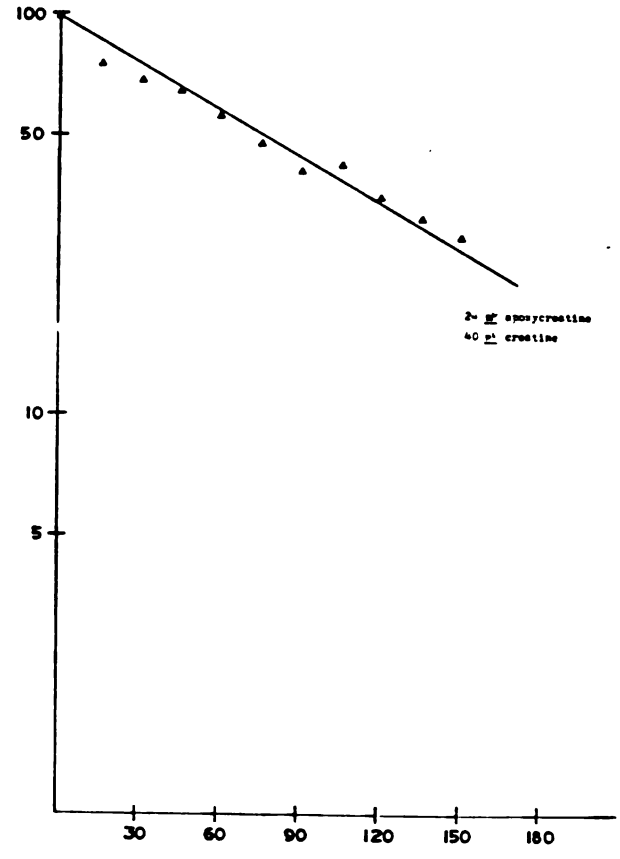
33.3 μ epoxycreatine
20 μ creatine



μ epoxycreatine
20 μ creatine


100
50
10
5
30
60
90
120
150
180


100
50
10
5
30
60
90
120
150
180


100
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60
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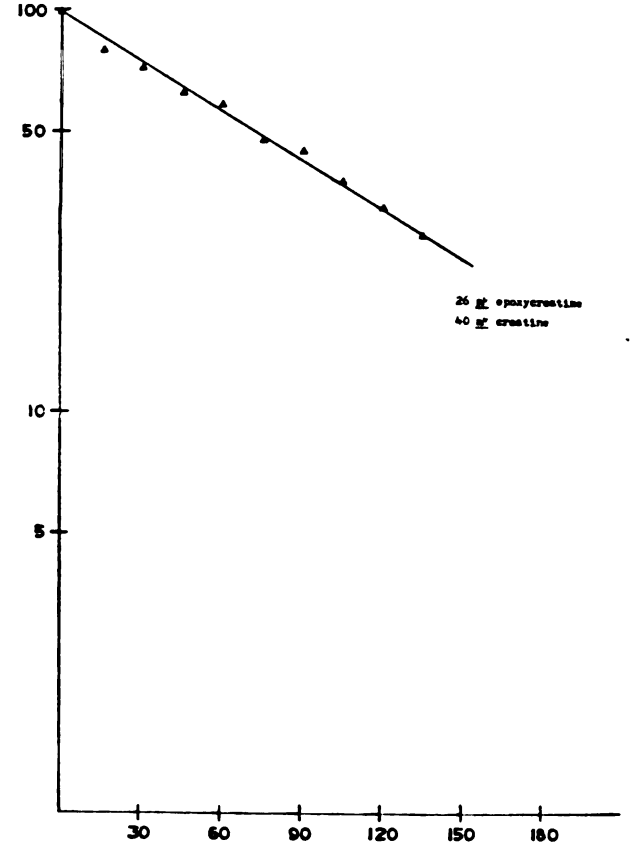
120
150
180


100
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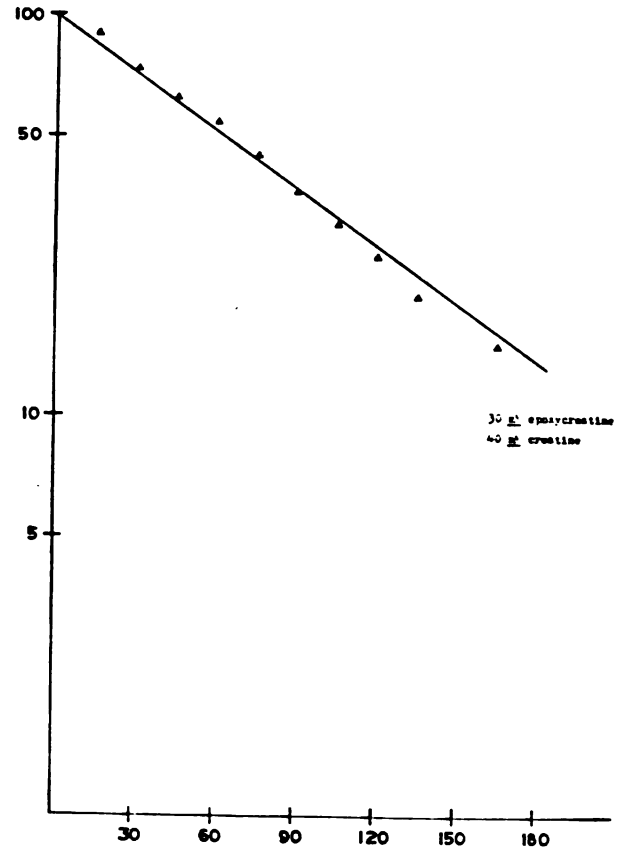
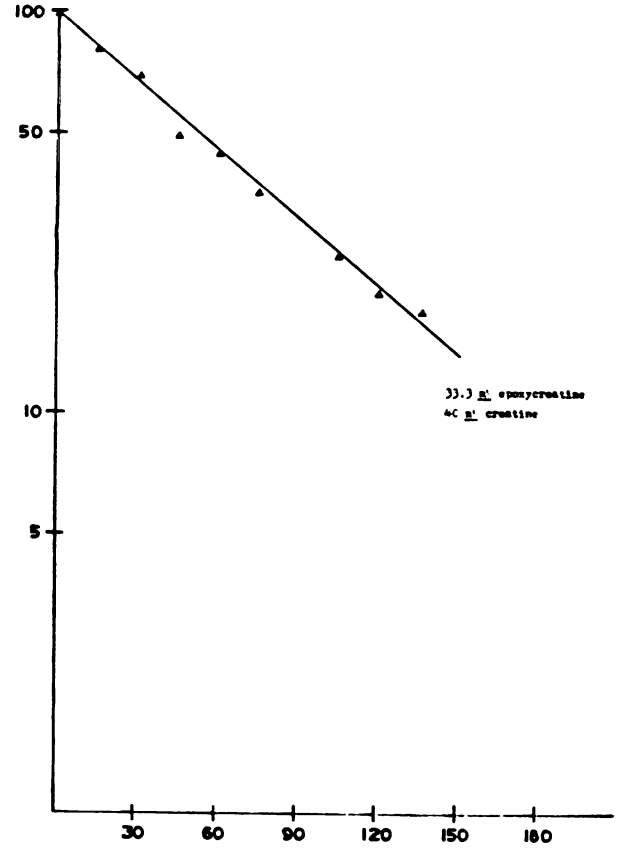
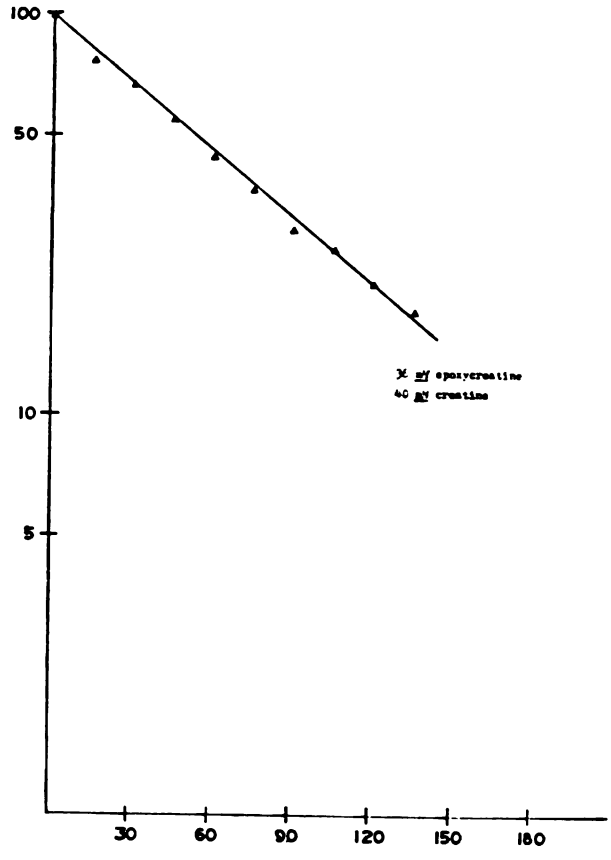
10
5
30
60
90
120
150
180


100
50
10
5
30 30 epoxycreatine
40 40 creatine
30
60
90
120
150
180


100
50
10
5
33.3 33.3 epoxycreatine
40 40 creatine
30
60
90
120
150
180


100
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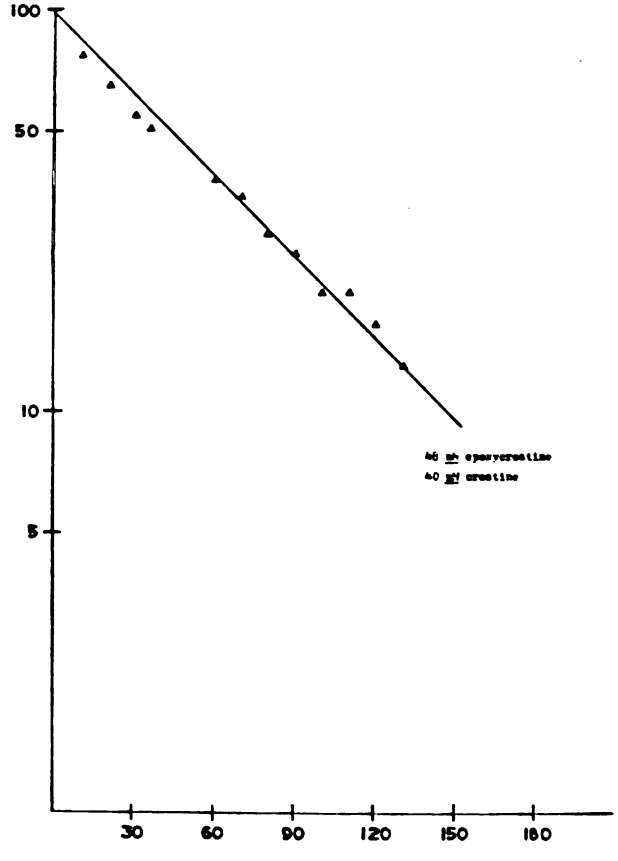
100
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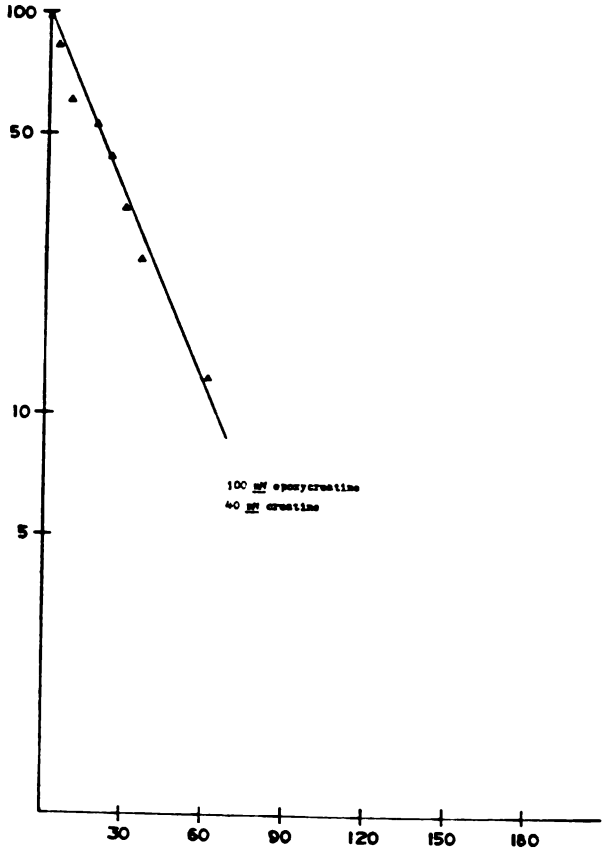
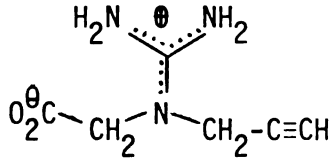
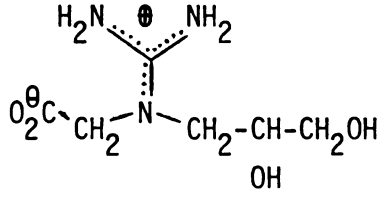
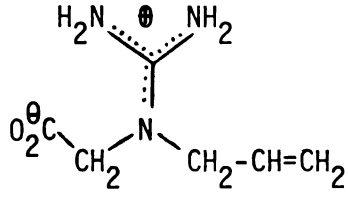
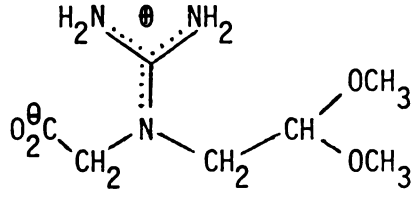
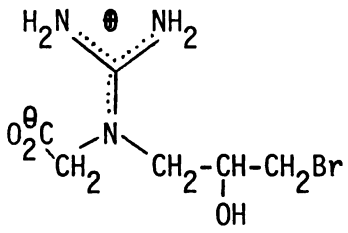
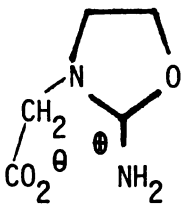
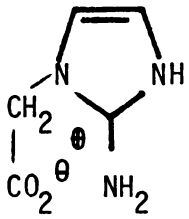

100
50
10
5
100 μM epoxycreatine
40 μM creatine
30
60
90
120
150
180

Appendix C

This table summarizes the preliminary testing of new creatine analogs as substrates in the enzymatic reaction. Unless otherwise stated, the analogs were assayed by the pH-stat method with the concentration being 40 mM.

Compound	Initial rate % (relative to creatine)
	5.8
	3.8
	3.2
	2.0

Compound	Initial rate % (relative to creatine)
	not a substrate
	not a substrate
	not a substrate (analyzed spectrophotometrically)

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