MECHANISTIC STUDIES ON KINASES

by

Rebecca Elaine Reddick

B.A., University of Kansas, Lawrence, KS, 1979

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACEUTICAL CHEMISTRY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

Date University Librarian

Degree Conferred: JUN 15 1986

This thesis is dedicated to
my husband, Terry,
who gave me the freedom
to quit
and the support and encouragement
to finish.

Acknowledgements

I would like to thank Professor George Kenyon for his well-rounded approach to teaching and research which has given me a broader outlook on scientific research. I also thank him for for instruction in English composition.

I would also like to thank Professor Norman Oppenheimer for helpful advice, comments and discussions throughout graduate school.

I also wish to thank Professor Dan Santi for reading this thesis on relatively short notice and for helpful discussions on kinetics. Thanks are also due to Drs. Frank Raushel and Paul Cook for helpful discussions during the writing of this thesis.

I would like to thank Tom Marshner, Mike Wendland, Terry Matsunaga and Max Keniry. They were most often available and of assistance during late night NMR sesssions. Special thanks to Tom Marschner for frequent mechanical, electronic and computer assistance in all the labs. I would also like to thank Vince Powers for running my GC-MS samples.

I would like to thank Dr. and Mrs. Roger Ketcham for sharing their home with me during my last month at UC. I would also like to thank Dr. Fred Rudolph who generously loaned word processing and drawing facilities at Rice University so that I could live in Houston while writing. Special thanks to Drew Minnick who did all my bureaucratic paperwork for the past six months.

I thank all my friends in the Kenyon and Oppenheimer groups for many lively social and political discussions. I will always be thankful for Lisa Peterson and Terry Matsunaga who watched out for my social life after "my" Terry had to go to Houston. I would also like to thank Eastern Airlines and GTE Sprint for the cheap flights and phone calls to Houston. I hope they invested the money wisely as I did.

Finally, I would like to thank my parents and grandparents for their very special support during all my years of college education. They made my education possible and taught me that I can and should do whatever I want to do.

I would like to acknowledge the American Foundation for Pharmaceutical Education, the Johnson and Johnson Foundation, the PEO and the UC Regents for financial support.

Abstract

We have synthesized [2-^{15}N]creatine, [2-^{15}N]creatinine, [2/3-^{15}N]creatinine, [2-^{15}N]phosphocreatinine, [2/3-^{15}N]phosphocreatinine, [2-^{15}N]phosphocreatine, and [2/3-^{15}N]phosphocreatine. ^{1}H and ^{15}N NMR analyses of the labeled creatinines revealed that creatinine is protonated exclusively at the unmethylated ring nitrogen (N-3) and that in the predominant resonance form at low pH, conjugation to the carbonyl is present. ^{31}P and ^{15}N NMR analyses of the labeled phosphocreatinines confirm unequivocally that the site of phosphorylation of phosphocreatinine is at the exocyclic nitrogen as reported by Zeile and Meyer (Zeile, 1938). ^{31}P and ^{15}N NMR analyses indicate that no spontaneous ^{15}N/^{14}N positional isotope exchange occurs in specifically labeled phosphocreatine and that the 18 Hz ^{31}P-^{15}N one-bond coupling constant in [2-^{15}N]phosphocreatine established here is in disagreement with the 3 Hz value recently published by Brindle et al. (Brindle, 1984). As a result of these two findings, [2-^{15}N]phosphocreatine was used in an investigation of the stereochemistry of the reaction catalyzed by creatine kinase.

We found that [2-^{15}N]phosphocreatine undergoes complete ^{15}N/^{14}N PIX when the creatine kinase reaction is allowed to come to equilibrium. However, this PIX does not occur from the enzyme-bound form of creatine at pH 8.0 even under conditions where the amount of exchange should be maximized. This suggests that the reaction proceeds with regioselectivity and that

there is a significant barrier to rotation of the guanidino group of creatine at the enzyme's active site. Alternatively, it may simply indicate that the rate of partitioning of the ternary product complex creatine kinase•creatine•MgATP to phosphocreatine is less than 0.15 times the rate of partitioning of this complex to form creatine. Another isotope exchange experiment is proposed that will indicate which of these explanations is correct. An analogous isotope experiment was run using [2-^{15}N]phosphocreatine to examine the ratio of partitioning of ternary product complex creatine kinase•creatine•MgATP to MgATP *vs* partitioning to phosphocreatine. We found that in this case the ratio of partitioning rates is indeed quite small (0.20±0.11).

The specificity of pyruvate kinase for the non-nucleotide substrate has been investigated. Pyruvate kinase was found to catalyze the phosphoryl transfer between ATP and two α-hydroxy acids, isoserine and vinylglycolate. The products, phosphovinylglycolate and phosphoisoserine, were characterized by ^{31}P NMR spectroscopy. The rates of these reactions are very low compared to the physiological reaction. V_{max}/K_m for vinylglycolate is approximately 0.0015 sec^{-1}. No initial rate studies could be carried out for the reaction of isoserine because prohibitively large amounts of enzyme would have been needed to produce the data needed for such kinetic analyses. Phosphovinylglycolate was found to be a competitive inhibitor with a K_i values of 2.5 m*M*. ^{1}H NMR assignments for three compounds prepared for the screening studies are reported here for the first time.

George L. Kenyon

Table of Contents

List of Tables

List of Schemes

List of Figures

List of Figures (continued)

List of Figures (continued)

List of Figures (continued)

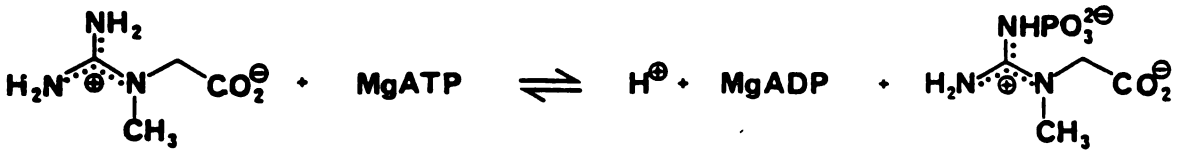
Part II.

Chapter 3.

List of Figures (continued)

Historical Background–Creatine Kinase

Creatine kinase (CPK) catalyzes the following reaction:



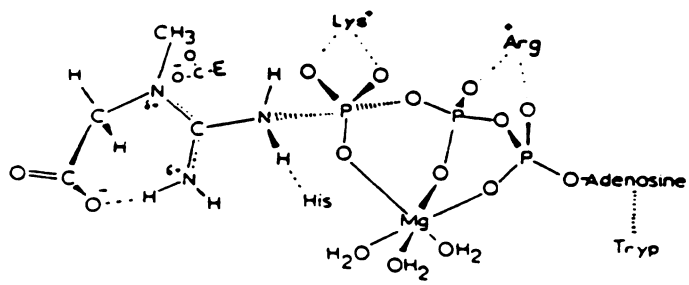
Creatine kinase is present in relatively high levels in vertebrate muscle, heart and brain tissues. However, it now appears that it may be present in nearly all tissues, albeit in much lower quantities. It has been demonstrated that CPK is present at the mitotic spindle of even non-muscle cells and may, therefore, be involved in the ubiquitous function of cell division (Silver, 1980)(Koons, 1983). The enzyme catalyzes the reversible ATP-dependent phosphorylation of creatine (Cr). The physiological role of the product, phosphocreatine (PCr), is that of an energy reservoir. Whenever cellular ATP consumption is larger than its production via glycolysis and oxidative phosphorylation, PCr is catabolized via the reverse reaction to maintain a constant level of ATP needed to drive various ATP-dependent reactions.

This role has been verified by the use of *in vivo* ^{31}P NMR techniques. These techniques have been used to examine steady state levels of PCr, ATP and ADP in cells, organ, and whole animals (Hoult, 1974)(Ingwall, 1982)(Dawson, 1977.). More recently, the technique of magnetization transfer has been used to examine the

kinetics of the CPK reaction in resting skeletal muscle (Meyer, 1982) and ischemic rabbit heart (Nunnally, 1979). In fact, ^{31}P-NMR studies of the reaction *in vivo* have been used to establish the role of CPK deficiency in certain clinical disease states. As a result, these disease states can now be more readily diagnosed.

CPK has a much wider clinical role in the diagnoses of myocardial infarction and Duchenne muscular dystrophy (Sjovall, 1964). Because the levels of CPK are also relatively low in most tissues, the presence of measurable quantities in the bloodstream is diagnostic for lesions in tissues rich in CPK. CPK exists in solution as a dimer of molecular weight 81,000. Most CPK is formed from basic subunits of three types, M, B, and Mi. The three major isozymes from muscle, brain and heart tissue are MM, BB, and MB, respectively. A fourth major isozyme, MiMi, is produced by mitochondria (Blum, 1983). Since these isozymes can be separated by electrophoretic techniques, the source of a sample of CPK can be determined. (Sjovall, 1964) (Prellwitz, 1981) (Phillips, 1980)

Creatine kinase was first isolated from rabbit muscle by Kuby (Kuby, 1954). Over the course of the next thirty years investigations into the catalytic mechanism led to the development of the following picture of the active site of the central complexes.



The features of this model are reviewed thoroughly in the recent chapter by Kenyon and Reed (Kenyon, 1983), but they are outlined here briefly.

The stereochemistry of phosphoryl transfer with respect to ATP in the CPK reaction has been established through studies with isotopically labeled ATP. All available evidence points toward a mechanism in which direct phosphoryl transfer occurs in a single associative step (Lowe, 1980)(Hansen, 1981).

The stereochemistry of phosphoryl transfer with respect to the guanidino substrate has been investigated by the use of restricted analogs of creatine. The creatine kinase catalyzed phosphorylation of cyclocreatine is regiospecific (Rowley, 1971)(Struve, 1977). Thus, it has been inferred that creatine itself also may be phosphorylated regiospecifically on the $-NH_2$ group that is trans to the carboxymethyl group for creatine in the extended conformation. The question of whether this stereochemistry is exactly analogous to that of creatine, the true substrate, is one of the subjects under investigation in this thesis.

Several specific amino acids have been shown to be involved in the catalytic mechanism. For example,. ^1H NMR and pH rate studies suggest that a histidine residue acts as the acid-base catalyst that deprotonates the $-NH_2$ groups of creatine upon phosphrylation by MgATP (Cook, 1981)(Rosevear, 1981). Based on NOE studies of the formate transition state analog complex (James, 1974)(James, 1976) in conjunction with chemical modification studies, it was concluded that both a Lys residue and an Arg residue were

involved in binding phosphate groups at the active site. In addition, it appears that there is a Trp residue in the ADP binding site. Affinity labeling experiments with epoxycreatine suggest that a carboxyl group is present at the creatine binding site possibly to help neutralize the partial positive charge at the nitrogen that becomes phosphorylated (Marletta, 1979).

One Cys residue reacts selectively with -SH modification reagents. Modification of this residue with any one of twelve thiol reagents results in complete inactivation of the enzyme (see review by Kenyon and Reed) (Kenyon, 1983). Thus, this Cys was labeled an "essential" Cys. Later, it was shown that the use of small modifying groups reduced, but did not eliminate, catalytic activity(Maggio, 1977). It was established that this was not the result of incomplete reaction of the enzyme being modified. Nevertheless, it has been countered that the binding of substrates may induce the transfer of the modifying group to another group on the enzyme, presumably in a reversible manner (Reddy, 1979). Site-specific mutagenesis experiments may be employed in the future to determine the exact role of this Cys residue.

Many analogs of creatine have been examined with respect to activity as substrates and inhibitors of the CPK catalyzed reaction. (Rowley, 1971) (McLaughlin, 1976) (Dietrich, 1980) (Dietrich, 1984) (Ngan, 1984) (Gercken, 1974). Briefly, these have established that the enzyme tolerates steric bulk added at the methylene and methyl carbons, but not at the guanidino nitrogens. Substitution of the carboxyl group by other charged groups are also tolerated. Studies with restricted analogs have shown that the enzyme has distinct

preferences for certain relative orientations of the functional moieties in creatine. Thus it has been possible to define many of the three dimensional steric requirements of the creatine binding site. For example, substitutions at the pro-R hydrogen of the $-CH_2$ group are better tolerated than those of the pro-S hydrogen. These preferences may be the result of simple steric interaction (i.e. the shape of the active site). Alternatively, they may be related to relative strengths of electrostatic interactions with charged groups at the active site, e.g. the carboxyl groups of the analogs.

It has been found that a wide variety of planar anions have a stabilizing effect on the dead-end complex, enzyme•creatine•MgADP. These complexes are considered to be transition state analog complexes because the anions appear to play the role of the missing PO_3 group(Milner-White, 1971). These complexes have proven to be useful in the investigation of many aspects of the CPK reaction (Reed, 1978)(Travers, 1980)(Barman, 1985).

A transition from Mg^{2+} coordination of the α,β and γ phosphoryl groups of ATP to Mg^{2+} coordination of the PCr phosphate group and the α and β phosphoryl groups of ADP is consistent with the results of two analog studies. The use of bidentate and tridentate isomers of CrATP and bidentate isomers of CrADP(Dunaway-Mariano, 1980) and of phosphorothioate analogs (Burgers, 1980) revealed distinct preferences of the enzyme for certain analogs and isomers thereof. In epr studies on the transition state analog complex with formate, all 6 ligands to Mn(II) have been identified(Reed, 1980). Three are waters not in exchange with

bulk solvent. Two of the remaining three are from the α and β phosphate groups of ADP and the last is from the formate oxygen which mimics the phosphoryl group transferred in the normal reaction. More recently, Leyh et al. (Leyh, 1985) found that the Mn(II) epr spectrum for a complex of enzyme, Mn(II)ATP and the non-reactive creatine analog, 1-(carboxymethyl)-imidazolidin-4-one, closely matches that for the reactive complex of enzyme, Mn(II)ATP and creatine in the equilibrium mixture. They used this epr data to obtain a clear interpretation of the epr spectrum for central complexes in an equilibrium mixture of the creatine kinase reaction. The results show that Mn(II) is coordinated to all three phosphates of ATP and that it remains coordinated to them even after the γ-phosphoryl group has been transferred to creatine to form PCr at the active site. Very recently they have used chirally labeled α-^{17}O-ATP and β-^{17}O-ATP to more completely define the stereochemistry of the active metal-nucleotide complex(Reed, unpublished results). The active isomer has now been determined to be α, β, γ tridentate α-(S),β-(S)-MgATP.

The complete amino acid sequences for CPK from *Torpedo californica* electric organ, rabbit brain and muscle, rat brain and muscle, and chick brain and muscle have recently become available via recombinant DNA techniques(West, 1984)(Ordahl, 1985)(Kwiatkowski, 1984)(Giraudat, 1984)(Benfield, 1984)(Putney, 1984) These data have revealed an extremely high degree of homology across species. In fact, the sequence differences between tissue types within the same species exceed those of different species within

tissue-types. Most recently, efforts to express the gene from *T. californica* has been successful (Kenyon, 1986).

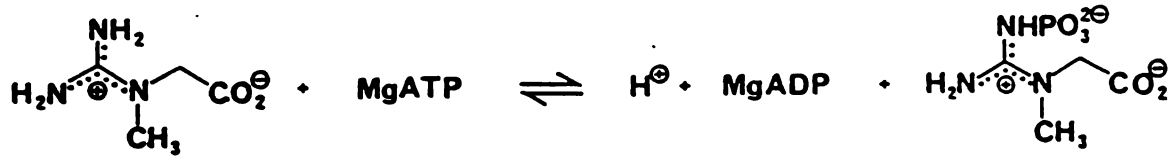
^{31}P NMR has been used to determine a value of 1 for the equilibrium constant of the enzyme-bound intermediates (Rao, 1979). More recently, Leyh et al. (Leyh, 1985) have confirmed these measurements. Keq for the overall reaction is 16 at pH 8.0 (Lawson, 1979). The creatine kinase reaction follows a rapid equilibrium, random, bimolecular kinetic scheme at least above pH 8 (Morrison, 1967) (Morrison, 1966). Equilibrium isotope exchange measurements have shown that the ATP⇄ADP, ATP⇄ADP, and PCr⇄Cr exchange rates are all equal at pH 8.0. Thus, at pH 8.0 the association and dissociation steps in the mechanism of creatine kinase are more rapid than the chemical step (Engelborghs, 1975). Travers et al. used stopped-flow methods measure rate constants for the chemical steps (Travers, 1979). The model for this study was a random mechanism that included a third intermediate between the two ternary substrate and product complexes. In addition, k_{off} for the binary complexes of MgADP and CrATP have been determined (350 sec^{-1} and 517 sec^{-1}, respectively) (Rosevear, 1981) (Dunaway-Mariano, 1980) and k_{on} values were calculated from the dissociation constants (3.2 x 10^6 $M^{-1}sec^{-1}$ and 7.6 x 10^6 $M^{-1}sec^{-1}$, respectively). The magnitudes of the rates for other steps have not yet been measured explicitly, although measurements of exchange to turnover ratios and equilibrium flux studies have given information about relative rates. It is known that at pH 7.0 phosphocreatine is "sticky". This means that the ternary substrate complex, E•PCr•MgADP, partitions to creatine much faster than

back to phosphocreatine (Cook, 1981). Presumably, this means that at pH 7.0 the exchange rates will no longer be the same. At pH 8.0 phosphocreatine is no longer sticky because catalysis is slower, but the release of phosphocreatine is not. Recently, there have been attempts to establish these relative exchange rates at pH 7.0 using saturation transfer and phase-inversion NMR techniques in order to confirm the conclusions of Cook, et al., but the results so far are not conclusive (Brindle, 1985). Interestingly, one explanation proposed to explain the results of Brindle, et al. is that there is a third intermediate between the two ternary substrate and product complexes as reported by Travers et al. Cryoenzymic kinetic studies carried out using stopped flow techniques have revealed that the steps leading to formation of the transition state analog complex with nitrate ion are kinetically similar to those leading to the productive E•MgATP•Cr complex (Travers, 1980). Soon it should be possible to begin combining the information regarding rate constants and relative rate constants to build up a detailed reaction energy profile. One purpose of the isotope exchange experiments performed for this thesis was to determine rate constants for some of the steps not yet defined kinetically such as k_{off} for MgATP and Cr in the ternary product complexes. Such a reaction profile will be an invaluable tool for the interpretation of the results of site-specific mutagenesis experiments with the newly sequenced and expressed creatine kinase genes.

Chapter 1.
Syntheses and NMR Studies of [^{15}N]Creatine, [^{15}N]Creatinine, [^{15}N]Phosphocreatine and [^{15}N]Phosphocreatinine

Introduction

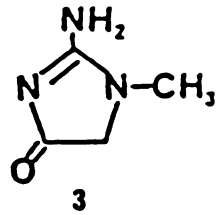
Creatine (1) and phosphocreatine (2) are of great importance in the energy fluxes involved in the muscle contractile process. When muscle ATP levels are high, energy is stored by the reversible creatine kinase-catalyzed transfer of the gamma phosphoryl group of ATP to creatine (**Equation 1**). The reverse reaction provides a ready source of ATP for muscle activity.



1 (eq. 1) 2

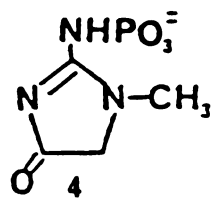
Creatinine (3) is an important end product of nitrogen metabolism in vertebrates and appears in the urine of normal adult humans. Creatinine is mostly formed from non-enzymatic cyclization of creatine. However, according to a recent report, non-enzymatic hydrolysis of phosphocreatinine (4) to give creatinine (3) may account for a significant fraction of the daily loss of phosphocreatine and creatine from muscle tissue (Iyengar, 1984).

These compounds and numerous analogs have been used extensively in investigations into the mechanism of creatine kinase over the course of the last 75 years

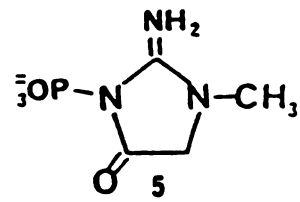


3

Figure 1 Creatinine

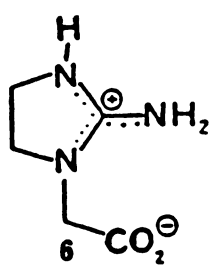


4

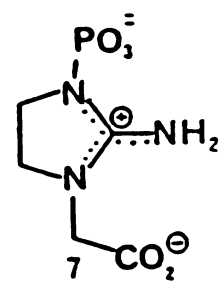


5

Figure 2 Possible Structures of Phosphocreatinine.



6



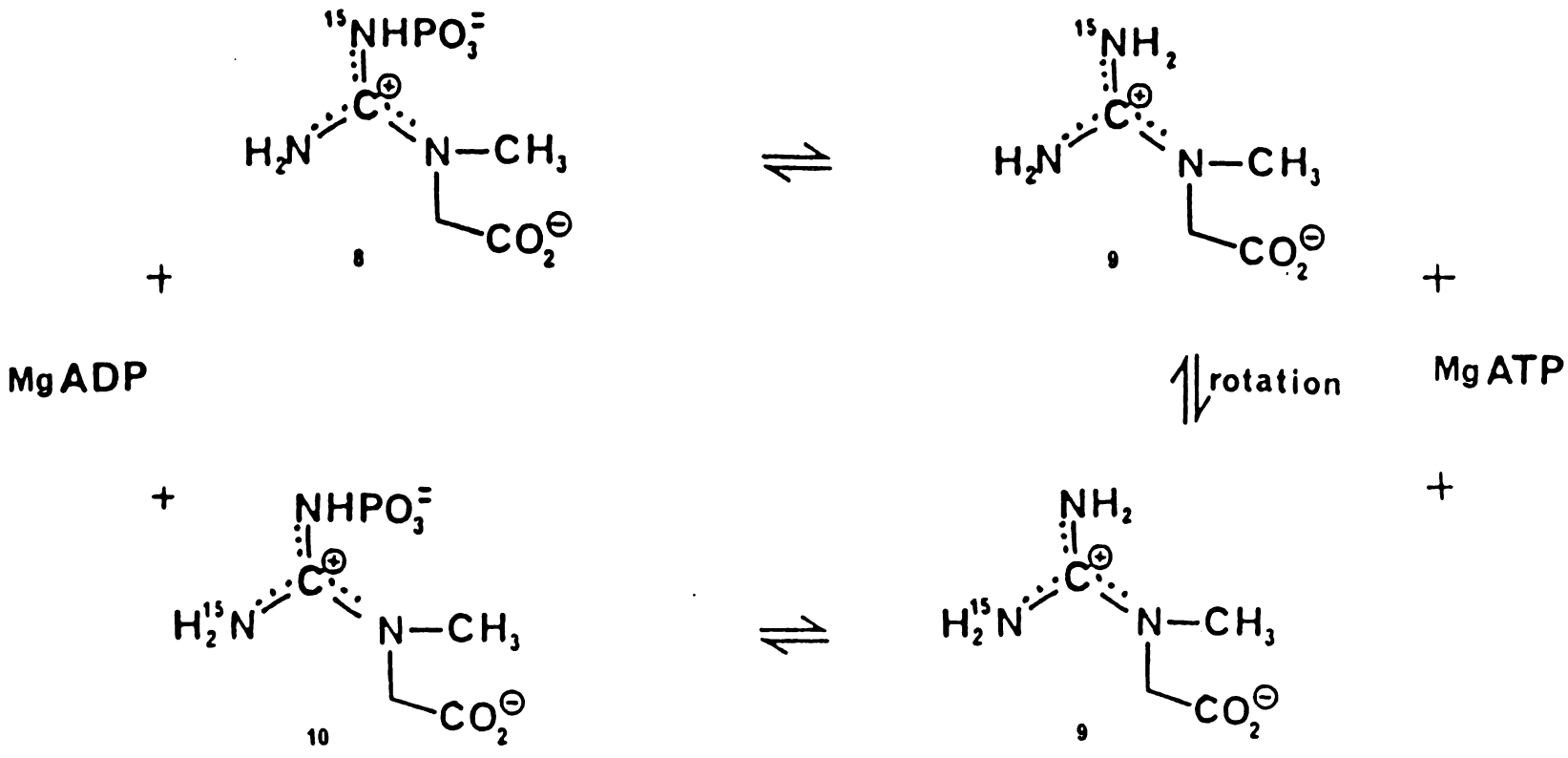
7

Figure 3 Structures of Phosphocyclocreatine and cyclocreatine

(Rowley, 1971) (McLaughlin, 1976) (Dietrich, 1980) (Nguyen, 1984) The chemical and structural properties of these molecules have been investigated as well. However, there are still unanswered questions about the properties of these molecules. Some of these are important to a better understanding of the reaction catalyzed by creatine kinase. Others pertain more to fundamental organic chemistry. In these investigations we have addressed three topics.

The first is whether phosphocreatinine is phosphorylated at N-2 or N-3 (**Figure 2**). Phosphocreatinine (**4/5**) and creatinine (**3**) are inhibitors of the enzymatic reaction catalyzed by creatine kinase (**Equation 1**) while the closely related compounds 1-carboxymethyl-2-iminoimidazolidine (cyclocreatine) (**6**) and phosphocyclocreatine (**7**) (**Figure 3**) are substrate analogs (McLaughlin, 1972) (Rowley, 1971). Therefore, it is important to determine the sites of phosphorylation in both phosphocreatinine and phosphocyclocreatine. The structure of phosphocyclocreatine (**7**) has been firmly established (Struve, 1977) (Phillips, 1979). Zeile and Meyer (Zeile, 1938) deduced by a degradative method that phosphocreatinine is the ring-cyclized form of phosphocreatine and that the phosphoryl group is attached to the exocyclic nitrogen (**4**). However, as will be discussed later, their proof is not unequivocal.

The second topic is the question of whether [2-^{15}N]phosphocreatine (**8**) might be useful as a probe of the stereospecificity and kinetics of the creatine kinase reaction. When phosphocreatine labeled with ^{15}N at N-2 (**8**) is incubated with ADP and creatine kinase and converted to [2-^{15}N]creatine (**9**) and



Scheme I $^{15}N/^{14}N$ Positional Isotope Exchange.

ATP, the two guanidino-NH_2 groups are capable of equilibration via rotation about the carbon-nitrogen bond **(Scheme I)**. Any phosphocreatine resynthesized will be labeled with ^{15}N at the N-3 position **(10)** 50% of the time. However, before this positional isotope exchange reaction of [2-^{15}N]phosphocreatine can be used in studies of creatine kinase two preliminary conditions must be met. The first is that the phosphorus resonances of [2-^{15}N]phosphocreatine and ^{15}N-3-phosphocreatine can be resolved in order to quantitate their relative amounts in solution. Although doubly labeled [$^{15}N_2$]phosphocreatine has previously been reported, data on NMR properties such as the ^{31}P-^{15}N one-bond coupling constant were suspect (Brindle, 1984). The second prerequisite was that the specifically labeled phosphocreatine does not undergo $^{14}N/^{15}N$ positional isotope exchange all by itself. Intramolecular phosphoryl transfers have been demonstrated in systems where a five-membered ring transition state was necessary (O'Neal, 1983), but a four-membered ring transition state would be required for such a transfer in phosphocreatine.

The third topic was the consideration of certain structural features in protonated creatinine. Protonation could conceivably occur at any one of the three guanidino nitrogens present **(Figure 4)**, but the exact site of protonation has never been established for creatinine. It was also possible that a tautomeric equilibrium existed between two or more of the structures in **Figure 4 (11, 12, and 13)**. In addition, structure **(11)** can be represented by the three resonance forms in **Figure 5**. The tautomeric preferences and resonance forms of an extensive series of

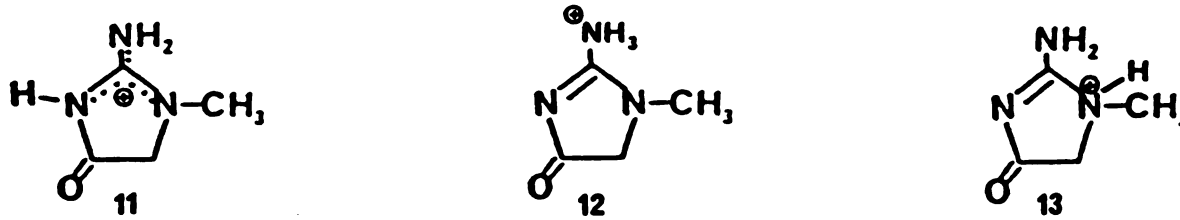


Figure 4 Proposed Tautomeric Structure of Protonated Creatinine.

11. where N-3 protonation is favored over either N-2 or N-1 protonation.; 12. where N-3 protonation is favored over either N-2 or N-1 protonation.; 13. where N-3 protonation is favored over either N-2 or N-1 protonation.

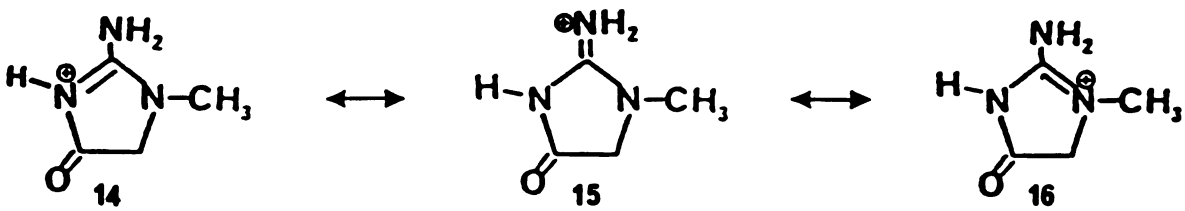
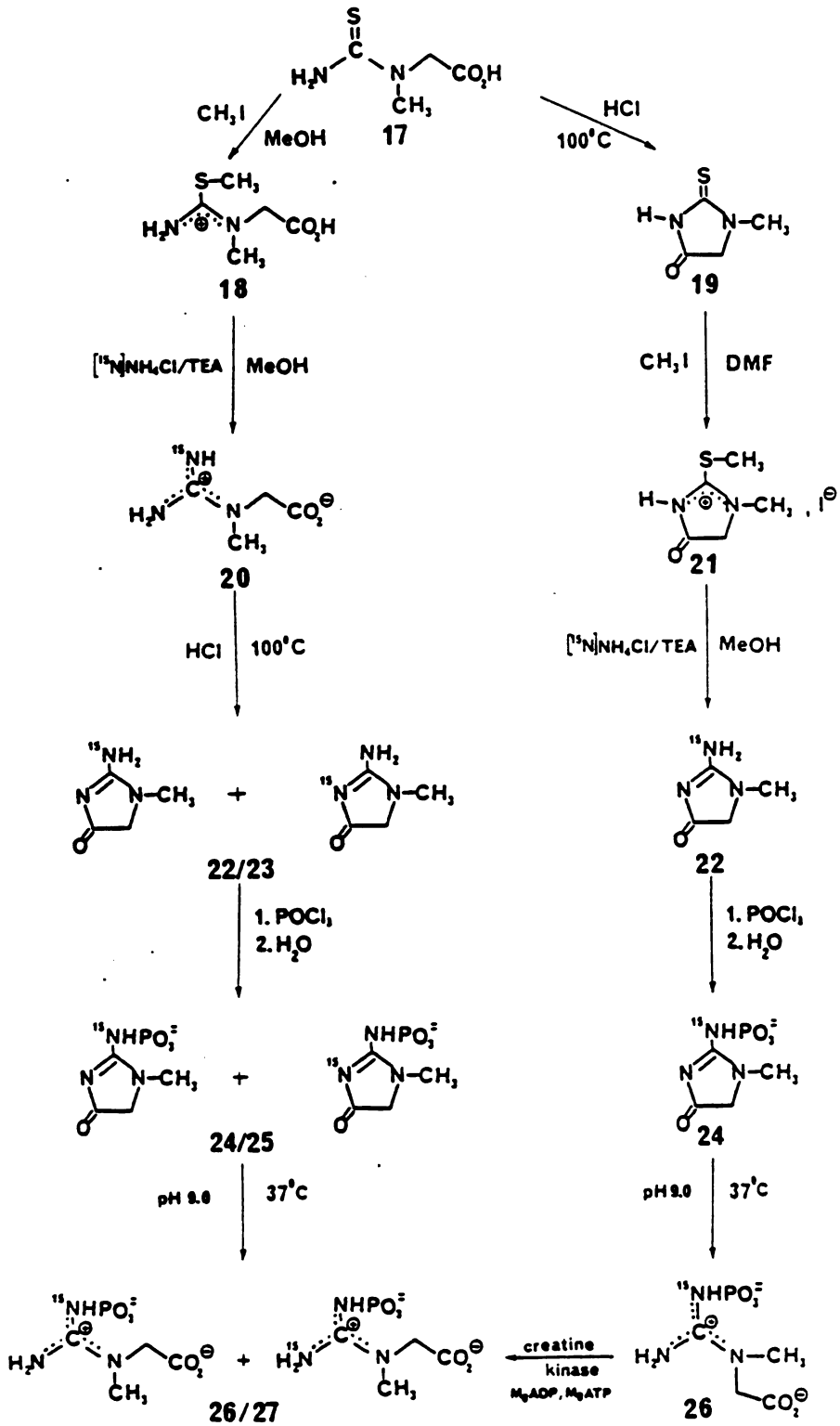


Figure 5 Resonance Forms for Creatinine Protonated at N-3

glycocamidines at pH 12 have been examined (Matsumoto, 1968)(Rowley, 1971). However, no information about the structures at low pH could be derived from the methods used by Rowley et al.

Finally, many compounds in which P-N bonds are present have been characterized by ^{15}N NMR. However, a search of the literature revealed no ^{15}N NMR data for the phosphoguanidines. In addition, ^{15}N-induced chemical shift changes have received only limited attention even though this phenomenon could be exploited in the study of chemical or biochemical mechanisms.

The isotopic syntheses and NMR experiments described in this paper have been used in all these investigations. To date no syntheses of phosphocreatine, phosphocreatinine, or creatinine have been reported with exclusive ^{15}N labels at the N-2 positions. We outline a synthetic method for the preparation of ^{15}N labeled phosphocreatine, phosphocreatinine, creatinine and creatine specifically labeled at N-2 and also for the preparation of analogs in which the label is completely scrambled between the N-2 and N-3 positions (**Scheme II**). In this chapter we report an unequivocal determination of the phosphorylation site in phosphocreatinine, a determination of the exclusive protonation site in creatinine and evidence to suggest which of the three possible resonance forms predominates. We further report that two requirements for the use of specifically labeled [2-^{15}N]phosphocreatine as a probe of the creatine kinase-catalyzed reaction (Chapter 2) have been met. These are 1) that no $^{15}N/^{14}N$ positional isotope exchange occurs non-enzymatically, and 2) that



Scheme II Synthetic Scheme for Labeled Substrates.

the one-bond ^{15}N-^{31}P coupling constant is sufficiently large that [2-^{15}N]phosphocreatine and [3-^{15}N]phosphocreatine can be readily distinguished by ^{31}P NMR. Finally, the data in this work will contribute to the body of knowledge of the magnetic resonance properties for compounds in the glycocyamidine and phosphoguanidine classes.

Materials and methods

[^{15}N]Ammonium chloride was purchased from MSD Isotopes in 99% isotopic purity. ATP, ADP, NADH, NADP, sarcosine, glycine, α-ketoglutarate, magnesium acetate, Trizma base, creatine kinase, creatinase, Chelex-100(Na^+), unlabeled creatine, creatinine, phosphocreatinine and phosphocreatine were the products of Sigma. Iodomethane and $POCl_3$ were purchased from Aldrich. Dowex AG 1-8x(Cl^-) (200-400 mesh) was purchased from Bio-Rad. $POCl_3$ was distilled under vacuum at 37°C. MeOH was refluxed over Mg turnings and distilled just prior to use. DMF was shaken with KOH and distilled from CaO. Urease and glutamate dehydrogenase were purchased from Boehringer-Mannheim.

Synthesis of Labeled Compounds.

3-Methyl-4-thiohydantoic acid (17) was synthesized according to the procedure of Rowley et al. **(Scheme I)** (Rowley, 1971).

N-carboxymethyl-N-methyl-S-methylisothiuronium hydroiodide (18) was synthesized according to the procedure of Rowley et al **(Scheme I)** (Rowley, 1971).

1-Methyl-2-thiohydantoin (19) was prepared by heating a suspension of 3-methyl-4-thiohydantoic acid (1.77 g, 12 mmol)

in 11 mL of concentrated HCl and 3 mL of H_2O for 24 hr on a steam bath. White, needle-like crystals (1.3 g, 10 mmol, 83.3% yield) were isolated by filtration, washed with ethanol and mixed hexanes and used successfully without further purification. Both the ^{1}H-NMR(s, 4.74 δ; s, 3.20 δ) and m.p.(227-230°C) were in good agreement with literature values (Rowley et al., 1971).

[2-^{15}N]Creatine (20) and [2/3-^{15}N]-Creatinine (22/23) were both prepared by the reaction of [^{15}N]NH_4Cl (100 mg; 1.83 mmol) and N-carboxymethyl-N-methyl-S-methylisothiuronium hydroiodide **(18)** (674 mg; 2.32 mmol) dissolved in 1.75 mL dry MeOH and triethylamine (880 µl; 4.2 meq). The reaction mixture was stoppered, covered with aluminum foil and stirred for 48 hr at room temperature. The product **(20)** precipitated out as a white powder which was filtered and used without further purification.

The [2-^{15}N]creatine **(20)** (196 mg) was heated in a stoppered flask at 100°C for 24 hr in 1.64 mL conc. HCl with 0.44 mL H_2O. Water and HCl were removed under vacuum and the residue was passed through a Dowex anion exchange column in H_2O and dried under vacuum. The product was recovered as a white powder and identified as creatinine **(22/23)** as described above (125.6 mg; 60.6% yield based on [^{15}N]NH_4Cl).

1-Methyl-2-methythio-2-imidazolin-4-one hydroiodide (21) was prepared by a modification of the procedure of Rowley et al. **(Scheme I)** (Rowley, 1971). A solution of 1-methyl-2-thiohydantoin **(19)** (650 mg, 5 mmol) in 5 mL of DMF at 0°C was treated with iodomethane (0.8 mL, 10.6 mmol). The solution

was warmed slowly to 35°C over a 30 min period and stirred for an additional 30 min while the product precipitated as a white powder. Unreacted iodomethane was removed in vacuo in a hood, and the product was filtered and washed with ethanol and mixed hexanes. NMR(s, 2.81 ppm; s, 3.33 ppm; methylenes probably under either the HOD peak or exchanged as in literature) and melting point (226-227°C) were in good agreement with the literature values (Rowley, 1971). Yield: 806 mg; 2.78 mmol, 55.6% of theoretical.

Creatinine (3) (Method I) To 40 mL of a 1 M methanolic NH_3 solution was added freshly prepared 1-methyl-2-methylthio-2-imidazolin-4-one·HI **(21)** (14.1 g, 51.8 mmol) and then 6 mL of dry triethylamine. The flask was stoppered, covered with aluminum foil and stirred at 0°C until nearly all of the solid had dissolved and the solution had turned dark brown. The reaction mixture was stirred 12 hr at room temperature while the product precipitated as a light-colored solid. After 7 more hr of stirring, the precipitate was filtered and rinsed with $CHCl_3$. The filtrate was shaken with an equal volume of $CHCl_3$ and the resulting precipitate was filtered and rinsed with $CHCl_3$. The Benedict-Behre (Benedict, 1936) test gave a positive result with both precipitates. The crude creatinine was passed through a Dowex anion exchange column in H_2O. The eluate was evaporated to dryness, and white crystals of creatinine were obtained (1.1 g, 23.7% yield based on 1 L of gaseous NH_3). The product was identified as creatinine by comparison with commercial samples using both ^1H-NMR and TLC on silica gel (Shoptaugh, 1978).

Creatinine (Method II) **[2-^{15}N]Creatinine (22)** was prepared by the reaction of 1-methyl-2-methylthio-2-imidazolin-4-one hydroiodide **(21)** (4.2 g; 15.4 mmol) and [^{15}N]NH_4Cl (0.5 g; 9.17 mmol) dissolved in 8.5 mL dry methanol and triethylamine (3.5 mL; 25.1 mmol). The solution was stoppered, covered with aluminum foil and stirred for 48 hr at room temperature. The solution darkened to deep amber within 1 hr. After 48 hr the crude product which had precipitated as a light-colored powder was filtered (549 mg). Solvent was removed from the filtrate under vacuum, and the residue was stirred with 25 mL $CHCl_3$ for 15 min. An additional 115 mg of crude creatinine was recovered by filtration. The combined crude products were passed through the Dowex anion exchange column in H_2O. The product was recovered as a white powder (556 mg; 4.9 mmol; 50% yield based on [^{15}N]NH_4Cl) and identified as creatinine by TLC on silica gel (BuOH/HOAc/H_2O;2:1:1)(Shoptaugh, 1978), by ^{1}H-NMR and by GC-MS of the di-trifluoroacetate of the (2-hydroxy-2-methyl)ethyl derivative **(30)**(Bjorkhem, 1977).

[2-^{15}N]Phosphocreatinine (24) was prepared by a method that closely paralleled the procedure of Zeile and Meyer (Zeile, 1938) for the phosphorylation of creatine.

To a stirred suspension of [2-^{15}N]creatinine **(22)** (545 mg; 4.3mmol) in 115 ml freshly distilled $POCl_3$ was added 79 µl(4 mmol) H_2O. The suspension was heated at reflux for about 45 min to dissolve the creatinine. Reflux was continued an additional 3 hr before the $POCl_3$ was removed under vacuum at 37°C. Residual $POCl_3$ was removed by repeated evaporation of 10 mL

aliquots of anhydrous ether under reduced pressure. Finally, the residue was hydrolyzed and neutralized to a phenolphthalein endpoint with 4 N NaOH at 0°C. The sample was purified by anion-exchange chromatography at 4°C in a modification of the method of Martonosi (Martonosi, 1960) by using first a 250 mL linear gradient of 0-0.3 M TEA/HCO_3^- (pH 7.8) and then 200 mL of 0.3 M TEA/HCO_3^- as eluent. Fractions were assayed for acid-labile phosphates according to the procedure of Ames (Ames, 1966) and for the guanidinium moiety according to the procedure of Benedict-Behre (Benedict, 1936). Phosphocreatine elutes from the column at 0.25 M TEA/HCO_3^- under these conditions. The pooled fractions were concentrated under vacuum below 30°C to remove water and buffer, then stored at 0°C (2.55 mmol; yield: 59% based on [2-^{15}N]creatinine). The product was identified by ^{31}P-NMR, by TLC on silica gel and by hydrolysis to form phosphocreatine (26).

[2/3-^{15}N]Phosphocreatinine (24/25) was prepared from [2/3-^{15}N]creatinine (22/23) in the same manner as [2-^{15}N]phosphocreatinine (22) in a yield of 31.8%.

[2-^{15}N]Phosphocreatine(26) was synthesized using a slight modification of the procedure of Iyengar et al. (Iyengar, 1984) by mild hydrolysis of 320 mL of 8 mM [2-^{15}N]phosphocreatinine (24) (2.55 mmol) in 30 mM glycine buffer (pH 9.0). This solution was incubated at 37°C for 24 hr. The sample was purified by anion-exchange chromatography at 4°C on the Dowex anion exchange column by using first a 250 mL linear gradient of 0-0.3 M TEA/HCO_3^- (pH 7.8) and then 200 mL of 0.3 M TEA/HCO_3^- as eluent. Fractions were assayed for acid-labile phosphates according

to the procedure of Ames (Ames, 1966). Phosphocreatinine is eluted from the column at 0.3 M TEA/HCO_3^- under these conditions. The pooled fractions were concentrated under vacuum below 30°C to remove water and buffer, then stored at 0°C (655 μmol, 25.7% yield). The product was identified as [2-^{15}N]phosphocreatine **(26)** by comparison of the ^{31}P chemical shift at pH 9.0 (d, 3.1 δ upfield from a 0.85% H_3PO_4 external reference; J_{NP}= 18 Hz) with that for the unlabeled phosphocreatine, by co-spotting with unlabeled phosphocreatine on silica gel TLC with BuOH/HOAc/H_2O(2/1/1) (Rf=0.34) (Shoptaugh, 1978) and by enzymatic reaction with ADP in the reaction catalyzed by creatine kinase. The product was contaminated by a small amount of glycine buffer but this did not interfere with quantitation, enzymatic reactions, ^{31}P-NMR, or ^{15}N-NMR spectra.

[2/3-^{15}N]Phosphocreatine (26/27) (Method I) was prepared from [2/3-^{15}N]phosphocreatinine **(26/27)** in the same manner as [2-^{15}N]phosphocreatinine in an overall yield of 3.3%.

[2/3-^{15}N]Phosphocreatine (26/27) (Method II) was synthesized by enzyme-catalyzed positional isotope exchange of [2-^{15}N]phosphocreatine **(26)**. [2-^{15}N]Phosphocreatine **(26)** (350 μmol) was dissolved in 35 mL of 6.0 mM ADP, 15.2 mM ATP, 20.5 mM $Mg(OAc)_2$ and 50 mM Tris/HOAc. Enzymatic reaction was initiated by the addition of 1225 units of creatine kinase. It was estimated from data reported in the literature (Lawson, 1979), (Morrison, 1965) that equilibrium would be achieved in 2-3 hr after 50% of the initial phosphocreatine was consumed. After 8 hr, the reaction mixture was diluted to 112 mL with H_2O and

poured over a 117 mL slurry of 25% acid-washed charcoal in 91 mL H_2O and filtered through Celite. The sample was purified by anion-exchange chromatography at 4°C by using a 250 mL linear gradient of 0.15-0.3 M TEA/HCO_3^- (pH 7.8) and then 200 mL of 0.3 M TEA/HCO_3^- as eluent. Fractions were assayed for acid-labile phosphates according to the procedure of Ames (Ames, 1966). Phosphocreatine elutes from the column at 0.25 M TEA/HCO_3^- under these conditions. The pooled fractions were concentrated under vacuum below 30°C to remove water and buffer and stored at -20°C. Yield: 75 μmol.

Kinetics of Hydrolysis of Phosphocreatinine

The time course of the hydrolysis of unlabeled phosphocreatinine under conditions listed in "Preparation of Phosphocreatine" (Iyengar, 1985) was analyzed by ^{31}P-NMR spectroscopy. Aliquots of a typical reaction mixture were removed at 30 min intervals, frozen, lyophilized and stored at 0°C until analysis by ^{31}P NMR spectroscopy.

NMR Sample Preparation

[2-^{15}N]Creatine (20), [2-^{15}N]creatinine (22) and [2/3-^{15}N]creatinine (22/23) were dissolved in 0.4 mL of 20% D_2O to give solute concentrations of 0.08, 0.55, and 0.24 M, respectively. The pH of each solution was adjusted with NaOH and/or HCl.

[2-^{15}N]Phosphocreatinine (24), [2/3-^{15}N]phosphocreatinine (24/25), [2-^{15}N]phosphocreatine (26) and [2/3-^{15}N]phosphocreatine (26/27) were dissolved in 0.4 mL of 20% D_2O to concentrations of 80 mM to 1 M, and the pH was adjusted with NaOH.

When necessary, the ^{15}N labeled samples were percolated through columns of Chelex-100 (sodium) into acid-washed vials and lyophilized to prepare samples of 50-200 mM in 0.4 mL 20% D_2O . The 5-mm NMR tubes were made metal-free by soaking overnight in 1/1 concentrated HNO_3/ concentrated H_2SO_4 and rinsing thoroughly with distilled, deionized water.

NMR Measurements

^{15}N NMR spectra were obtained at 24.426 MHz on the UCSF wide-bore 240-MHz spectrometer. A spectral width of 6300 Hz and either 8192 or 16384 data points were used to acquire the free induction decay; a 4 sec time delay between pulses was employed in most cases. An exponential line-broadening factor of 2-10 Hz was applied to the total free induction decay prior to Fourier transformation. Up to 10,000 data acquisitions were collected. Exponential line-broadening of 10-20 Hz was applied in obtaining the spectra for [2-^{15}N]creatine (20) and for [2/3-^{15}N]phosphocreatine (26/27) as the low concentrations made it impossible to obtain good signal-to-noise in a reasonable time period. No proton decoupling was used. Chemical shifts were measured relative to [^{15}N]NH_4NO_3, pH 2.0, used as an external standard.

^{31}P NMR spectra were taken at 97.5 MHz on the UCSF wide-bore 240 MHz spectrometer. A spectral width of 2000 Hz and 8196 data points were used to acquire the free induction decay; a 44° tip angle and no time delay between acquisitions were employed in most cases. These conditions permitted the full relaxation between acquisitions of all nuclei present for quantitation

of relative amounts of phosphocreatinine, phosphocreatine, and inorganic phosphate in each spectrum. An exponential line-broadening factor of 0.5-2.0 Hz was applied to the free induction decay prior to Fourier transformation. When necessary, a double exponential apodization factor of up to 10 was applied to the free induction decay prior to Fourier transformation to improve resolution for peak integration. Chemical shifts were measured relative to an 0.85% solution of H_3PO_4 (in D_2O) as external standard. Typically 500-2000 data acquisitions were collected.

Routine ^1H NMR spectra in 99% D_2O were taken at 80 MHz on a Varian FT-80 spectrometer. High resolution ^1H-NMR spectra in 20% D_2O were measured at 500.04 MHz on a General Electric 500 MHz spectrometer. A spectral width of 6000 Hz and 8194 data points were used to acquire the free induction decay. A 1:3:3:1 solvent suppression sequence (Hore, 1983) with a tip angle of 25° and a τ-value of 370 μsec was used. There was no delay between acquisitions. Typically, four data acquisitions were collected.

Preparation of GC-MS Samples of Creatinine:

The derivatization of creatinine for GC-MS analysis was as described by Bjorkhem et al. (Bjorkhem, 1977). One mg of creatinine in 0.5 mL of MeOH was treated with 0.5 mL of 1,2-epoxypropane and incubated at 70°C for 30 min in a Teflon-sealed screw-capped vial. The solvent was evaporated under $N_{2(g)}$, and then the residue was dissolved in 200 μl of EtOAc and treated with 100 μl trifluoroacetic anhydride for 30 min at 37°C. The solvent

and excess reagent were evaporated under $N_{2(g)}$, and the residue was taken up in one mL of mixed hexanes for GC-MS analysis of the derivatized samples on the UCSF MS-25 mass spectrometer.

Results and Discussion

The syntheses of [2-^{15}N]creatinine (22) and [2-^{15}N]creatine (20) from [^{15}N]NH_4Cl were quite similar to the synthesis of N-methylcreatinine from methylamine (Rowley, 1971). The cyclization of creatine to form creatinine has been reported previously (Edgar, 1932). The synthesis of phosphocreatinine was achieved by direct phosphorylation of creatinine in contrast to the method of Zeile and Meyer, where creatine was used as starting material. It was found that phosphorylation of creatinine did not occur when freshly distilled $POCl_3$ was used; only when one equivalent of water was added to the mixture was the reaction successful with creatinine. The reason for this is not known. However, since the reaction was not accompanied by complete scrambling of the label, it is unlikely that it proceeds through a mechanism that involves ring opening. Instead, it is more likely that either the active species in the phosphorylation is a partial hydrolysis product of $POCl_3$ or that the reaction is catalyzed by HCl released in the hydrolysis of $POCl_3$. Thus, when Zeile and Meyer used creatine as the starting material, creatinine was most likely formed *in situ* by dehydration, followed by phosphorylation.

The hydrolysis of phosphocreatinine in borate buffer, as described by Iyengar and co-workers (Iyengar, 1984), was accompanied by the formation of unwanted side-products. Since

borate is known to form complexes with phosphoryl compounds, the reaction was conducted in glycine buffer. To assess whether changing the buffer affected either the kinetics of the reaction or the ratio of products, the reaction was analyzed using ^{31}P-NMR spectroscopy. The relative concentrations of phosphocreatinine, phosphocreatine, and inorganic phosphate were estimated by integration of the ^{31}P NMR spectra. The results are listed in **Table I**. These concentrations were plotted against time to show relative rates of hydrolysis via ring opening (to form phosphocreatine) and via cleavage of the P-N bond (to form phosphate and creatinine). A first order rate constant for the disappearance of phosphocreatinine of $(1.07 \pm 0.03) \times 10^{-3}$ min^{-1} was determined from a plot of ln [PCrn] vs. time (see **Figure 6**). Phosphocreatine was formed approximately three times faster than creatinine under these conditions. The results were in good agreement with those of Iyengar and co-workers (Iyengar, 1984) for the reaction in borate buffer as monitored by HPLC. Thus, although borate buffer interfered with the isolation of products from preparative hydrolysis of phosphocreatinine, it apparently had no effect on the kinetics of the reaction. This method was used to prepare both [2-^{15}N]phosphocreatine (**26**) and [2/3-^{15}N]phosphocreatine (**26/27**).

Complete scrambling of [2-^{15}N]phosphocreatine was catalyzed by creatine kinase in the presence of MgADP. When phosphocreatine labeled with ^{15}N at N-2 is converted to creatine and ATP, the two guanidino-NH_2 groups may equilibrate via rotation about the carbon-nitrogen bond. The phosphocreatine

Table I

Timecourse of the Hydrolysis of Phosphocreatinine at pH 9.0

Time (hr)	Concentration (mM)			ln[PCrn]
	[PCrn]	[PCr]	[Crn]	
0.0	10.0	0.0	0.0	2.3
1.0	9.3	0.5	0.2	2.2
3.3	8.1	1.3	0.5	2.1
6.1	7.0	2.4	0.6	1.9
9.0	5.7	3.7	0.7	1.7
12.0	4.8	4.2	1.0	1.6
24.0	2.1	6.7	1.2	0.8

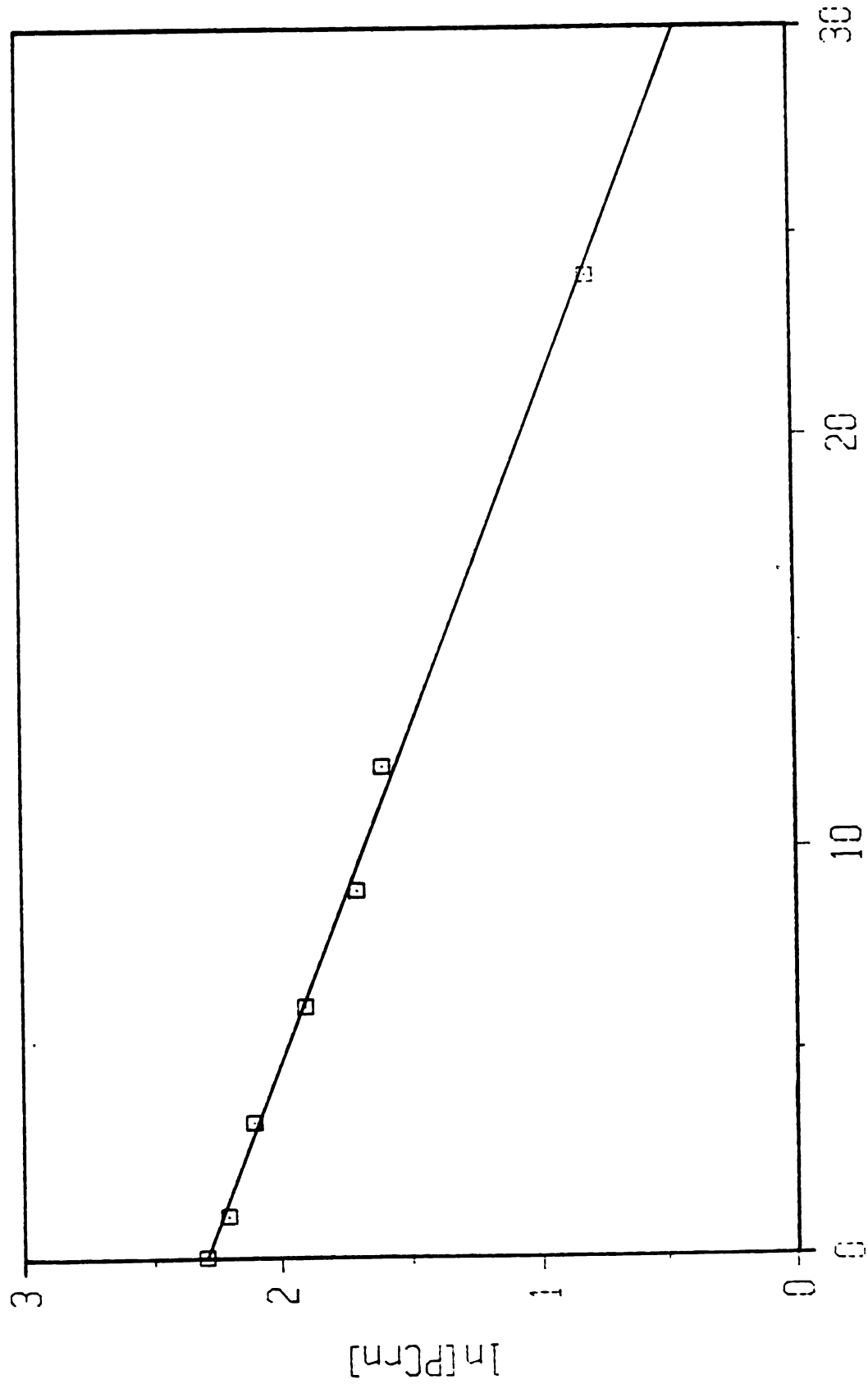


Figure 6 Kinetics of hydrolysis of phosphocreatinine in glycine pH 9.0

resynthesized is labeled with ^{15}N at the N-3 position 50% of the time (Chapter 2). This enzymatic scrambling was used as an alternate synthetic route to [2/3-^{15}N]phosphocreatine.

The ^{15}N NMR data for [2-^{15}N]phosphocreatinine, [2/3-^{15}N]phosphocreatinine, [2-^{15}N]phosphocreatine and [2/3-^{15}N]phosphocreatine are shown in **Table II**. The ^{15}N NMR data for [2-^{15}N]creatinine and [2/3-^{15}N]creatinine at pH 2.0 and 11.0 and for [2-^{15}N]creatine are also included in **Table II**. All peak assignments were made on the basis of comparison of the spectra of the specifically labeled material with the material in which the label is scrambled. The ^{15}N spectra for [2-^{15}N]phosphocreatinine, [2/3-^{15}N]phosphocreatinine, [2-^{15}N]phosphocreatine and [2/3-^{15}N]phosphocreatine are shown in **Figures 7-10**, respectively. The ^{15}N NMR spectra of [2-^{15}N]creatinine and [2/3-^{15}N]creatinine at pH 11.0 and for [2-^{15}N]creatinine and [2/3-^{15}N]creatinine at pH 2.0 are shown in **Figures 11-14**, respectively. The ^{31}P NMR data for [2-^{15}N]phosphocreatinine, [2/3-^{15}N]phosphocreatinine, [2-^{15}N]phosphocreatine and [2/3-^{15}N]phosphocreatine are summarized in **Table III**. The ^{31}P NMR spectra are shown in **Figures 15-18** The ^{1}H NMR data for [2-^{15}N]creatinine, [2/3-^{15}N]creatinine and [2-^{15}N]creatine at low pH are summarized in **Table IV**. The ^{1}H NMR spectra for [2-^{15}N]creatinine, [2/3-^{15}N]creatinine, [2-^{15}N]creatine and for the impurity in [2/3-^{15}N]creatinine are displayed in **Figures 19-22**.

The N-3 resonances for phosphocreatinine and creatinine at high pH are 184.6 ppm and 180.3 ppm, respectively (**Table II**). The nitrogen resonances of unprotonated amides are usually in the

Table II.

^{15}N NMR Spectra of ^{15}N Labeled Compounds

Compound	pH	Chemical Shift (ppm) N-2	Chemical Shift (ppm) N-3	J_{NP} (Hz)	J_{NH} (Hz)
2-^{15}N-phospho-creatinine	9.0	84.7	---	21.4	---
2/3-^{15}N-phospho-creatinine	9.0	84.8	184.6	20.9	---
2-^{15}N-phospho-creatine	9.0	81.2	---	18.3	---
2/3-^{15}N-phospho-creatine	9.0	81.1	57.3	N.D.	---
2-^{15}N-creatinine	11.0	53.4	---	---	---
2-^{15}N-creatinine	2.0	56.3	---	---	93.0
2/3-^{15}N-creatinine	11.0	53.6	180.3	---	---
2/3-^{15}N-creatinine	2.0	55.9	122.9	---	93.0
2-^{15}N-creatine	7.0	56.5	---	N.D.	---

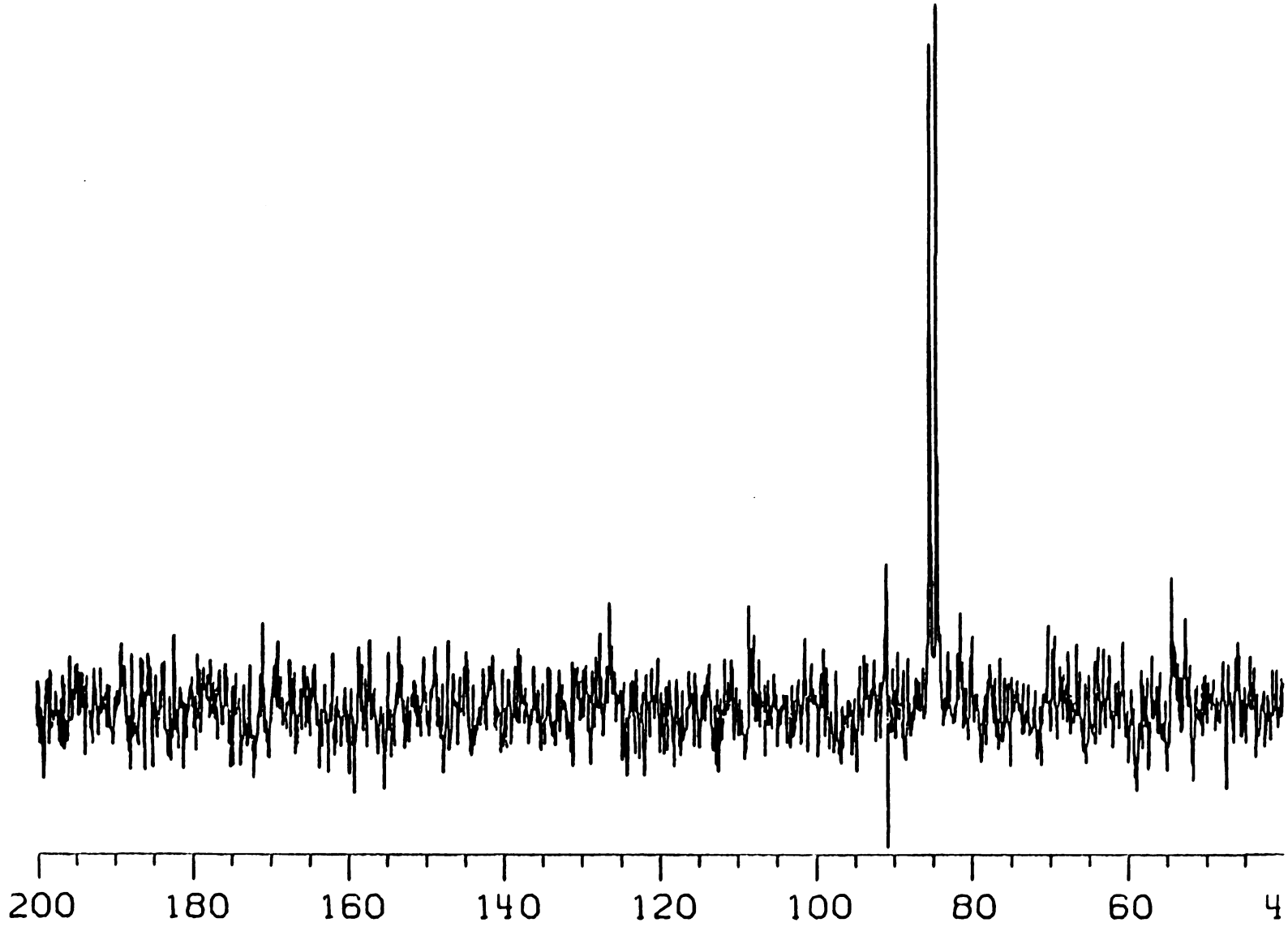


Figure 7 ^{15}N NMR Spectrum of [2-^{15}N]Phosphocreatinine pH 9.0.

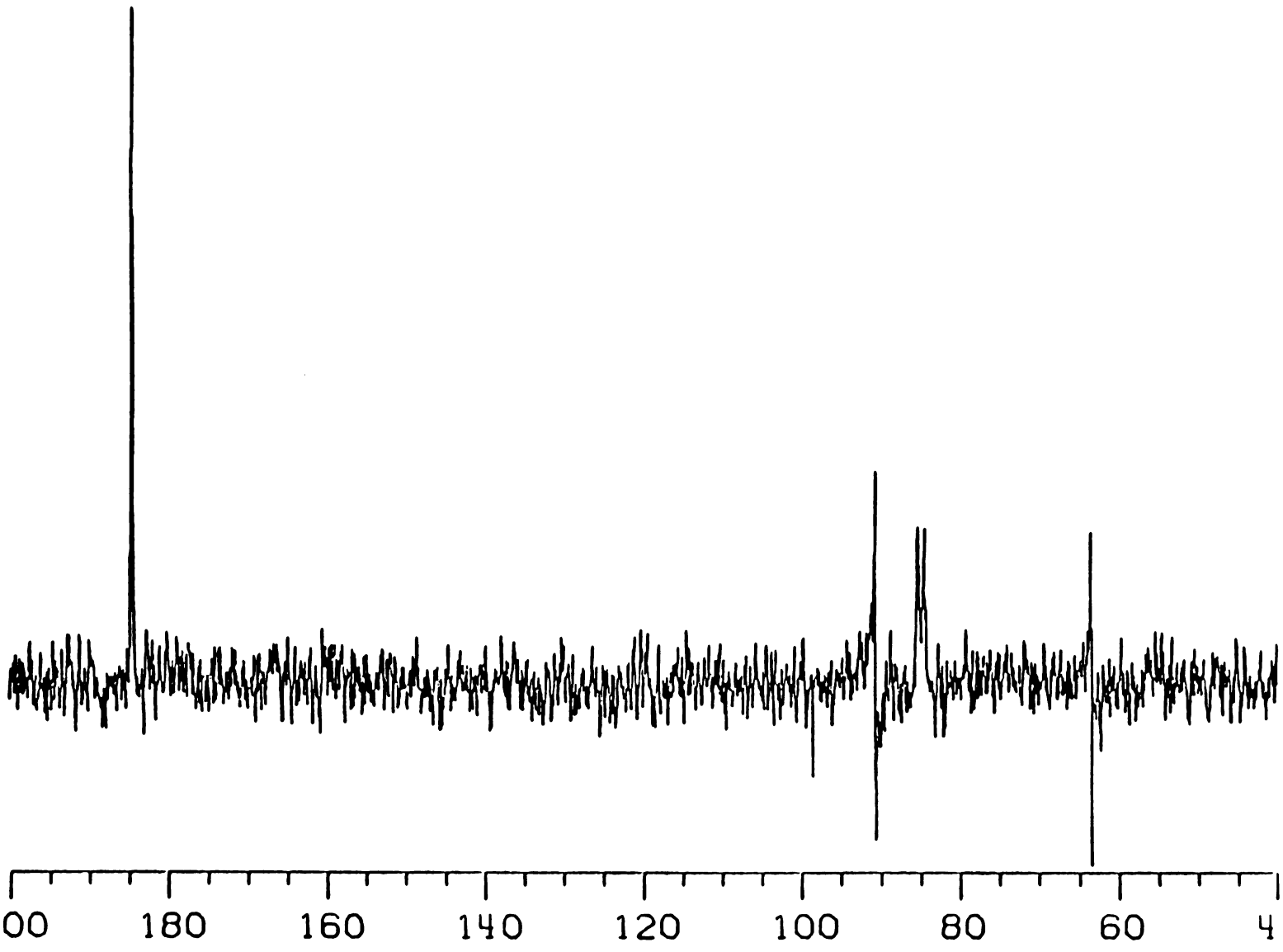


Figure 8 ^{15}N NMR Spectrum of [2/3-^{15}N]Phosphocreatinine pH 9.0.

Figure 9 ^{15}N NMR Spectrum of [2-^{15}N]Phosphocreatine pH 9.0.

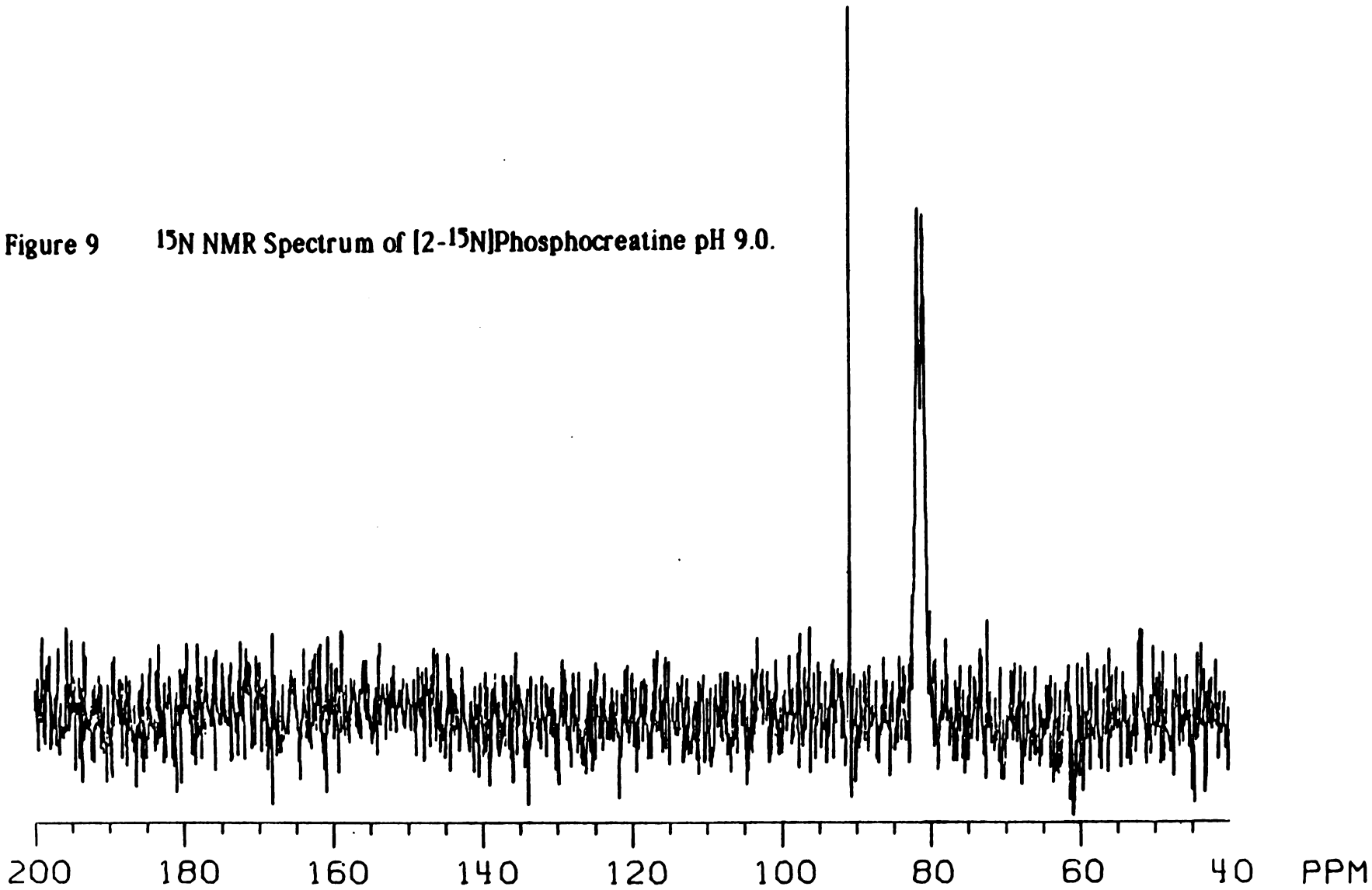


Figure 10 ^{15}N NMR Spectrum of [2/3-^{15}N]Phosphocreatine pH 9.0.

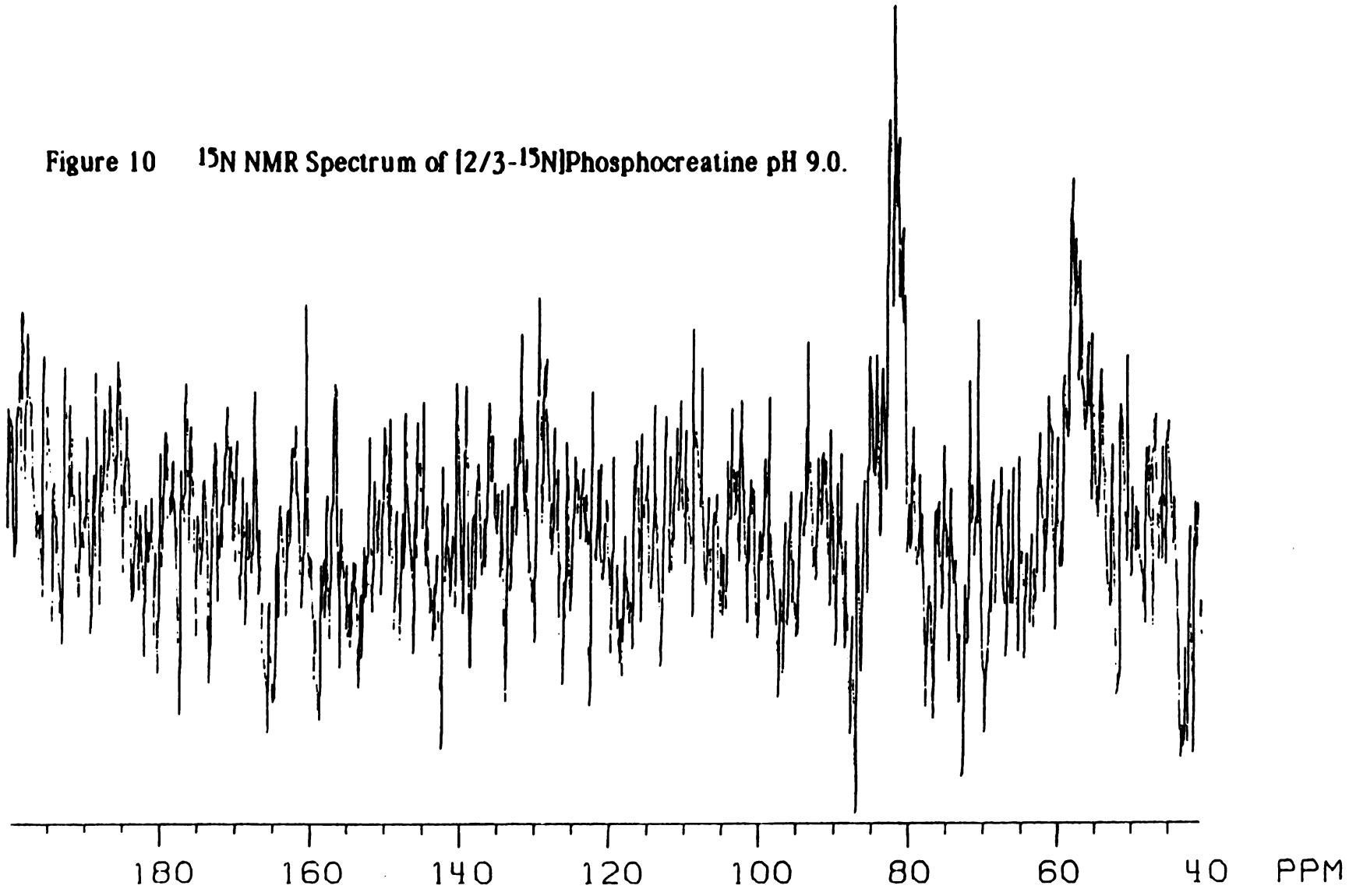


Figure 11 ^{15}N NMR Spectrum of [2-^{15}N]Creatinine pH 11.0.

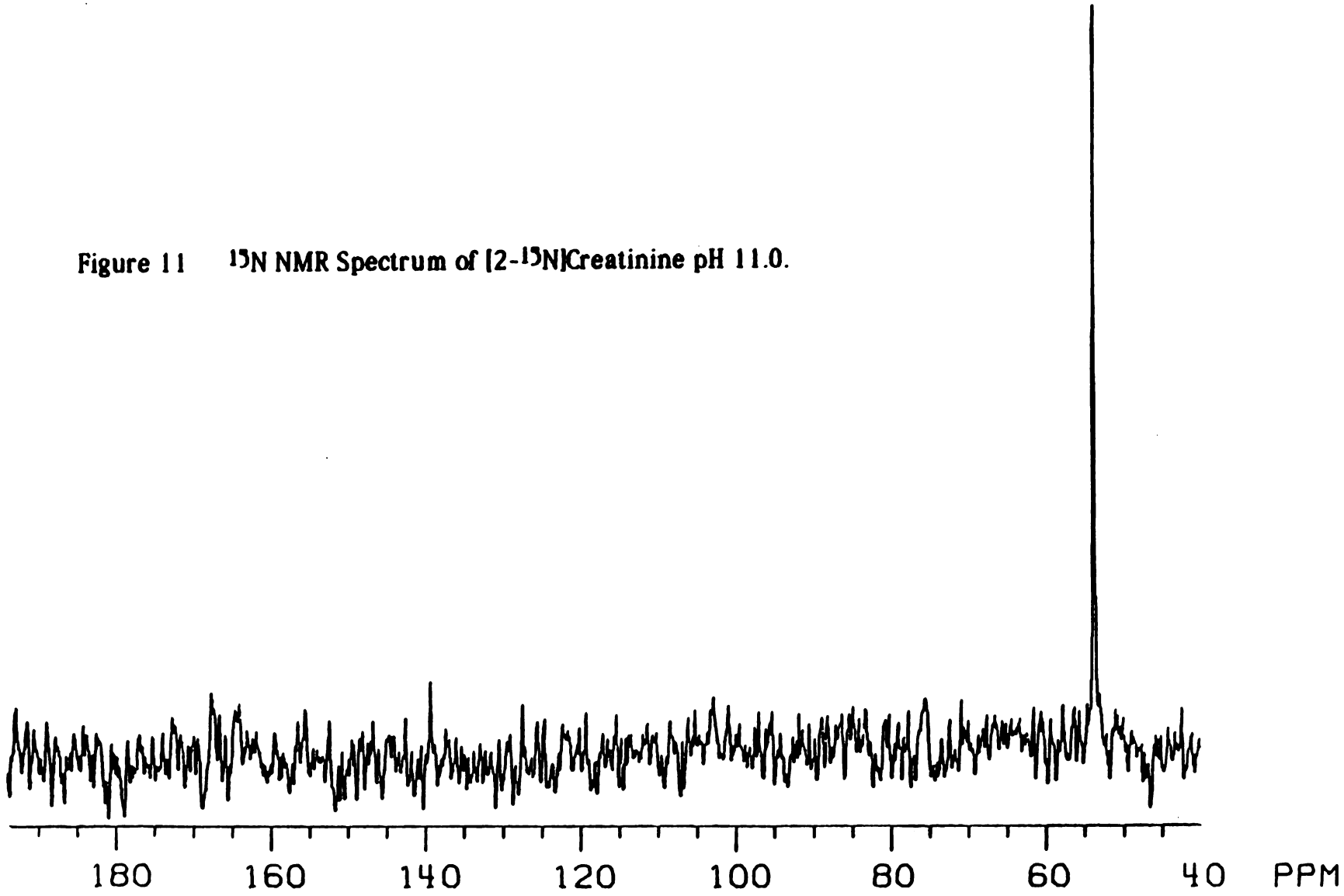


Figure 12 ^{15}N NMR Spectrum of [2/3-^{15}N]Creatinine pH 11.0.

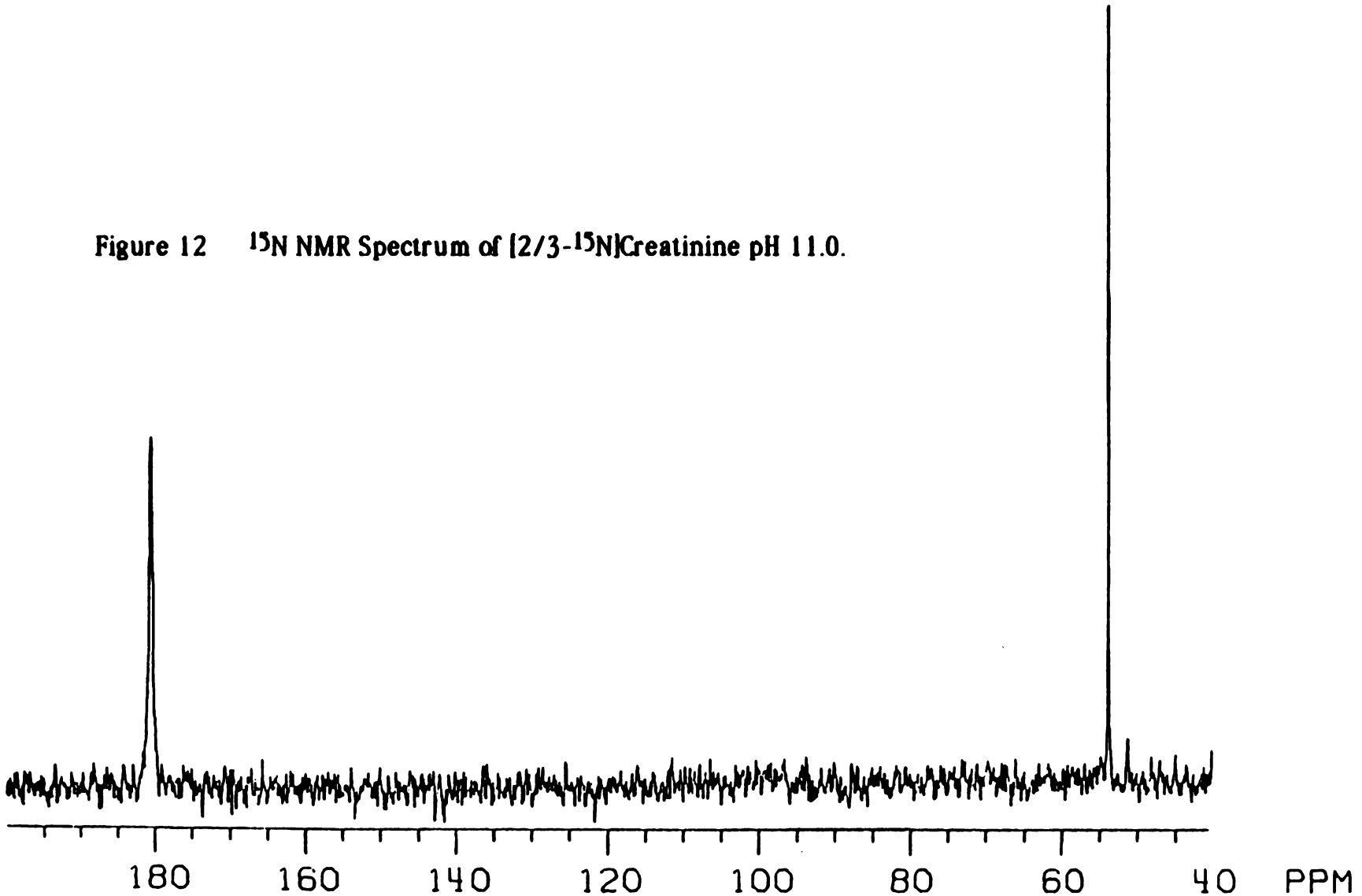
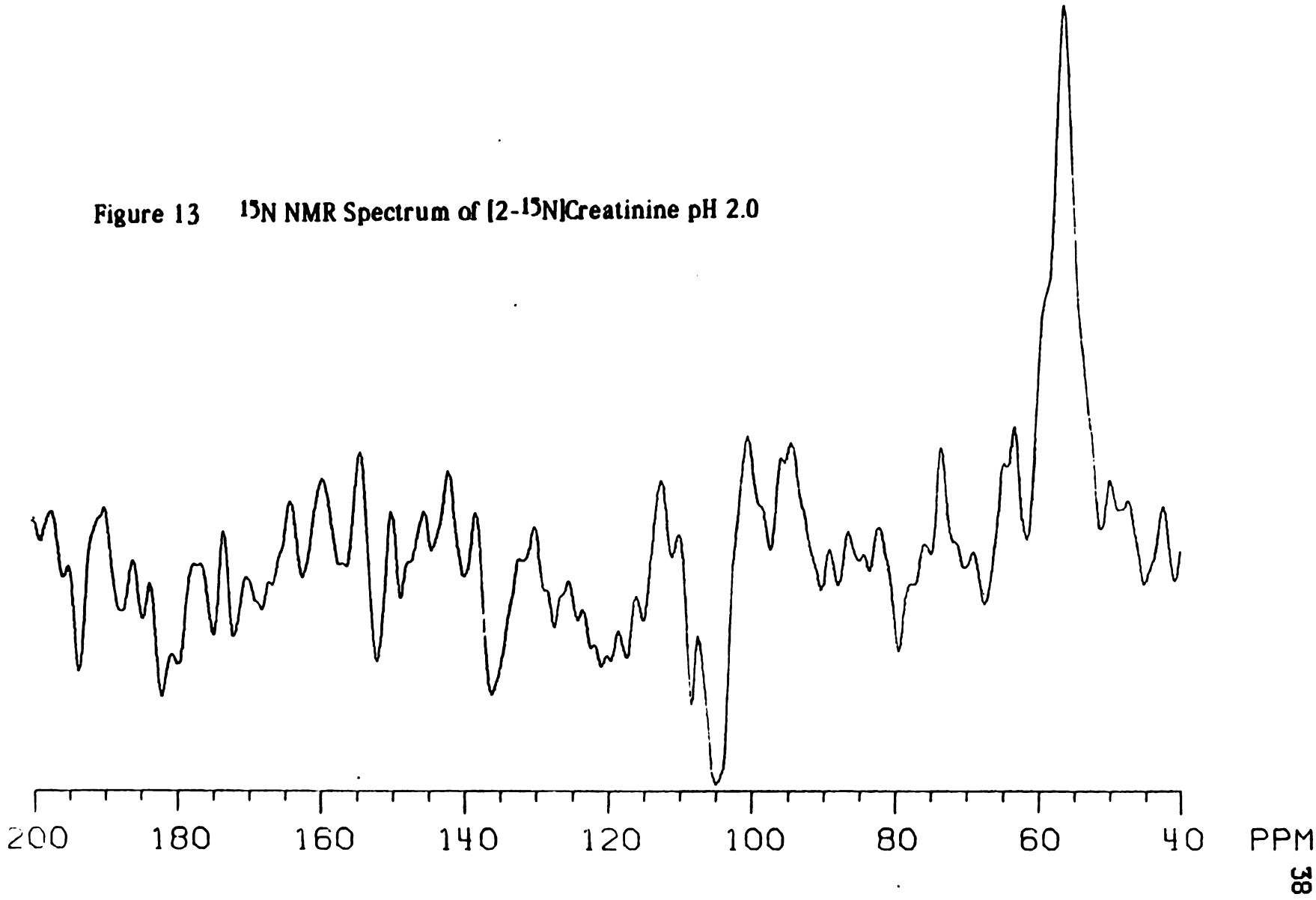


Figure 13 ^{15}N NMR Spectrum of [2-^{15}N]Creatinine pH 2.0



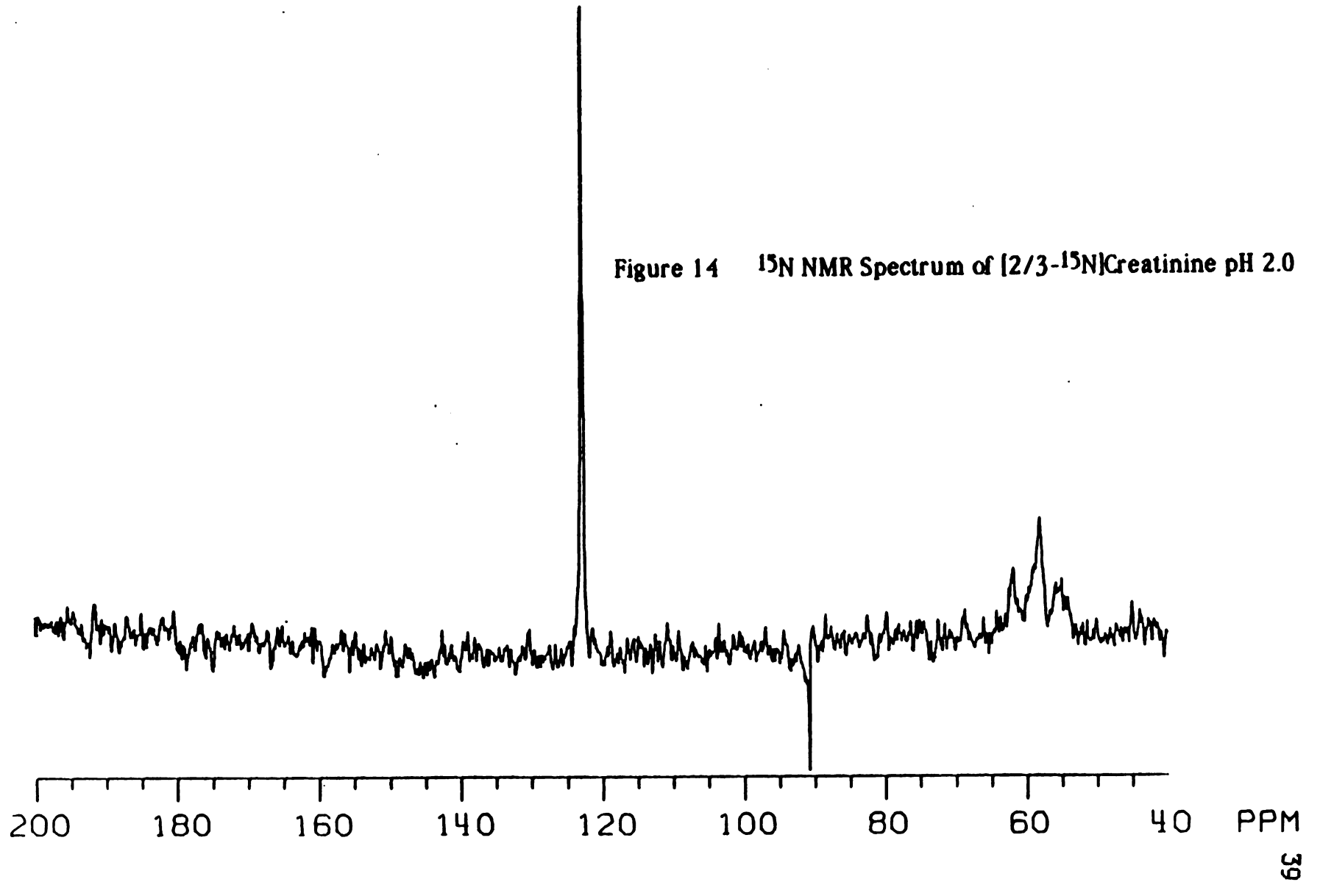


Figure 14 ^{15}N NMR Spectrum of [2/3-^{15}N]Creatinine pH 2.0

200-230 ppm range (Witowski, 1973). The fact that the nitrogens of phosphocreatinine and creatinine have both guanidino and amide character may help to explain the upfield shift as compared to simple amides. The amide characters of N-3 in creatinine and of N-3 in phosphocreatinine are lost upon ring opening. A dramatic upfield shift of over 120 ppm (from 180.3 ppm to 56.5 ppm) is observed in the nitrogen resonance for N-3 of creatinine as compared to the resonance for N-2 in creatine, the ring-opened product. A shift of the same magnitude and direction in the N-3 nitrogen resonances (from 184.6 ppm to 57.3 ppm) accompanies the ring-opening of phosphocreatinine to form phosphocreatine. Since both creatine and phosphocreatine are zwitterionic guanidines, the chemical shifts for N-2 of creatine and N-3 of phosphocreatine should be in the range found for guanidium nitrogens in other zwitterionic guanidines. The nitrogen chemical shifts in argininosuccinate are 69 and 51 ppm for the substituted (N-3) and unsubstituted (N-2) nitrogen resonances, respectively (Raushel, 1984). Thus, the chemical shifts of N-2 in creatine and N-3 in phosphocreatine are consistent with the values observed for similar molecules. Ring opening has only a small effect on the chemical shifts of the N-2 resonances of creatinine and phosphocreatinine. For both transformations the accompanying upfield changes in ^{15}N chemical shifts at N-2 are less than 5 ppm. This reflects the fact that the chemistry at these positions is not altered so dramatically by ring-opening.

No ^{15}N chemical shift values for phosphoguanidines have been published although several phosphoramides have been studied using

^{15}N NMR. We report for the first time the direct measurement of ^{15}N NMR resonances and one-bond ^{31}P-^{15}N coupling constants of phosphoguanidines (Tables II and III). We have observed that when a guanidino nitrogen is directly phosphorylated its ^{15}N resonance is shifted significantly downfield. This can be seen by comparison of the N-2 chemical shifts of creatine and phosphocreatine (Table II). The difference in nitrogen chemical shifts is more than 24 ppm. In contrast, the difference between resonances for N-2 of creatine and for the unphosphorylated nitrogen (N-3) of phosphocreatine is less than 1 ppm. Similar changes in nitrogen resonances accompany the phosphorylation of creatinine to give phosphocreatinine.

Isotope-induced chemical shift changes, such as the ^{18}O-induced shift of about 0.02 ppm per ^{18}O on ^{31}P resonances, are widely known (Cohn, 1982). The magnitude of the chemical shift change depends upon the size of the fractional change in mass upon isotopic substitution (Cohn, 1982). Therefore, the chemical shift change induced by ^{15}N on phosphorus resonances should be less than 0.02 ppm per ^{15}N-^{31}P. In fact, an upfield chemical shift change of 0.01 ppm does appear in the spectrum of [2/3-^{15}N]phosphocreatine (Figure 16). There, the phosphorus resonance appears as a triplet due to the superposition of the singlet due to ^{31}P bound to ^{14}N upon the doublet due to ^{31}P bound to ^{15}N. However, the triplet is slightly asymmetric because of the ^{15}N induced chemical shift change. The same phenomenon is observed in the spectrum of [2/3-^{15}N]phosphocreatinine (Figure 15). Although some isotope-induced chemical shift change might

Table III.

^{31}P NMR Spectra of ^{15}N Labeled Molecules

Compound	Chemical Shift (ppm)		J_{NP} (Hz)	Isotope-Induced Chemical Shift Changes
	^{14}N-P	^{15}N-P		
2-^{15}N-phosphocreatinine	-4.03	-4.04 (minor)	20.2	0.01
2/3-^{15}N-phosphocreatinine	-4.08	-4.09	20.2	0.01
2-^{15}N-phosphocreatine	-3.26	-3.27 (minor)	18.2	0.01
2/3-^{15}N-phosphocreatine	-3.27	-3.28	18.3	0.01

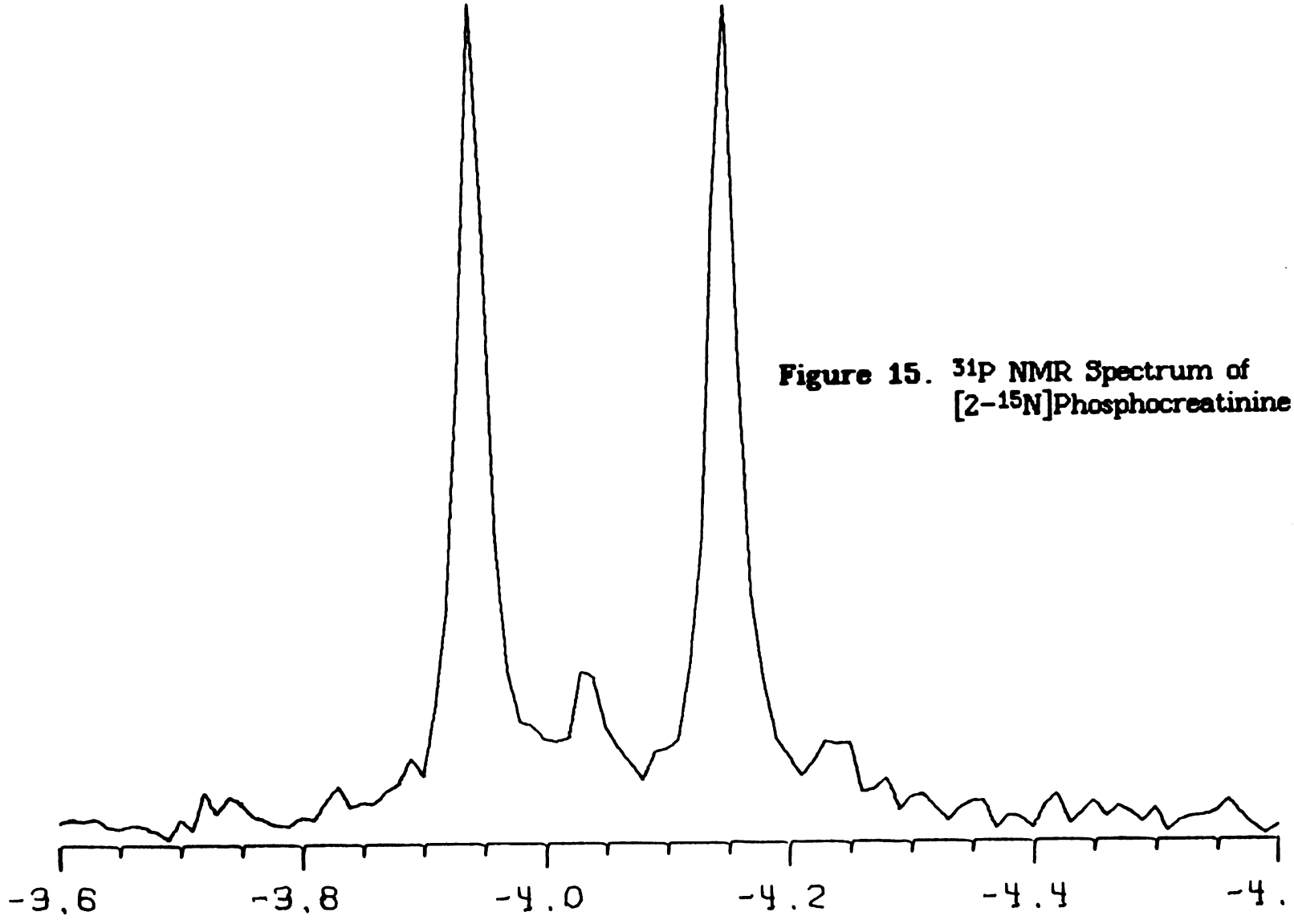
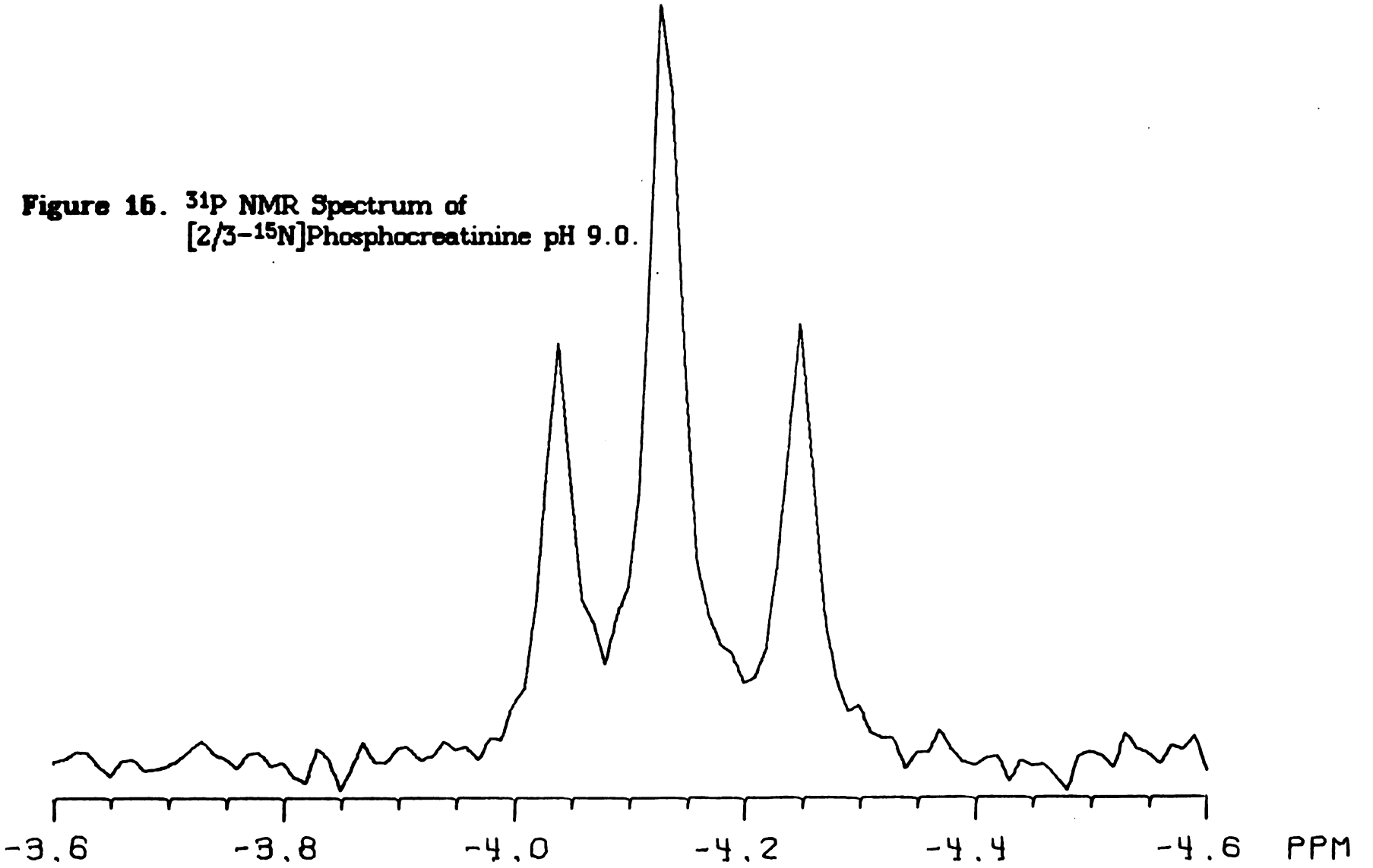


Figure 15. ^{31}P NMR Spectrum of [2-^{15}N]Phosphocreatinine pH 9.0.

Figure 16. ^{31}P NMR Spectrum of [2/3-^{15}N]Phosphocreatinine pH 9.0.



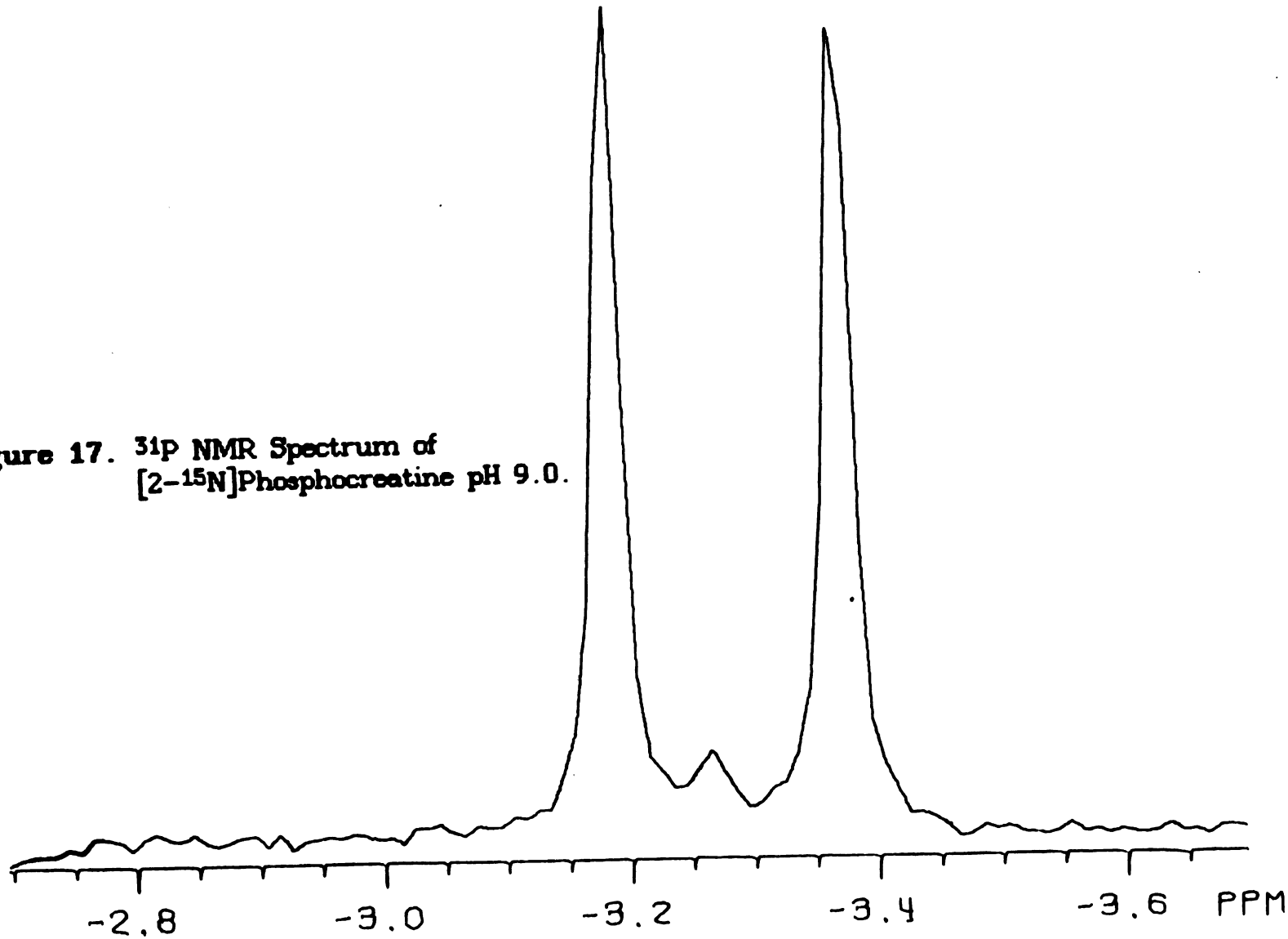
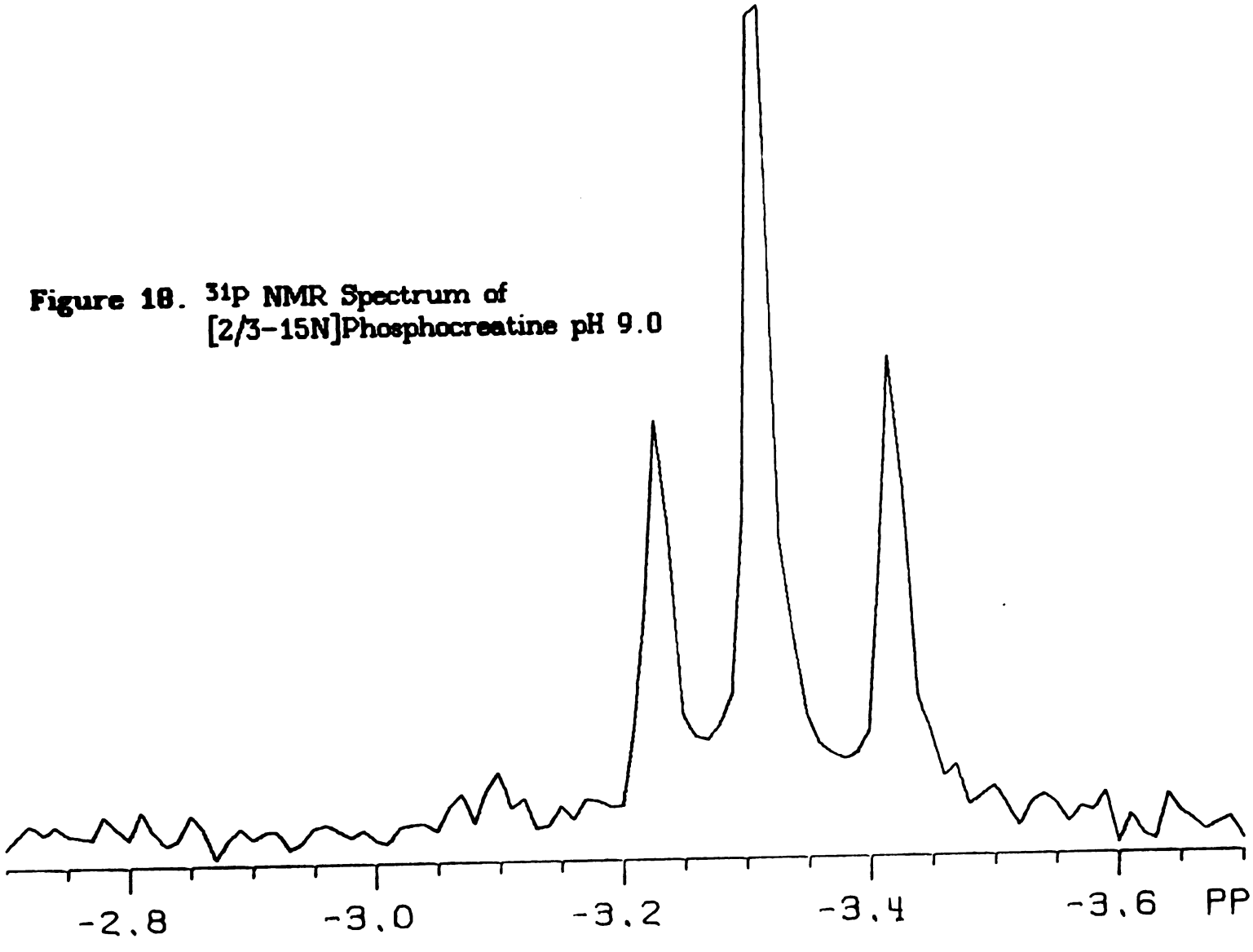


Figure 17. ^{31}P NMR Spectrum of [2-^{15}N]Phosphocreatine pH 9.0.

Figure 18. ^{31}P NMR Spectrum of [2/3-15N]Phosphocreatine pH 9.0



be expected for ^{1}H bonded directly to ^{15}N, none was detected. This is probably so because the magnitude of the shift is also a function of the range of shifts of the resonant nucleus (Cohn, 1982). ^{1}H has a very small range of shifts (about 12 ppm) compared to other nuclei for which such effects have been observed. For example, the chemical shift ranges for ^{31}P and ^{15}N are about 320 ppm and 1000 ppm, respectively (Witowski, 1973).

In the ^{1}H NMR spectrum of [2-^{15}N]creatinine (Figure 19), the signal for the guanidino-NH_2 protons at 8.5 ppm appears as a doublet as a result of the one-bond ^{15}N-^{1}H coupling constant of 93 Hz. The resonance for protons bound to N-2 in [2/3-^{15}N]creatinine (Figure 20) is an apparent triplet formed by the superposition of the singlet due to protons bonded to ^{14}N at N-2 upon the doublet due to protons bonded to ^{15}N at N-2. Pronounced line-broadening of the resonance of ^{14}N is produced by the presence of an electric quadrupole moment in that nucleus. The same quadrupole moment can also produce line-broadening in the spectra of the neighboring nuclei (Witowski, 1973). Because of this effect of the electric quadrupole, the resonance of ^{1}H bound to ^{14}N would be expected to be much broader than the same proton bound to ^{15}N. In fact, a large line-broadening due to ^{14}N is evident in the proton NMR spectrum of [2/3-^{15}N]creatinine. In this spectrum, the center peak of the apparent triplet is obviously much broader than the outer peaks. This is so because the narrower outer peaks are a doublet due to protons bound to ^{15}N, whereas the broad center peak is due to protons bound to ^{14}N. The same type of line-broadening induced by an ^{14}N quadrupolar

Table IV

^{1}H NMR Spectra of ^{15}N-Labeled Creatine and Creatinines

Compound	pH	Chemical Shift (ppm)	J_{NH}(Hz)
2-^{15}N-creatinine	2	8.5	93
2/3-^{15}N-creatinine	2	8.5	93
2-^{15}N-creatine	7	6.8	92
impurity in 2/3-^{15}N-creatinine	2	6.7	92

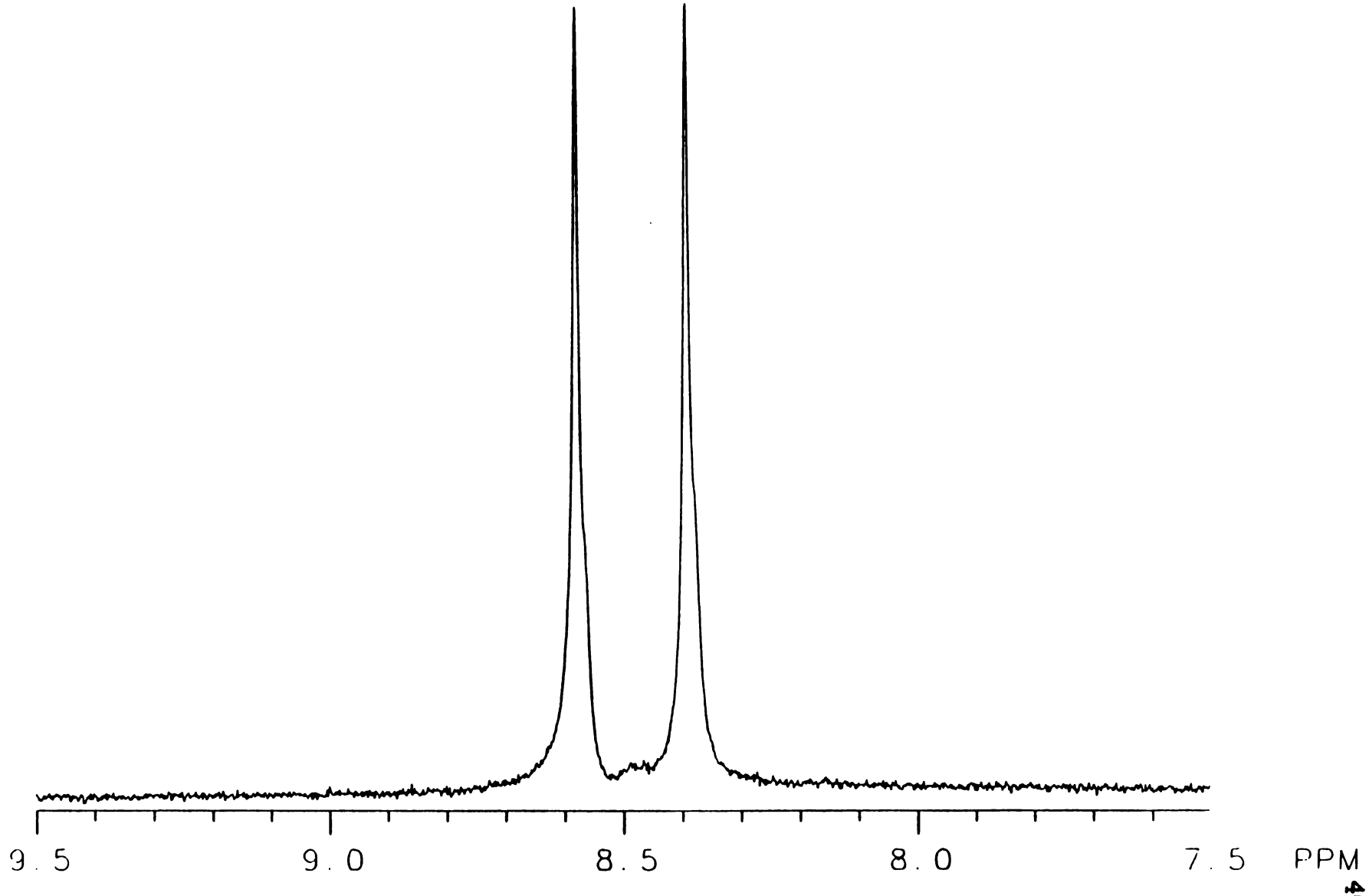


Figure 19 ^{1}H NMR Spectrum of [2-^{15}N]Creatinine pH 2.0.

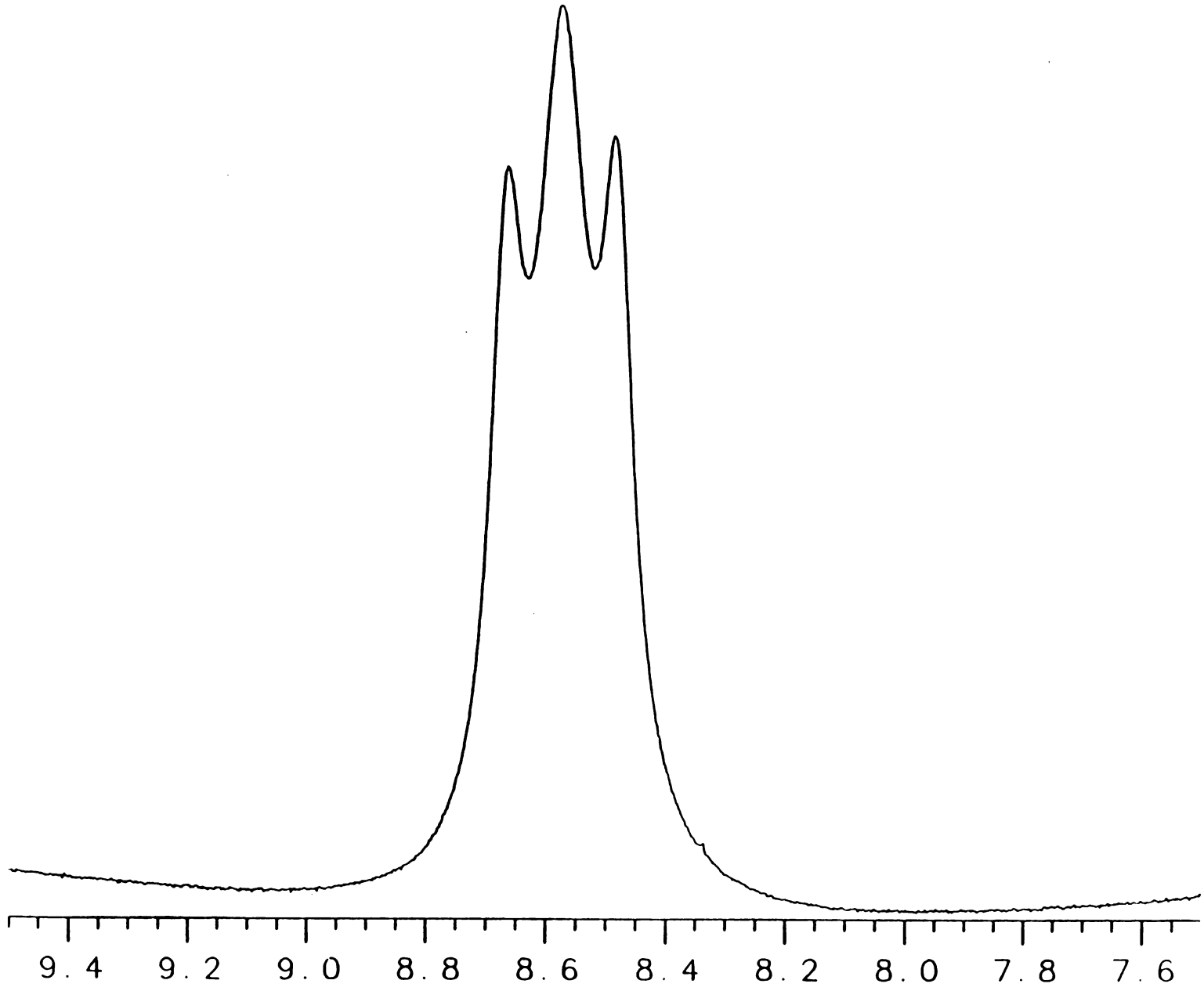


Figure 20 ^{1}H NMR Spectrum of [2/3-^{15}N]Creatinine pH 2.0.

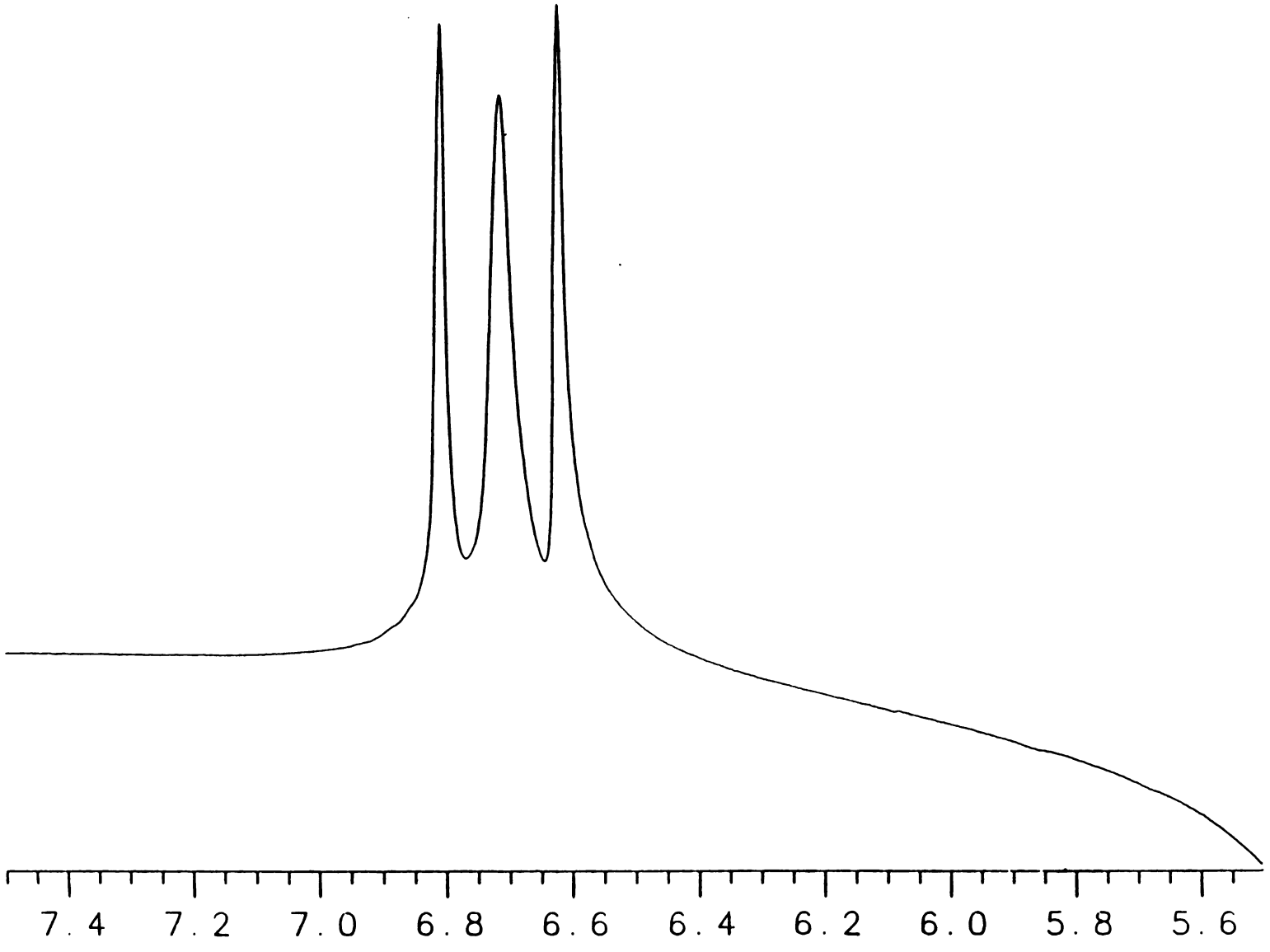


Figure 21 ^{1}H NMR Spectrum of Impurity in [2/3-^{15}N]Creatinine pH 2.0.

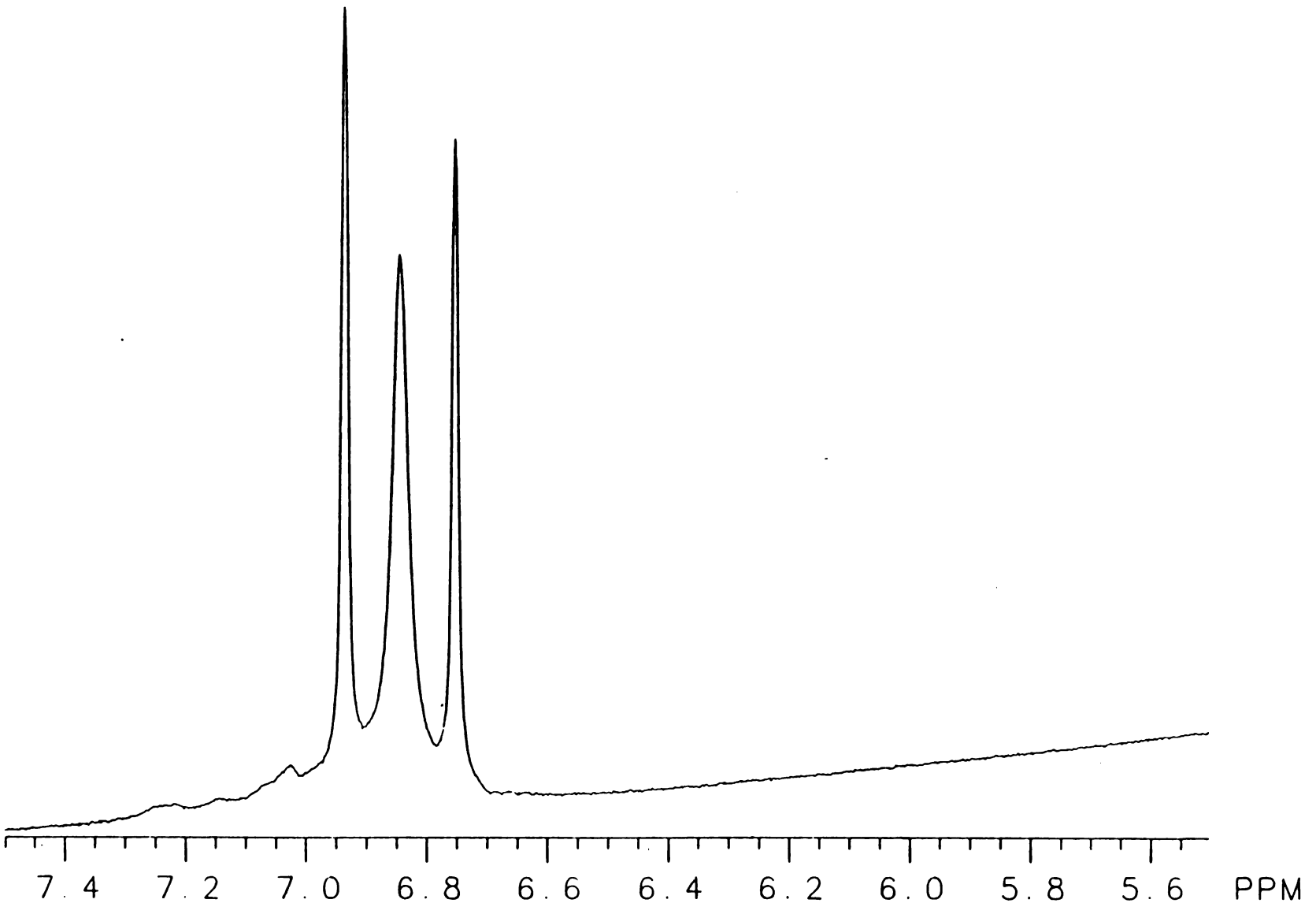


Figure 22 ^{1}H NMR Spectrum of [2-^{15}N]Creatine pH 7.0

nucleus occurs in creatine, but to a lesser extent than in creatinine.

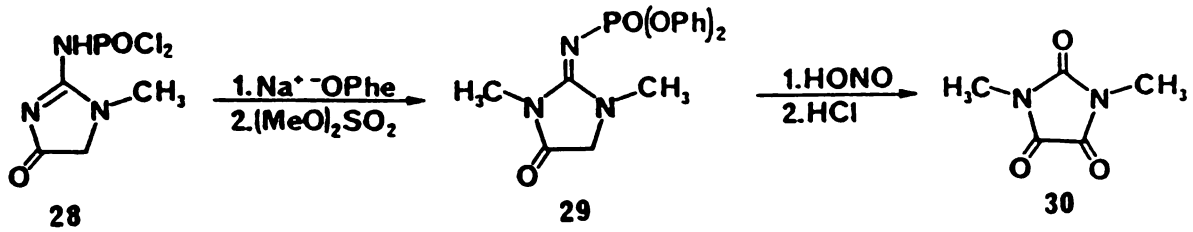
No large line-broadening of phosphorus resonances in phosphocreatine or phosphocreatinine was produced by ^{14}N. Even so, this effect of ^{14}N may be partially responsible for difficulties experienced in obtaining complete resolution of the phosphorus resonances of [2/3-^{15}N]phosphocreatine and [2/3-^{15}N]phosphocreatinine.

The ^{1}H NMR spectrum of [2/3-^{15}N]creatinine contained a small amount of material giving an apparent triplet at 6.8 ppm (**Figure 21**), similar to the pattern at 8.5 ppm. However, the intensity of this apparent triplet was less than one-half the intensity of the resonance at 8.5 ppm for protons bound to N-2 of [2/3-^{15}N]creatinine. Even when the 1:3:3:1 pulse sequence was altered so that the relative signal at 6.8 ppm was maximized, the intensity remained much smaller. Therefore, the peaks were not due to any protons bound to ^{15}N at N-3. Instead, the impurity was identified as creatine by comparison with the ^{1}H-NMR of [2-^{15}N]creatine (**Figure 22**). The ^{1}H NMR spectrum of [2-^{15}N]creatine at pH 7.0 is an apparent triplet formed by the superposition of the singlet due to protons bonded to ^{14}N at N-2 upon the doublet due to protons bonded to ^{15}N at N-2 at 6.7 ppm.

Phosphocreatinine (4/5)(**Figure 2**) is an inhibitor of the reaction catalyzed by creatine kinase with K_i= 5 mM compared to a K_m= 0.5 mM for creatine (Gercken, 1974). However, a related compound, 1-carboxymethyl-2-iminoimidazolidine (cyclocreatine) (6)(**Figure 3**), is a substrate analog with a V_{max} 90% of that for

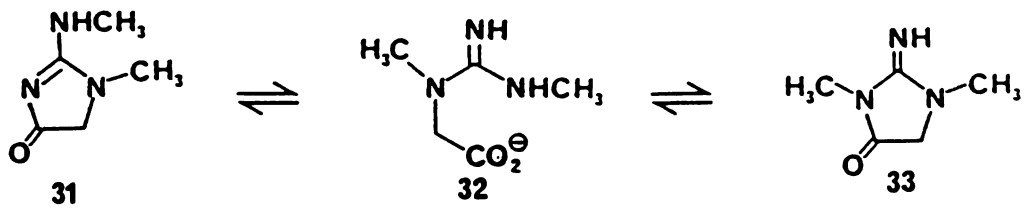
creatine (1) (McLaughlin, 1972)(Rowley, 1971). Therefore, it is of considerable interest to establish the sites of phosphorylation in both phosphocreatinine (5) and phosphocyclocreatine (7)(Figure 3), the product of the creatine kinase-catalyzed phosphorylation of cyclocreatine (6). The structure of phosphocyclocreatine was correctly established by NMR studies (Struve, 1977) and by X-ray crystallographic determination (Quiocho, 1979) after initially being incorrectly deduced from ^1H NMR data (Rowley, 1971). Zeile and Meyer (Zeile, 1938) established the structure of phosphocreatinine by the degradation of the acid dichloride of phosphocreatinine outlined in Scheme III. The ultimate product in this series of steps is dimethylparabanic acid (30). However, this proof is not unequivocal.

Zeile and Meyer employed a similar degradative method to determine that 1,3-dimethyl-2-imidazolidin-4-one (33) is the methylcreatinine isomer produced when creatinine is treated with dimethyl sulfate. Later, it was shown that 1,3-dimethyl-2-imidazolidin-4-one hydrolyzes spontaneously in water to generate the acyclic creatine analog (32) (Kenyon, 1971). Kenyon and Rowley noted that if this reaction provided a pathway for the interconversion of methylcreatinine isomers (Scheme IVa) under the conditions used by Zeile and Meyer, their structure proof would be negated. An independent synthesis of the isomeric methylcreatinine (31) ultimately proved that such an isomerization does not occur in the methylcreatinines. Nevertheless, a similar uncertainty still surrounds the existing structure proof for phosphocreatinine. If the structure of



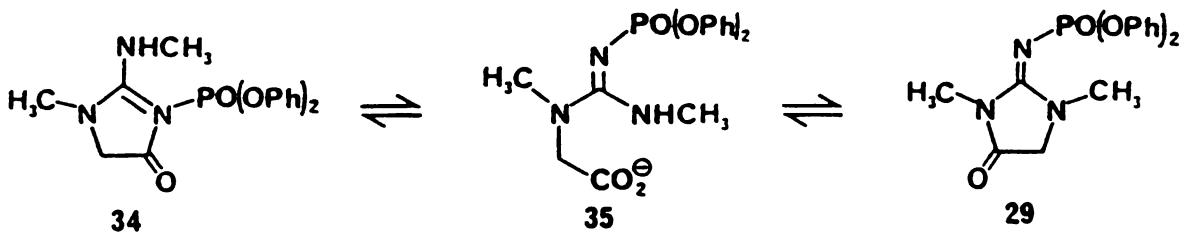
28 29 30

Scheme III Chemical degradation of the acid dichloride of phosphocreatinine to dimethylparabanic acid.



31 32 33

a



34 35 29

b

Scheme IV Isomerization of (a) methylcreatinine (b) methylcreatinine phosphate diphenyl ester.

phosphocreatinine with phosphorus at N-3 were correct, the isomeric methyldiphenylphosphocreatinine (34) shown in Scheme IVb would be an intermediate in the degradative phosphocreatinine structure proof of Zeile and Meyer. If this intermediate underwent the hydrolysis to the acyclic phosphocreatine analog (35), bond rotation, and recyclization outlined in (Scheme IVb), the isomer with an exocyclic phosphoryl group (29) would be produced. Dimethylparabanic acid (30) would still be the end-product of the degradative pathway. Therefore, the existing structure proof for phosphocreatinine is inadequate. We report the unequivocal determination of the site of phosphorylation in this molecule, based on the presence of the one-bond $^{15}N-^{31}P$ coupling constant in phosphocreatinine specifically enriched in ^{15}N at N-2 and on the absence of such coupling in phosphocreatinine enriched in ^{15}N at N-3. The data in Tables II and III were used in this determination.

Upon phosphorylation of creatinine to give phosphocreatinine, the N-2 resonance is shifted downfield by approximately 30 ppm (from 53.6 ppm to 84.8 ppm) and is a doublet due to the one-bond $^{15}N-^{31}P$ coupling constant of 21 Hz (Table II). In contrast, the resonance of N-3 is shifted downfield by only approximately 5 ppm (from 180.3 ppm to 184.6 ppm) and is observed as a singlet. Since the large chemical shift change in the ^{15}N resonance and the $^{31}P-^{15}N$ coupling are observed exclusively for N-2 and not for N-3, these results are consistent only with a structure for phosphocreatinine in which the phosphorus is bonded directly to N-2. Corroborating evidence is contained in the ^{31}P NMR spectra of

[2-^{15}N]phosphocreatinine and [2/3-^{15}N]phosphocreatinine which are shown in **Figures 15** and **16**. At pH 9.0, the phosphorus resonance of [2-^{15}N]phosphocreatinine appears as a doublet at 3.2 ppm, as the result of the 20 Hz one-bond ^{15}N-^{31}P coupling constant. The phosphorus resonance of [2/3-^{15}N]phosphocreatinine appears as a triplet formed by the superposition of the signal due to the ^{31}P bound to ^{14}N upon the signal due to ^{31}P bound to ^{15}N. The one-bond heteronuclear coupling between ^{15}N and ^{31}P is observed only when ^{15}N is at N-2. This confirms unequivocally the conclusions of Zeile and Meyer (Zeile, 1938) based on degradation studies and proves that the site of phosphorylation in phosphocreatinine **(4)** is opposite to the analogous site of phosphorylation in phosphocyclocreatine **(7)**.

With the structures of phosphocreatinine and phosphocyclocreatine unequivocally established, more detailed questions can be asked about the specificity and stereoselectivity of the reaction catalyzed by creatine kinase. The lack of reactivity for phosphocreatinine and creatinine as substrates for creatine kinase has been attributed to the lack of a carboxyl moiety in these molecules rather than on any other differential properties with the normal substrates (Gercken, 1974). However, since the site of phosphorylation in phosphocyclocreatine is opposite to the analogous site in phosphocreatinine, the lack of reactivity in creatinine could also be attributed to unfavorable positioning of the exocyclic nitrogen (N-3) and to lower nucleophilicity of the nitrogens conjugated to the ring carbonyl (N-2 and N-3).

All of the numerous analogs examined to date ((Rowley, 1971) (McLaughlin, 1976) (Dietrich, 1980) (Nguyen, 1984) (Gercken, , 1974) contain a carboxyl group, except for the glycocyamidine derivatives. Absence of activity in these glycocyamidines might also be explained in the same way as for phosphocreatinine and creatinine. In fact, substitution of a carboxyl group for the methyl of creatinine(as in 1-carboxymethyl-2-iminoimidazolidine-4-one) does not restore substrate activity lost on cyclization (**Figure 23**). Thus, the conclusion of Gercken that the carboxyl group is necessary for activity has not been completely established. Substitutions of the carboxyl group with other charged groups have been made. However, these substitutions have established only a tolerance, not a need, for a negatively charged group at this site. The apparent dependence of activity on the stereochemical position of the carboxyl group (Rowley, 1971) (McLaughlin, 1976) (Dietrich, 1980) might be produced by steric effects alone. Although the carboxyl may be important, this fact remains to be established experimentally. N-Ethyl-N-methylguanidine and N,N'-ethylene-N-methylguanidine represent the smallest possible perturbations of the creatine system in which the carboxyl is omitted. Testing these compounds for activity might help to establish the role of the carboxyl group in the specificity of the creatine kinase reaction.

One prerequisite for the use of specifically labeled [2-^{15}N]phosphocreatine (**8**) as a probe of the creatine kinase catalyzed reaction is that ^{14}N/^{15}N positional isotope exchange must not occur by itself (Chapter 2) (**Scheme I**). Therefore, we sought to

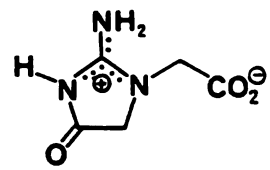


Figure 23 Structure of 1-carboxymethyl-2-iminoimidazolidine-4-one.

determine whether intramolecular transfer of the highly reactive phosphoryl group of phosphocreatine would occur in aqueous solution. Although this process would not seem likely based on examination of molecular models of phosphocreatine, i.e., a four-membered ring transition state would be required for attack of the $-NH_2$ on the phosphoryl group, related intramolecular phosphoryl transfers have been proposed. In the case of phosphoenolpyruvate, an intramolecular phosphoryl transfer requiring a five-membered ring transition state was shown to explain the observation of unusually facile exchanges of the carboxyl and phosphoryl oxygens (O'Neal, 1983). In the case of phosphocreatine, only specific labeling of the phosphoramide nitrogen (N-2) would allow us to find whether such a reaction occurs. The data in **Tables II and III** were used.

At pH 9.0, the phosphorus resonance in [2-^{15}N]phosphocreatine is a doublet at 4.0 ppm as the result of the one-bond ^{15}N-^{31}P coupling constant of 18 Hz (**Figure 17**). A small peak, which integrates for 6.3± 3.0% of the total peak area, appears slightly off center of the doublet. Thus, some scrambled material is present in the product. However, in the spectrum of [2-^{15}N]phosphocreatinine (**Figure 15**), a small peak, which integrates for 6.3± 3.0% of the total peak area also appears slightly off center of the doublet. Thus, the same amount of scrambled material is present in the [2-^{15}N]phosphocreatinine from which the [2-^{15}N]phosphocreatine was prepared, and the requirement that $^{15}N/^{14}N$ positional isotope exchange does not occur in [2-^{15}N]phosphocreatine (26) by itself is met.

We then sought to determine the ultimate source of the scrambling in [2-^{15}N]phosphocreatinine. One possibility was that, if the precursor to [2-^{15}N]creatinine (22) (Scheme II) were not competely cyclized, a small amount of [2-^{15}N]creatine (20) would be formed in the synthesis of the [2-^{15}N]creatinine. If this material were present during the phosphorylation step, some of the phosphocreatinine formed would be scrambled. However, there was no evidence of contaminating [2-^{15}N]creatine in the samples of [2-^{15}N]creatinine as judged by ^{1}H NMR. Instead, the spectrum exhibited a small broad peak in the center of the doublet due to the presence of ^{14}N at N-2 (Figure 19). Accurate integration is difficult because the resonance of these protons is broadened by the quadrupolar ^{14}N nucleus and because the use of the 1:3:3:1 pulse sequence leads to distortion of peak phase and intensity in a chemical shift dependent way. Nevertheless, it is evident that there is a small amount of ^{14}N at N-2. This small amount of creatinine with ^{14}N at N-2 in the supply of [2-^{15}N]creatinine was probably responsible for the apparent scrambling in [2-^{15}N]phosphocreatinine.

Two sets of circumstances would lead to a small amount of ^{14}N at N-2 in [2-^{15}N]creatinine. The first is an isotopic enrichment in ^{15}N lower than that expected on the basis of the isotopic purity of [^{15}N]NH_4Cl (99% isotopic enrichment). However, this was quickly ruled out by GC-MS analysis of a creatinine derivative which showed that the isotopic purity was 99± 0.5%. The second hypothesis is that creatinine itself undergoes ring-opening and ring-closure reactions under the conditions of its

synthesis and isolation . There is some evidence in the literature to suggest that even at very low pH there is some creatine in equilibrium with creatinine. Such an equilibrium would eventually lead to complete scrambling of the label. In fact, the spectrum of partially purified [2/3-^{15}N]creatinine which was formed by cyclization of [2-^{15}N]creatine in mineral acid did show evidence that creatine was present (Figure 20-22).

The second prerequisite for the use of specifically labeled [2-^{15}N]phosphocreatine as a probe of the creatine kinase-catalyzed reaction (Chapter 2) is that the relative amounts of phosphocreatine labeled at N-2 and at N-3 can be quantitated. If the ^{31}P resonances of [2-^{15}N]phosphocreatine (8) and [3-^{15}N]phosphocreatine (10) can be resolved, then ^{31}P NMR spectroscopy could be used to quantitate them. Recently, an NMR study on the kinetic mechanism of creatine kinase has been published in which J_{NP} for [2,3-$^{15}N_2$]phosphocreatine was found by an indirect method to be 3 Hz (Brindle, 1984). The ^{31}P NMR spectrum of a mixture of [2-^{15}N]phosphocreatine and [3-^{15}N]phosphocreatine is a doublet of width J_{NP} superimposed upon the singlet of [3-^{15}N]phosphocreatine. In addition, an isotope-induced chemical shift change in the ^{31}P NMR resonance in [2-^{15}N]phosphocreatine of about 0.01 ppm (1 Hz at 97 MHz) was expected. Thus, the necessary linewidths could be well under 0.25 Hz. It is unlikely that such resolution could be achieved, especially since some line-broadening of the ^{31}P signals by the ^{14}N quadrupolar nucleus to which it is attached might be expected. Fortunately, however, the spectra in Figures 17 and 18 show

that this coupling constant is 18 Hz. Thus, the needed resolution is much easier to achieve and this analog is useful for the study of the creatine kinase catalyzed reaction.

There is no evidence from the spectrum of the [2/3-^{15}N]phosphocreatine to suggest long-range ^{31}P-^{15}N coupling between the phosphorus and N-3 on the order of 3 Hz. Further, the discrepancy between our observations and the measurement of Brindle et al. (Brindle, 1984) cannot be explained by pH effects since the coupling constant was found to be pH-independent at least down to pH 5.5. A detailed discussion of the reasons for the discrepancy is outside the scope of this work, but some observations can be made. Briefly, the pulse sequence of the technique used to measure J_{NP} involves a delay, τ, between pulses. In this experiment the intensity of the phosphocreatine resonance in a mixture of ^{15}N-labeled and unlabeled material is at a minimum when $\tau = (2n+1)/2J$ (where n=1,2,3,...). If J_{NP}= 18 Hz, then the minimum value of τ which satisfies this condition is 27.8 msec. Minima should also be observed at τ = 83.3, 138.9, 194.4, etc. msec. Brindle and co-workers collected datapoints beginning at a τ-value of about 50 msec and continuing at 15-25 msec intervals thereafter. Thus, it is not surprising that they did not observe minima at e.g., 27.8 and 83.3 msec and that the calculated value of J_{NP} is low. The coupling constant had been determined in order to establish experimental conditions for measuring isotope exchange rates in the reaction catalyzed by creatine kinase. An accurate determination of J_{NP} is not strictly necessary for these investigations. However, optimal spectral

conditions are defined by an equation that is a function of J_{NP}. From an inspection of the equations it is apparent that an incorrect determination of J_{NP} would decrease the accuracy of their measurements by decreasing the signal-to-noise ratios in individual ^{31}P NMR spectra.

The protonation of creatinine could conceivably occur at any one of the three guanidino nitrogens present (**Figure 4**). Two of these tautomers (**11** and **12**) allow conjugation with the carbonyl moiety, but for only one (**11**) is it possible to draw resonance forms for the guanidinium ion (**Figure 5**). A dramatic chemical shift change in the ^{15}N resonance upon protonation is widely accepted as a criterion for experimentally establishing the site of protonation in such systems (Markowski, 1977)(Cain, 1977)(Gonnella, 1983). The data in **Table II** were used to determine this site in creatinine.

The creatinine N-2 resonance is shifted slightly downfield by approximately 3 ppm (from 53.5 ppm to 56.6 ppm) upon protonation, but the resonance of N-3 shifts upfield nearly 60 ppm (from 180.3 ppm to 122.7 ppm)(**Table II**). The large upfield chemical shift change at N-3 is consistent only with protonation at N-3 (**11**), since protonation at either N-1 or N-2 (**12** and **13**) would not be likely to produce much change in the chemical shift of N-3. Thus our results show that creatinine is undoubtedly protonated at N-3 (**11**)

The fact that the shift change observed for N-3 is somewhat less than is observed for the nitrogen in pyridine (97 ppm) may mean either that N-3 is not the exclusive site of protonation or

that the formal positive charge on the N-3 nitrogen (14) is distributed partly by resonance to the exocyclic amino nitrogens (N-1 and N-2)(Figure 5). The small downfield shift of the N-2 resonance is consistent with either partial protonation of N-2 or with its involvement in conjugation with a protonated N-3. That is, contributions from either tautomer (12) or resonance form (15) would be expected to shift the N-2 resonance slightly upfield. Parallel arguments have been used in the study of the protonation site of adenosine (Markowski, 1977). We wanted to find which explanation of the data was correct. Therefore, we sought to determine whether some tautomeric equilibrium between forms (11) and (12) was present.

The tautomeric preferences of an extensive series of glycocyamidine analogs at pH 12 have been examined (Matsumoto,1968)(Rowley,1971). For creatinine at pH 12 the structure in Figure 1 is preferred. In that structure there is conjugation between the guanidinium and carbonyl moieties. At low pH this conjugation would still be expected to predominate. However, at low pH the UV spectra were ambiguous. Even the ^{15}N chemical shift data in Table II alone were insufficient to come to any conclusions about the tautomeric preference of protonated creatinine. Further analysis of the ^{15}N NMR spectra in Figure 4 and ^{1}H NMR spectra in Figures 19 and 20 gave us key information about the properties of the protons attached to N-2 and N-3. This information allowed us to draw conclusions about the structure of protonated creatinine.

At low pH, the ^{15}N resonance of N-2 (at 56 ppm) is split into a triplet by protons directly attached as a result of the one-bond $^{15}N-^{1}H$ coupling constant of 93 Hz, but the N-3 resonance remains a singlet (at 123 ppm)(**Table II**)(**Figure 14**). This heteronuclear coupling is also evident in the ^{1}H NMR spectra for both labeled creatinines (**Figures 19 and 20**). The resonance for the guanidino $-NH_2$ protons in [2-^{15}N]creatinine is a doublet at 8.5 ppm, the result of the one-bond $^{15}N-^{1}H$ coupling constant of 93 Hz. The resonance for protons bound to N-2 in [2/3-^{15}N]creatinine is a triplet formed by the superposition of the broadened singlet due to protons bonded to ^{14}N-2 upon the doublet due to protons bonded to ^{15}N-2. The ^{1}H resonance for the proton attached to N-3 was presumably too broad to be detected. These results indicate that there are only two protons bound to N-2 and that they are in slow exchange with solvent, whereas any protons bound to N-3 are in rapid exchange with solvent. This finding is consistent with our earlier conclusion that protonation occurs at N-3 and also indicates that there can be no contribution to the structure of protonated creatinine by structure (12). That structure has three protons bound to N-2. These three protons would undoubtedly be in rapid exchange with solvent. Additionally, if there were a tautomeric equilibrium between structure (12) and structure (11), a mechanism for the rapid exchange of the protons at N-3 would exist. If the protons exchanged rapidly, the ^{15}N resonance for N-2 would be observed as a singlet and no resonance for those protons would be observed at all in the ^{1}H spectrum. Therefore, a minor contribution by

resonance form (15) is the most likely explanation for the small downfield change in the resonance of N-2 when creatinine is protonated.

A major contribution to the structure of protonated creatinine by resonance form (15) is certainly ruled out. Two pieces of evidence point to this. The first is the magnitude of the chemical shift change in the ^{15}N resonance at N-3. It is about 67% of the dramatic shift of 97 ppm observed for the protonation of pyridine (Markowski, 1977). A second indication that the contribution of resonance form (15) is relatively small is the fact that the two protons attached to N-2 are isochronous (not resolved at 500 MHz). This is probably the result of rapid rotation about the C-N2 bond. Such a rotation would be impossible if the double bond character at that position were high. Thus, the expectation that resonance form (14) predominates is supported. No comment can be made about the relative importance of resonance form (16) because no ^{15}N NMR data were obtained for N-1.

Summary

A synthetic method for the preparation of ^{15}N labeled phosphocreatine, creatine, creatinine and phosphocreatinine specifically labeled at N-2 has been developed. A method was also developed for the preparation of analogs in which the ^{15}N label is completely scrambled between the N-2 and N-3 positions. The synthesis of specifically labeled creatinine is accompanied by a small amount of scrambling. We believe this is the result of a small amount of opening and reclosure of the creatinine ring during its synthesis and workup. No further scrambling was

observed in the syntheses of phosphocreatinine and phosphocreatine from the [^{15}N]creatinine. A notable exception is the alternate route to the synthesis of [2/3-^{15}N]phosphocreatine. This route is the enzyme-catalyzed ^{15}N/^{14}N positional isotope exchange of [2-^{15}N]phosphocreatine by creatine kinase in the presence of MgADP. The mechanism for this scrambling of the label in phosphocreatine involves ^{15}N-labeled creatine as an intermediate.

^{15}N NMR data for phosphoguanidines are reported here for the first time. As expected, the ^{31}P NMR data reflect an ^{15}N-induced chemical shift change of 0.01 ppm. No such change was observed in the ^{1}H resonances of protons bound to N-2 of creatinine. The line-broadening effect of the ^{15}N quadrupolar nucleus is evident in comparing the proton spectra of [2-^{15}N]creatine and [2/3-^{15}N]creatinine. Such line broadening is not apparent, however, from ^{31}P NMR spectra of the labeled phosphocreatinines and phosphocreatines.

The ^{15}N and ^{31}P NMR data were used to determine unequivocally that the site of phosphorylation in phosphocreatinine is at N-2, i.e., on the exocyclic nitrogen. The phosphoryl group of phosphocreatinine thus has the opposite stereochemistry from that required for phosphoryl transfer to MgADP in the reaction catalyzed by creatine kinase. The fact that phosphocreatinine and creatinine are not substrates in the creatine kinase reaction has been a major piece of evidence that the presence of a carboxyl group is important for substrate activity. The role of the carboxyl group is now called into question.

[2-^{15}N]Phosphocreatine was found by this investigation to be potentially useful as a probe of the stereospecificity and kinetics of the creatine kinase reaction. Two prerequisites for this use were met. First, the ^{31}P-^{15}N one-bond coupling constant was found to be 18 Hz, not 3 Hz as reported by Brindle et al (Brindle, 1984). A J value of 18 Hz is more than sufficient to allow resolution of ^{31}P NMR signals in spite of both the ^{15}N-induced chemical shift change and any line-broadening induced by the ^{14}N quadrupolar nucleus. Therefore, the phosphorus resonances of [2-^{15}N]phosphocreatine and [3-^{15}N]phosphocreatine can be resolved in order to quantitate their relative amounts in solution. Secondly, as stated above, the last step in the synthesis of [2-^{15}N]phosphocreatine was not accompanied by scrambling of the label. This proves that specifically labeled phosphocreatine does not undergo ^{14}N/^{15}N positional isotope exchange all by itself.

Finally, the ^{15}N and ^{1}H NMR studies reported in this chapter are consistent only with protonation exclusively at N-2 on creatinine at low pH. The evidence further suggests that the most important resonance form is that in which conjugation with the carbonyl moiety is present, although the other two forms are likely to make small contributions.

Acknowledgments

We express our appreciation to Dr. Vladimir Basus, Dr. Max Keniry, Mr. Thomas Marschner and Dr. Michael Moseley for assistance with the NMR measurements at UCSF. We are also grateful to Professors Norman Oppenheimer and Raja Iyengar for

helpful discussions. We also thank Mr. Vincent Powers for obtaining GC-MS data on the MS-25 mass spectrometer.

This research was supported by a grant from the NIH AM 17323 to GLK. RER is grateful to the American Foundation for Pharmaceutical Education and the Johnson & Johnson Foundation for support of graduate studies. Construction of the UCSF wide-bore 240-MHz spectrometer was funded by NIH Grant GM 27387.

Chapter 2

Isotope Exchange Experiments with Creatine Kinase

Introduction

Creatine kinase catalyzes the transfer of the γ-phosphoryl group of MgATP to creatine as shown in **Figure 1**. It has already been established by previous work in our laboratory that creatine kinase catalyzes the regiospecific phosphorylation of cyclocreatine (Rowley, 1971)(Struve, 1977) **(Figure 2)**. From this result, it can be inferred that creatine itself also may be phosphorylated regiospecifically on the $-NH_2$ group that is <u>trans</u> to the carboxymethyl group (i.e., the $-NH_2$ further removed from the negatively charged $-COO^-$ group, as shown in **Figure 1**). However, there are inherent problems in drawing conclusions about enzyme mechanisms based on analog studies. For example, it is possible that steric interactions of the ethylene bridge of cyclocreatine with the enzyme force the exocyclic nitrogen into a position less favorable for phosphorylation, but that in creatine the two nitrogens are positioned equally well for phosphoryl transfer. Kinetic(Rowley, 1971) and X-ray crystallographic analyses(Herriott, 1968)(Mendel, 1954)(Phillips, 1979) suggest that differences between substrate and analog interactions with the enzyme are minimal. Nevertheless, the argument that these differences are responsible for the stereochemistry involved in the reaction with cyclocreatine cannot be ruled out completely. Therefore, it is of considerable interest to examine the stereochemistry of this reaction using the normal substrates, phosphocreatine and creatine. One way to do this is to use [^{15}N]phosphocreatine specifically labeled at N-2

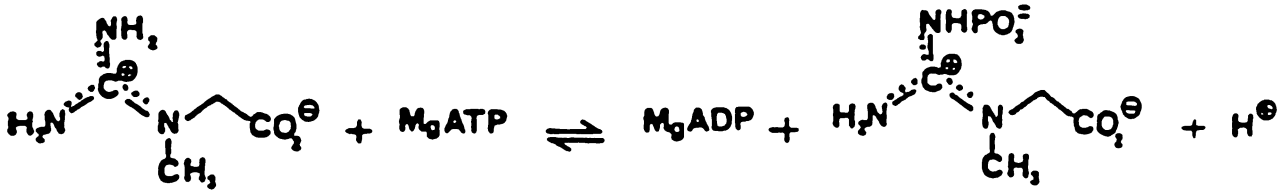


Figure 1 Reaction catalyzed by creatine kinase.

Figure 2 Structures of cyclocreatine and phosphocyclocreatine

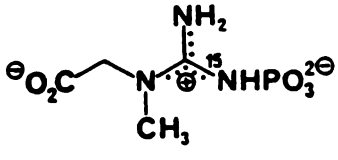


Figure 3 [2-^{15}N]Phosphocreatine

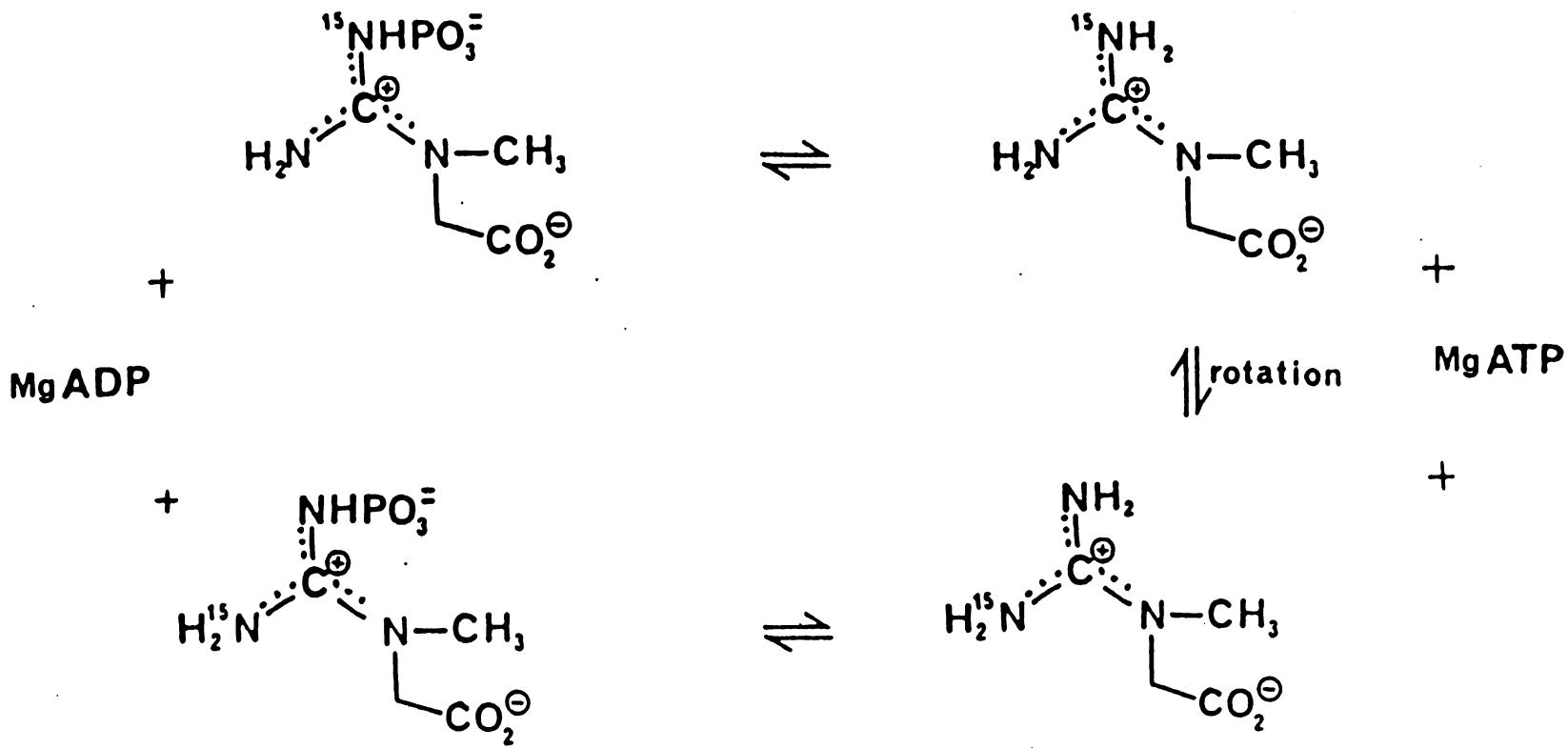
(Figure 3). The positional isotope exchange technique may be used in conjunction with other isotope exchange techniques to determine whether the phosphoryl group is transferred regiospecifically to one of the two guanidino $-NH_2$ of creatine.

Most applications of the positional isotope exchange techniques to enzymology have involved either $^{18}O/^{16}O$ or $^{17}O/^{16}O$ exchange in phosphoryl or carboxyl groups(Reynolds, 1983)(Midelfort, 1976)(Lowe, 1980). Recently, however, Raushel and Garrard have used $^{15}N/^{14}N$ exchange in the guanidino group of argininosuccinate to study argininosuccinate lyase(Raushel, 1984). The barrier to rotation of the guanidino group of arginine at the active site of argininosuccinate lyase is sufficiently small that PIX can occur via the enzyme-bound ligand. The PIX rate relative to the net chemical turnover of [3-^{15}N]argininosuccinate was found to be a function of fumarate concentration since the mechanism is random with respect to the two products, arginine and fumarate. By this positional isotope exchange enhancement technique, called PIXE, they determined individual rate constants (k_{off}) for the dissociation of fumarate and arginine from the ternary product complex. It was suggested that the PIXE experiment could be used to make estimates of individual rate constants for product dissociation (e.g., k_{off} for products from enzyme•MgATP•creatine) in any enzyme-catalyzed reaction in which PIX can be measured, provided that the V_{max} of the reverse reaction is significant relative to product dissociation.

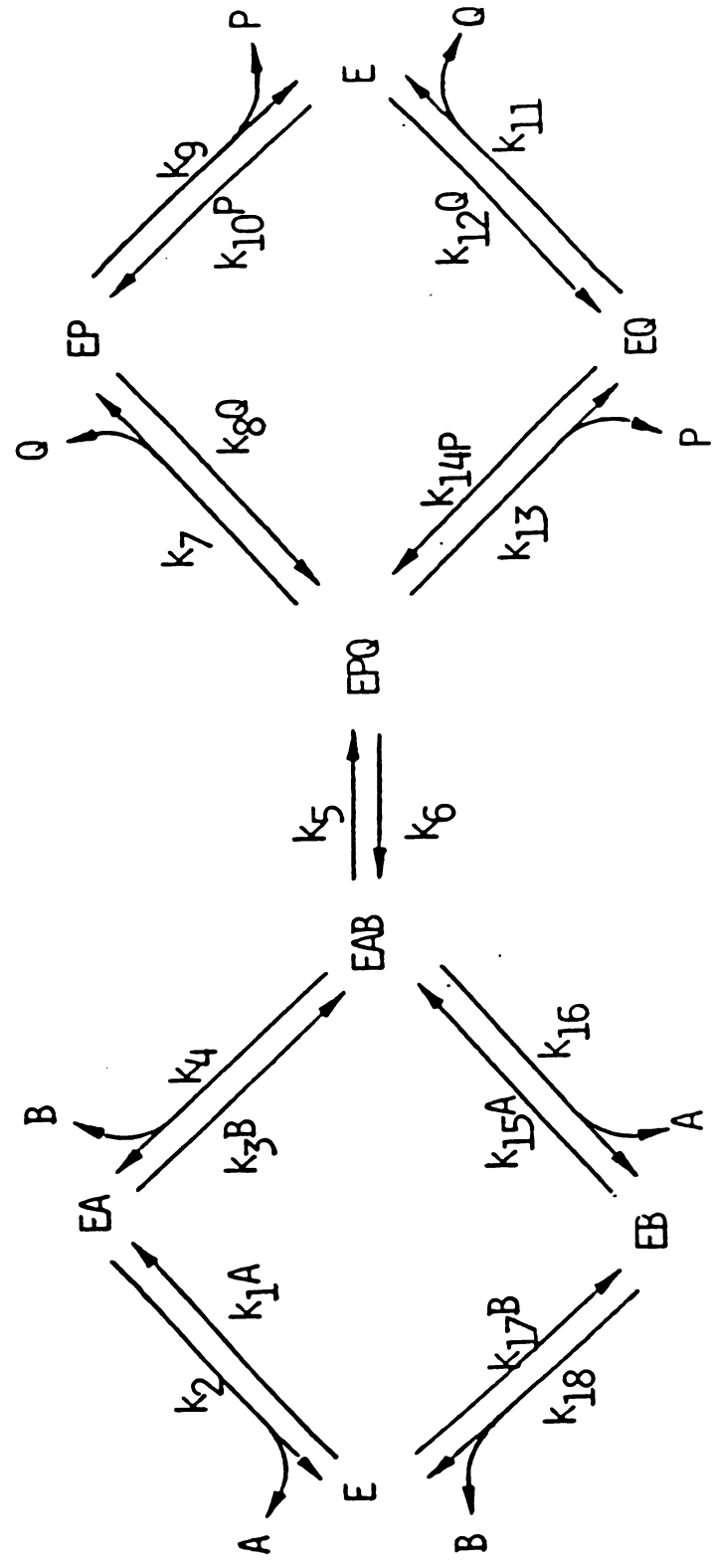
The mechanism of the reaction catalyzed by creatine kinase can be investigated by the use of the PIXE technique. First, a PIX reaction can occur in the guanidino group of creatine.

Creatine is a cosubstrate with MgATP in the reaction catalyzed by creatine kinase. The PIX for the enzyme-catalyzed reaction is outlined in **Scheme I**. When phosphocreatine labeled with ^{15}N at N-2 is incubated with MgADP and creatine kinase and converted to creatine and MgATP, the two guanidino $-NH_2$ groups are capable of equilibration via rotation about the carbon-nitrogen bond. Any phosphocreatine resynthesized will be labeled with ^{15}N at the N-3 position 50% of the time. Secondly, the creatine kinase reaction follows a rapid equilibrium, random, bimolecular kinetic scheme **(Scheme II)**, at least above pH 8 (Morrison, 1967; Morrison, 1966). As a result, the rate of any PIX occurring via the ternary complex (EPQ) relative to the net chemical turnover of phosphocreatine will be a function of MgATP concentration.

If PIX can occur in the enzyme-bound form of creatine, then PIX will occur even under conditions where the dissociation of labeled creatine from the enzyme is made essentially irreversible. The ratio of the rate of PIX to the rate of product formation (k_r/k_f) can be measured as a function of MgATP concentration under conditions where creatine that dissociates from the binary and ternary enzyme-creatine complexes is coupled away by the enzyme creatinase. This ratio reflects the relative rates of partitioning of the ternary complex in the forward direction (EPQ→P) and in the reverse direction via PIX (EPQ→A) on the enzyme. As the MgATP concentration is varied from zero to saturation, the k_r/k_f ratio should increase to a plateau value. A theoretical curve for a plot of k_r/k_f *vs* [MgATP] is shown in **Figure 4**. Using relationships among individual rate constants presented



Scheme I $^{15}N/^{14}N$ Positional Isotope Exchange.



Scheme II The kinetic mechanism of creatine kinase where A - PCr; B - MgADP; P - Cr; Q - MgATP.

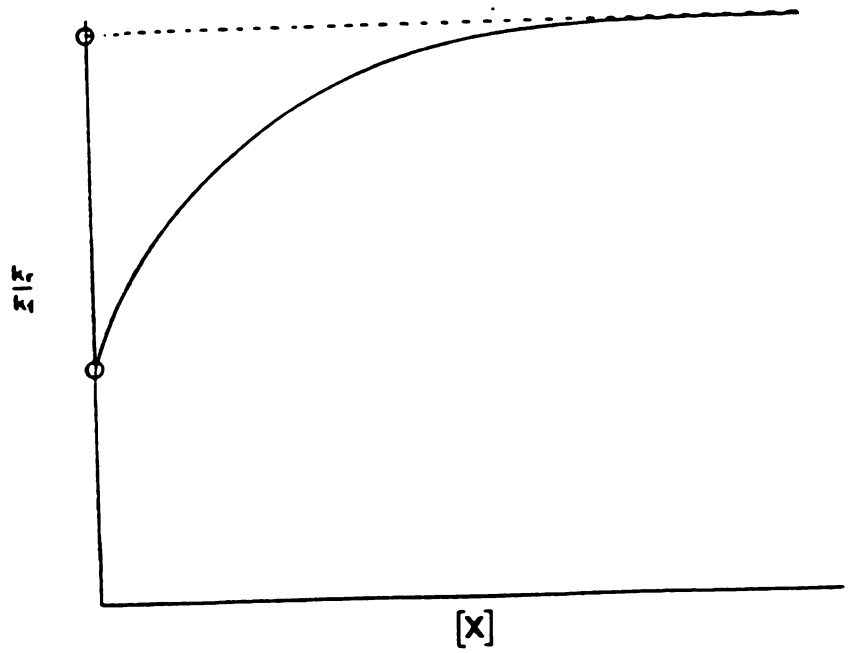


Figure 4 Theoretical curve for data derived from a PIXE or DIXE experiment.

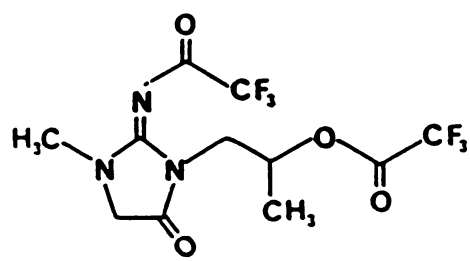


Figure 5 Structure of the di-trifluoroacetate of the (2-hydroxy-2-methyl)ethyl derivative of creatinine.

in the Theory section, it would therefore be possible to calculate an exact value for

$$[k_{13}(k_7+k_9)]/[k_9(k_{13}+k_7)]$$

and a lower limit for the values of k_{13}, the rate of release of creatine from the ternary enzyme–creatine–MgATP complex.

If, on the other hand, PIX cannot occur in the enzyme–bound form of creatine, then this technique could completely suppress PIX. This would suggest that the enzyme imparts a high barrier to the rotation about the C–N_{Me} bond of creatine and that the phosphoryl group is transferred with essentially complete regiospecificity. A lack of PIX in enzyme–bound creatine is thus a necessary piece of evidence in establishing the regiospecificity in the normal reaction catalyzed by creatine kinase.

Another isotope exchange experiment can be used to derive some of the same kinetic information provided by the PIXE technique of Raushel and Garrard. This technique is called dynamic isotope exchange enhancement (DIXE) and has been developed by Raushel (Raushel, 1986). In this experiment, the isotope exchange is from unlabeled product into a pool of labeled substrate. The ratio of the rate of this exchange to the rate of chemical turnover (k_r/k_f) can be measured as a function of the concentration of the other product. An important aspect of this technique is that no rotation of bonds in the enzyme–bound substrate is necessary. One such experiment involves the use of [2–^{15}N]phosphocreatine and was performed in this thesis.

In this experiment, the dissociation of MgATP from the ternary complex is made irreversible by coupling away free MgATP

with excess hexokinase. As the concentration of unlabeled creatine is raised from zero to saturation the rate of exchange of the unlabeled creatine into the ^{15}N-phosphocreatine pool will increase from zero to a plateau level. At saturating creatine concentration, this experiment is a measure of the ratio of the rates of partitioning of EPQ in the forward (EPQ→Q) and reverse (EPQ→A) directions. By measuring this ratio (k_r/k_f) at saturating creatine concentration it is possible to determine a lower limit to the value of k_7, the rate of release of MgATP from the ternary product complex. Such experiments were performed in this thesis, and will be described later.

Recently, we synthesized phosphocreatine labeled exclusively at N-2 (**Figure 3**). In addition, we established that the specifically labeled phosphocreatine does not undergo PIX all by itself. Although spontaneous PIX did not seem likely based on examination of molecular models of phosphocreatine, (i.e., a four-membered ring transition state would be required for attack of the $-NH_2$ on the phosphoryl group), related intramolecular phosphoryl transfers have been proposed(O'Neal, 1983). The synthesis of the precursor to [2-^{15}N]phosphocreatine, [2-^{15}N]creatinine, was accompanied by a small amount of PIX, but no further PIX accompanied subsequent steps in the synthetic pathway (Chapter 1).

We report the use of [2-^{15}N]phosphocreatine in isotope exchange experiments with creatine kinase. As expected, complete PIX accompanies the incubation of [2-^{15}N]phosphocreatine with creatine kinase and MgADP under conditions where the reaction is allowed to come to equilibrium. However, when [2-

^{15}N]phosphocreatine is incubated with creatine kinase and MgADP in the presence of creatinase to ensure that resynthesis of phosphocreatine occurs only via enzyme-bound creatine, no PIX is observed. This suggests that there may be a large barrier to rotation of creatine at the active site of creatine kinase and that the reaction is highly regiospecific. Alternatively, it is possible that the relative rate of partitioning of the ternary product complex back to starting materials (EPQ→A) is so low that the amount of PIX that accompanies partial reaction of the labeled phosphocreatine is too small to detect. In addition, when [2-^{15}N]phosphocreatine is incubated with creatine kinase and MgADP in the presence of hexokinase and an excess of unlabeled creatine, some unlabeled creatine exchanges in the reverse direction. This shows that the maximum rate of partitioning of the ternary product complex back to phosphocreatine (EPQ→A) relative to the rate of chemical turnover to form MgATP (EPQ→Q) is small and allows us to estimate a lower limit for the rate of dissociation of MgATP from the ternary complex, k_7.

Materials and Methods

ATP, ADP, NADH, NADP, glucose, α-ketoglutarate, unlabeled phosphocreatine, creatinine, creatine, creatine kinase, Chelex-100(Na^+), lactate dehydrogenase and pyruvate kinase were all purchased from Sigma Biochemicals. Hexokinase/glucose-6-phosphate dehydrogenase was obtained in a 500:190 (U/U) ratio as lyophilized protein from Sigma. Urease and glutamate dehydrogenase were purchased from Boehringer-Mannheim. Dowex AG 1-8x (Cl^-) 200-400 mesh was purchased from Bio-Rad.

[2-^{15}N]Phosphocreatine was synthesized as reported previously (Chapter 1).

Enzymatic Reaction of [2-^{15}N]Phosphocreatine with Creatine Kinase in the Absence of Coupling Enzymes: Equilibrium PIX

Thirty-five units of creatine kinase were incubated for 8 hr at 25°C with 10 mM [2-^{15}N]phosphocreatine, 6.0 mM ADP, 15.2 mM ATP, 20.5 mM $Mg(OAc)_2$ and 50 mM Tris/HOAc. It was estimated from data reported in the literature (Lawson, 1979), (Morrison, 1965) that equilibrium would be achieved in 2-3 hr after 50% of the initial phosphocreatine was consumed. The reaction mixture was diluted to 100 mL with H_2O, poured over a slurry of 3.3 mL of 22% (w/v) acid-washed charcoal and filtered through Celite. The sample was purified by anion-exchange chromatography at 4°C on a 10-mL Dowex AG1-8X column by first eluting with 100 mL H_2O and then with 0.3 M TEA/HCO_3^- (pH 7.8) in a modification of the procedure of Martonosi (Martonosi, 1960). Fractions were assayed for acid-labile phosphates according to the procedure of Ames (Ames, 1966). The pooled fractions were concentrated under vacuum below 30°C to remove water and buffer and stored at -20°C.

Enzymatic Reaction of [2-^{15}N]Phosphocreatine with Creatine Kinase Coupled to Creatinase: [Cr]=0

Creatine kinase (0.96 units) was incubated with an 8 mL-solution of 10 mM [2-^{15}N]phosphocreatine, 40 mM ADP, 300 mM ATP, 301 mM $Mg(OAc)_2$ and 50 mM Tris/HOAc (pH 8.0), which had been pre-incubated for 30 min at 25°C with 168 units

creatinase and 6.4 units creatininase. The reaction course was monitored by quantitation of urea in aliquots removed at various times (Kaltwasser, 1965). After 3 hr, 36±5% of the phosphocreatine had been consumed as determined by assay for urea.

The reaction mixture was diluted to 145 mLs with cold H_2O, poured into a 120 mL slurry of 25% (w/v) acid-washed charcoal (Switzer, 1978) in cold H_2O, stirred 2 min and then filtered through a Celite pad. The sample was purified by anion-exchange chromatography at 4°C on a 10-mL Dowex AG1-8X column. The column was washed with 200 mL of distilled and deionized water to remove Mg^{2+} ions completely. Then, both inorganic phosphate and phosphocreatine were eluted with 0.3 M TEA/HCO_3^- (pH 7.8) at a flow rate of 3 mL/min. The fractions were assayed for acid-labile phosphates according to the procedure of Ames (Ames, 1966). Solvent and buffer were removed under vacuum at under 30°C and stored at -20°C until analysis.

Enzymatic Reaction of [2-^{15}N]Phosphocreatine with Creatine Kinase Coupled to Hexokinase: [ATP]= 0

Creatine kinase (8 units) was incubated with 100 mL of 0.8 mM [2-^{15}N]phosphocreatine, 4 mM ADP, 105 mM creatine, 4.9 mM $Mg(OAc)_2$ (1 mM free Mg^{2+}), 0.8 mM NADP, 25 mM glucose, and 50 mM Tris/HOAc (pH 8.0) which had been preincubated at 25°C with 500 µl of stock HK/G6PDH until baseline was flat (approximately 10 min)(Swanson, 1972). The reaction was monitored by the decrease in absorbance at 340 nm. After 53 min, 36% of the phosphocreatine was consumed, and the reaction mixture was diluted into a slurry of 14% (w/v) acid-washed

charcoal in 50 mL cold H_2O, stirred 2 min and then filtered through a Celite pad. The sample was purified by anion-exchange chromatography at 4°C on a 10-mL Dowex AG1-8X column by eluting with 200 mL of distilled and deionized water, with a 250 mL linear gradient of 0-0.3 M TEA/HCO_3^- (pH 7.8), and then with the final buffer alone.

Fractions were assayed for acid-labile phosphates according to the procedure of Ames(Ames, 1966). Phosphocreatinine is eluted from the column at 0.3 M TEA/HCO_3^- under these conditions. The pooled fractions were concentrated under vacuum at below 30°C to remove water and buffer and stored at -20°C until analysis.

Hydrolysis and Cyclization of Phosphocreatine to Creatinine

The reisolated mixture of labeled and unlabeled phosphocreatine was treated with 2 mL of concentrated HCl and 400 µl of distilled and deionized water in a stoppered flask at 100°C for 24 hr, then evaporated to dryness under vacuum. The white residue was dissolved in water and passed through the Dowex anion exchange column. Creatinine was detected by TLC on silica gel by using the Benedict-Behre test(Benedict, 1936) Fractions containing creatinine were evaporated to dryness under vacuum, and the product was recovered as a white powder. It was further analyzed by GC-MS as the di-trifluoroacetate of the (2-hydroxy-methyl)ethyl derivative (Figure 5).

Preparation of GC-MS Samples

The derivatization of creatinine was as described by Bjorkhem et al.(Bjorkhem, 1977). One mg of creatinine in 0.5 mL of MeOH was treated with 0.5 mL of 1,2-epoxypropane and incubated at 70°C

for 30 min in a Teflon-sealed screw-capped vial. The solvent was evaporated under $N_{2(g)}$, then the residue was dissolved in 200 ul of EtOAc and treated with 100 µl trifluoroacetic anhydride for 30 min at 37°C. The solvent and excess reagent were evaporated under $N_{2(g)}$ and the residue was taken up in one mL of hexane for GC-MS analysis of the derivatized samples on the UCSF MS-25 mass spectrometer.

Preparation of NMR Samples

When necessary, the phosphocreatine samples were percolated through columns of Chelex-100 (sodium form) into acid-washed vials and lyophilized to prepare samples of 50-200 mM in 0.4 mL 20% D_2O. The 5-mm NMR tubes were made metal-free by soaking overnight in 1/1 concentrated HNO_3/ concentrated H_2SO_4 and rinsing thoroughly with distilled and deionized water.

NMR Measurements

^{31}P NMR spectra were taken at 97.5 MHz on the UCSF wide-bore 240 MHz spectrometer. A spectral width of 2000 Hz and 8196 data points were used to acquire the free induction decay; a 44° tip angle and no time delay between acquisitions was employed in most cases. An exponential line-broadening factor of 0.5 Hz was applied to the free induction decay prior to Fourier transformation. When necessary, a double exponential apodization factor of up to 10 was applied to the free induction decay prior to Fourier transformation to improve resolution for peak integration. Chemical shifts were measured relative to 0.85% H_3PO_4 in D_2O used as an external standard. Typically, 500-2000 data acquisitions were collected.

Theory

A general mechanism for the creatine kinase reaction is outlined in **Scheme II**. Above pH 8 the mechanism is random in both directions (Morrison, 1965)(Morrison, 1966). If there is free rotation in the ternary complex EPQ, measurement of the positional isotope exchange reaction relative to the net chemical turnover of phosphocreatine is a direct determination of the partitioning of the ternary complex EPQ (EPQ→P *vs* EPQ→Q). The steps for creatine addition (k_{10} and k_{14}) can be neglected when excess creatinase is added to keep the creatine concentration essentially zero. The partitioning of the complex EPQ can therefore be presented in terms of individual rate constants by using the theory of net rate constants of Cleland (Cleland, 1975). Thus the partitioning of EPQ forward to creatine (EPQ→P) can be represented as

$$V_f/EPQ = k_f = [k_7k_9+k_{13}(k_8Q+k_9)]/[k_8Q+k_7+k_9] \qquad (1)$$

and the partitioning of EPQ back to starting material (EPQ→A) is given by

$$V_r/EPQ = k_r = \frac{k_6[k_4k_2+k_{16}(k_3B+k_2)]}{[k_4(k_2+k_6)+(k_3B+k_2)(k_5+k_6+k_{16})]} \qquad (2)$$

At constant [B], k_r reduces to a constant, which we will call k'. Thus, the ratio of the PIX rate relative to the chemical rate (k_r/k_f) can be written as:

$$V_r/V_f = k_r/k_f = k'(k_8Q+k_7+k_9)/[k_7k_9+k_{13}(k_8Q+k_9)] \qquad (3)$$

If the concentration of B is kept a constant, then the value of k_r/k_f as a function of [Q] is given by equation (3), and a plot of k_r/k_f vs. [Q] will, in theory, yield data similar to that in **Figure 4**. The intercept on the vertical axis at [Q] = zero is:

$$k_r/k_f = [k'(k_7+k_9)]/[k_9(k_7+k_{13})] \qquad (4)$$

As [Q] is increased to saturating levels, the net chemical reaction through the top path decreases until all flux to creatine occurs through the lower path so that

$$V_r/V_f = k_r/k_f = k'/k_{13} = V_r/k_{13}(EPQ) \qquad (5)$$

From these two limiting values the exact value for the following relation can be calculated

$$[k_{13}(k_7+k_9)]/[k_9(k_{13}+k_7)]$$

Since e_t > EPQ this will give a lower limit to the value of k_{13}, k_{off} for the dissociation of creatine from EPQ. Thus,

$$k_{13} \geq V_f/e_t \qquad (6)$$

A related isotope exchange experiment, DIXE, is proposed at the end of this chapter. In this experiment, the exchange of unlabeled MgATP into a pool of [^{32}P]-phosphocreatine is measured relative to the net chemical turnover of phosphocreatine to creatine. This is a measurement of the partitioning of the binary complex EQ back to phosphocreatine (EQ→A), relative to the partitioning of the ternary complex EPQ in the direction of the product creatine (EPQ→P). As in the previous experiment, the steps for creatine addition (k_{10} and k_{14}) can be neglected when excess creatinase is added to keep the creatine concentration essentially at zero. The expression for partitioning of the complex EPQ in the forward direction is the same as in the PIX experiment (Equation 1). The expression for partitioning of EQ back to starting material (EQ→Q) can be derived using Cleland's theory of net rate constants:

$$V_r'/EP = k_r' = \frac{k_5k_8Q[k_4k_2+k_{15}(k_3B+k_2)]}{\begin{array}{c}k_4[k_8Q(k_2+k_5)+k_2(k_5+k_7)]+\\(k_3B+k_2)[k_8Q(k_5+k_5+k_{15})+k_5k_{15}+k_7(k_{15}+k_5)]\end{array}} \quad (7)$$

However, as the concentration of Q is increased to saturation, this expression reduces to the expression for V_f/EPQ in Equation (2). Thus,

$$V_r'/EP = k_r' = V_f/EPQ = k_r \quad (8)$$

At constant [B], k_r' reduces to the constant k' so the expression for the partitioning of EP back to PCr relative to the chemical rate (k_r'/k_f') is the same as that for (k_r/k_f) given in Equation 3. At saturating [Q], equations 5 and 6 also hold for this experiment and the limiting value obtained for k_{13} should be the same as that in Equation 6.

A different DIXE experiment was performed in this thesis. In this experiment, the exchange of unlabeled creatine into a pool of ^{15}N-phosphocreatine is measured relative to the net chemical turnover of phosphocreatine to MgATP. This constitutes a measurement of the partitioning of the binary complex EQ back to PCr (EQ→A) relative to partitioning of the ternary complex EPQ in the direction of the product MgATP (EPQ→Q). If excess hexokinase is added to the system, the steps for MgATP addition (k_8 and k_{12}) can be neglected in **Scheme II**. Thus, the partitioning of EPQ forward to MgATP (EPQ→Q) can be represented as

$$V_f^*/EPQ = k_f^* = [k_7(k_{14}P+k_{11})+k_{13}k_{11}]/(k_{14}P+k_{11}+k_{13}) \quad (9)$$

The expression for partitioning from EQ to PCr (EPQ→A) is analogous to that given in Equation (7):

$$V_r^*/EQ = k_r^* = \frac{k_6k_{14}P[k_4k_2+k_{16}(k_3B+k_2)]}{k_4[k_{14}P(k_2+k_6)+k_2(k_6+k_7)]+(k_3B+k_2)[k_{14}P(k_5+k_6+k_{16})+k_6k_{16}+k_7(k_{16}+k_5)]} \quad (10)$$

However, as the concentration of unlabeled P is increased to saturation, this expression reduces to the expression for V_r/EPQ given in Equation 2 and at constant [B], it reduces further to k'. Thus, the expression for the partitioning of EQ back to PCr relative to the chemical rate (k_r^*/k_f^*) can be written as:

$$k_r/k_f = [k'(k_{14}P+k_{11}+k_{13})]/[k_7(k_{14}P+k_{11})+k_{13}k_{11}] \qquad (11)$$

Since P is saturating, the net chemical reaction through the lower path has decreased until all flux to MgATP occurs through the upper path so that

$$V_r^*/V_f^* = k_r^*/k_f^* = k'/k_7 = V_r^*/k_7(EPQ) \qquad (12)$$

Since e_t > EPQ a lower limit for k_7 can be calculated. Thus,

$$k_7 \geq V_f^*/e_t \qquad (13)$$

The portion of the model for the argininosuccinate lyase reaction used by Raushel and Garrard that describes partitioning forward from EPQ to give fumarate is exactly the same as the portion of the model for the creatine kinase reaction shown in **Scheme II** that describes partitioning forward from EPQ to give creatine. Therefore, the expressions for the rates of the processes should be exactly analogous. However, there is a discrepancy between the expression derived by Raushel and Garrard (Raushel,1984) and that in Equation (1) in this chapter. For the sake of convenience and clarity, the numbering of the rate constants in argininosuccinate lyase model has been changed to that for the model of creatine kinase in **Scheme II**. Thus, according to Raushel and Garrard, when k_{10} and k_{14} can be neglected, the partitioning of the ternary complex forward to product (EPQ→P) can be expressed

$$V_f/EPQ = k_f = k_{13}+k_7' = k_{13}+[k_7k_9/(k_{11}+k_{10}Q)] \qquad (14)$$

where k' is the net rate constant for partitioning from EPQ to EQ. This conclusion results from assuming that the total rate of chemical turnover to form P is the same as the sum of the rate of the top path in the absence of the bottom path and the rate of the bottom path in the absence of the top path. However, Cleland (Cleland, 1975) has stated that for branched pathways this is not correct except for the purpose of calculating net rate constants for the previous steps. To do this "ignores the fact that the parallel paths must compete for the available enzyme and thus that the flux through each will be less than if that path were the only one operative"(Cleland, 1975). To get the net rate constant for partitioning forward through a branched pathway to a common product, the rate through each path in the presence of the other should be calculated and then these two rates should be added together. The apparent net rate constant for flow from EPQ→Q through the bottom path in the absence of the top path is given by:

$$k_{13(app)} = 1/(1/k_{13}) = k_{13} \qquad (15)$$

However, in the presence of the top path, one must allow for creatine species present as the intermediate EP. The concentration of EP depends upon the relative flow through the two paths, which in turn depends of the ratio k_7'/k_{13}. Thus, $(k_7'/k_{13})(1/k_9)$ must be added to the denominator to get:

$$k_{13(app)} = 1/[(1/k_{13})+(k_7'/k_{13})(1/k_9)] \qquad \text{Eq. (16)}$$

$$= k_{13}/[1+(k_7'/k_9)]$$

For the top path in the absence of the bottom, the apparent net rate constant (EPQ→P) is given by:

$$k_{7,9}' = 1/[(1/k_7')+(1/k_9)] = k_7'/[1+(k_7'/k_9)] \qquad (17)$$

Again, it is necessary to account for material piled up as intermediates between EPQ and Cr in the competing pathway. However, the conductance term in this case is zero because there are no intermediates. Thus Eq. (13) stands as is. Addition of these two expressions gives:

$$k_{7,13}' = (k_7'+k_{13})/[1+(k_7'/k_9)] \qquad (18)$$

By substituting in the expression for k_7' the expression for the partitioning of the ternary complex in the forward direction becomes

$$k_{7,13}' = \{[k_7k_9/(k_8Q+k_9)]+k_{13}\}/\{1+[k_7/(k_8Q+k_9)]\} =$$

$$= [k_7k_9+k_{13}(k_8Q+k_9)]/[k_8Q+k_7+k_9] \qquad (19)$$

This is the expression for k_f andV_f/EPQ given in Equation 1.

As it turns out, both Equation (1) and Equation (17) reduce to the same quantity at saturated product concentration so that

the results of the experiments reported by Raushel and Garrard are not affected by the discrepancy between expressions for k_f.

Results and Discussion

The equilibrium PIX experiment shows that creatine kinase does catalyze the $^{15}N/^{14}N$ positional isotope exchange reaction with [2-^{15}N]phosphocreatine. Figure 6 shows the ^{31}P NMR spectrum of ^{15}N-labeled phosphocreatine recovered from the incubation with MgATP, MgADP and creatine kinase in the absence of either any coupling enzymes or unlabeled creatine. The relative intensity of the central peak, corresponding to [3-^{15}N]phosphocreatine, has increased dramatically to approximately 50% of the total peak intensities. The label is completely scrambled under these conditions as expected. Therefore, PIX does occur when the system is allowed to come to equilibrium.

It remained to be determined whether enzyme-catalyzed PIX could be detected with ^{15}N-labeled phosphocreatine as formed from enzyme-bound creatine. As noted in the theory section, it is possible to increase the quantity k_r/k_f by adding in one of the products (either MgATP or creatine) so that the breakdown of EPQ via the pathway involving release of that product is inhibited. For this reason, the PIX experiment was conducted in the highest practical concentration of MgATP (300 m<u>M</u>). Figure 7 shows the ^{31}P NMR spectrum of ^{15}N-labeled phosphocreatine recovered from the incubation with MgADP, MgATP, creatine kinase, and creatinase. The relative intensity of the central peak, corresponding to [3-^{15}N]-phosphocreatine, remained the same as for the untreated labeled material, i.e., 6 ± 3% of total peak

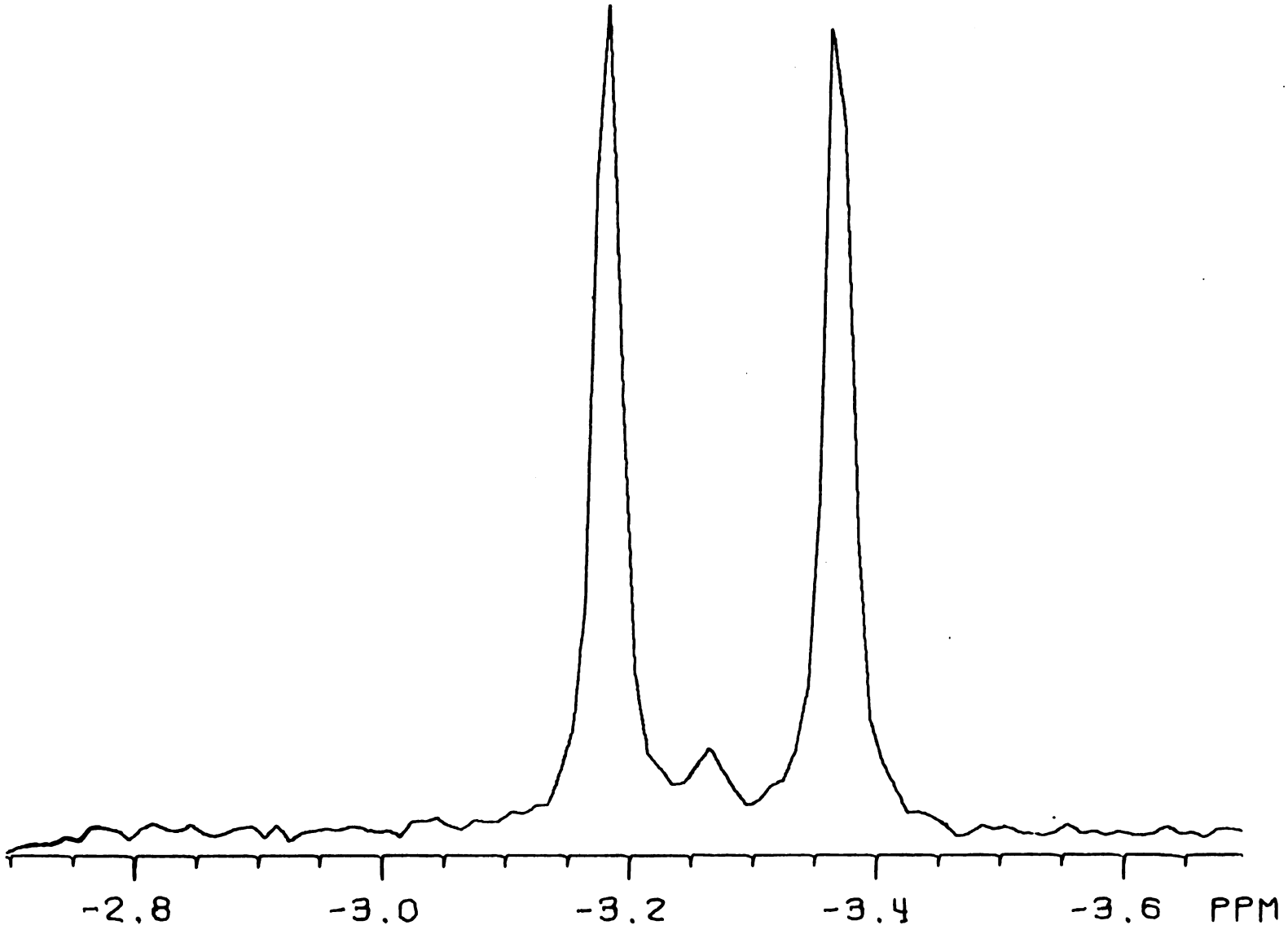


Figure 6A. ^{31}P NMR of [2-^{15}N]phosphocreatine before complete PIX.

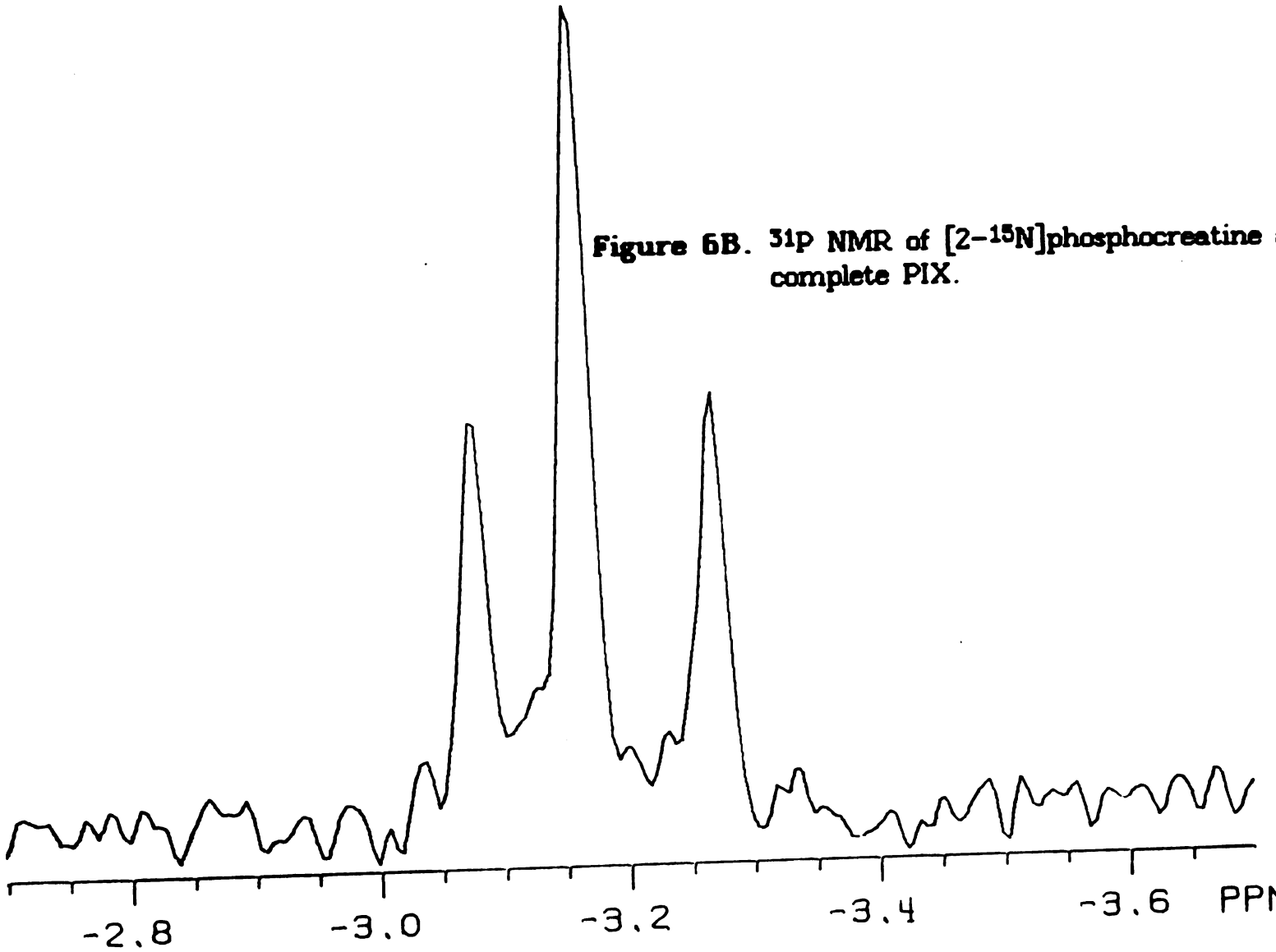


Figure 6B. ^{31}P NMR of [2-^{15}N]phosphocreatine after complete PIX.

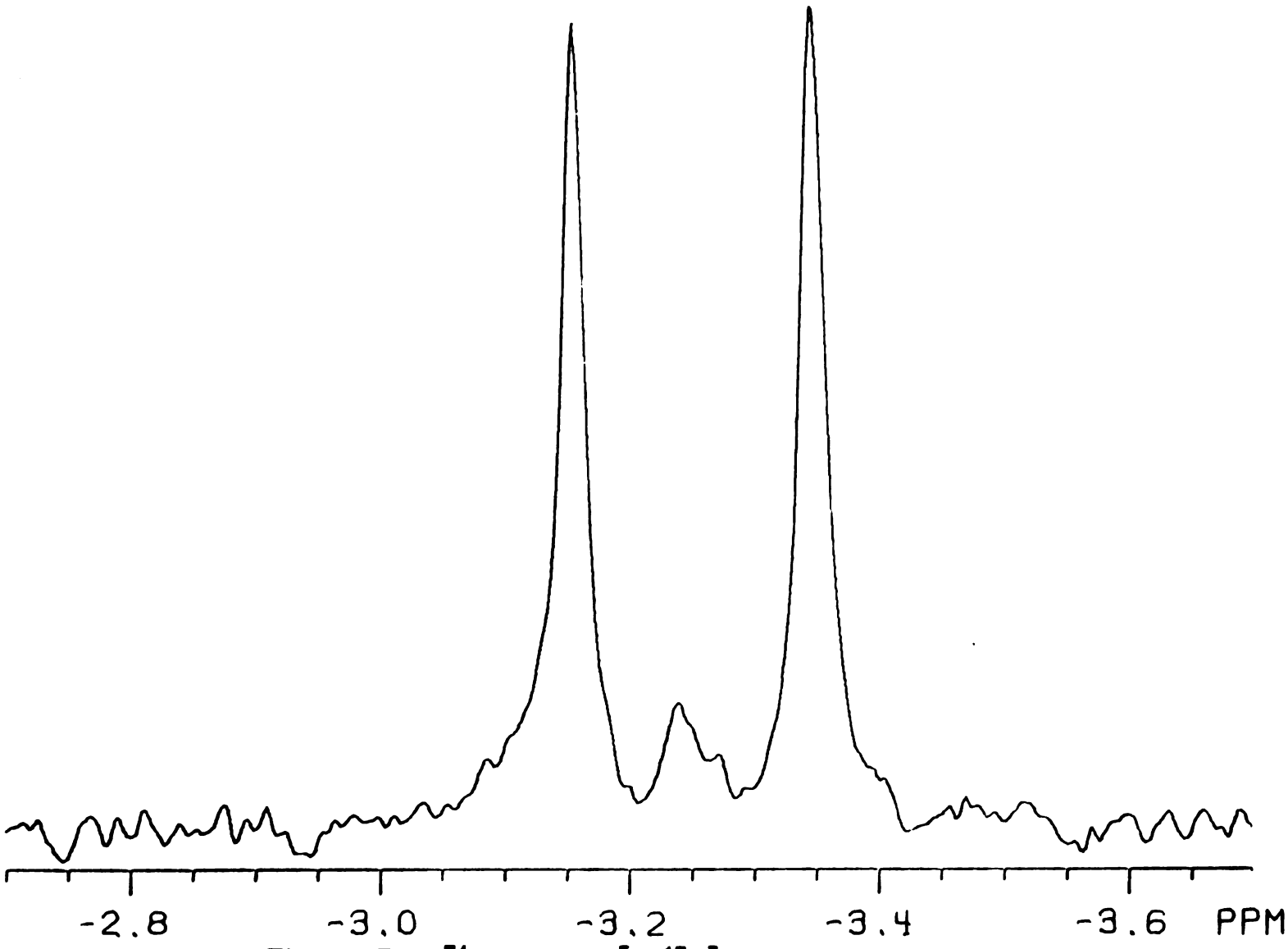


Figure 7. ^{31}P NMR of [2-^{15}N]phosphocreatine after partial reaction in the presence of creatinase.

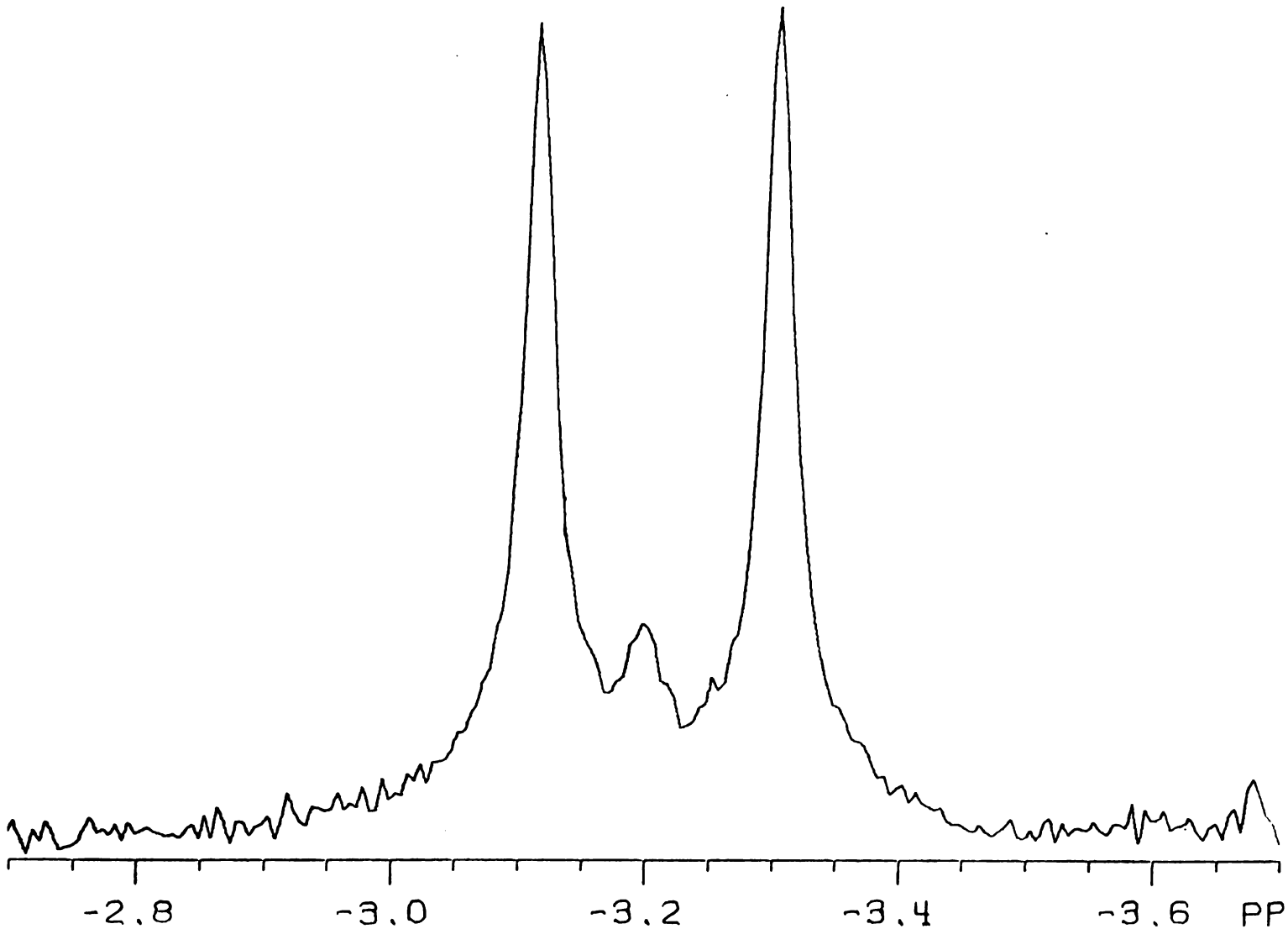


Figure 8. ^{31}P NMR of [2-^{15}N]phosphocreatine after partial reaction in the presence of hexokinase.

intensity. This corresponds to 95 % of the labeled creatine with ^{15}N at N-2, 5 % with ^{15}N at N-3 and 1% of total phosphocreatine unlabeled. Thus, no significant amount of positional isotope exchange has occurred in the enzyme-bound ligand. The kinetic data presented in Table I for the PIXE experiment are calculated from the limits of experimental error using the method of Litwin and Wimmer (Litwin, 1979):

$$v_{ex} = \frac{\chi \quad A_0}{[\ln(1-\chi)] \quad t} \ln(1-F) \qquad (20)$$

where χ = fraction of change of the original phosphocreatine pool, F = fraction of equilibrium value for exchange attained in the phosphocreatine pool at time t, and A_0 = the concentration of the original phosphocreatine pool.

Three parameters determine whether positional isotope exchange will occur. One is the degree of regioselectivity of the enzymatic reaction. Another is the magnitude of the rotational rate constant for creatine in the ternary product complex EPQ. The third is the magnitude of the ratio of the reverse partitioning rate constant to the forward partitioning rate constant (k_r/k_f). If it could be established that there is absolutely no PIX, it could easily be concluded that there is a large barrier to creatine rotation on the enzyme and that the phosphoryl group is transferred with complete regiospecificity. This is so because both k_r and EPQ must have non-zero values in order for the enzymatic reaction to take place. In contrast, neither rotation of creatine on

Table I

Results of Isotope Exchange Experiments

Experiment	ϵ (units)	T (hr)	F	V_f (μmol/hr)	V_{ex} (μmol/hr)	k_r/k_f
PIXE	0.96	3.0	0±.07	9.6±1.3	0±1.4	0±0.15
DIXE	8.0	0.88	---	32.6±4.5	6.4±2.9	0.20±0.11

the enzyme nor any lack of specificity is essential to the chemistry of this reaction. However, once it is hypothesized that a small, but undetected amount of PIX is possible, then insufficient back reaction must be considered as a possible reason for failure to observe PIX. Without further experimentation, however, we can only speculate as to which of these factors is responsible for our observations.

As noted earlier, there is already some evidence that the reaction is catalyzed in a regiospecific manner. If rotation about the C-N_{Me} bond of creatine in the EPQ complex is sufficiently free it would not be expected to be rate-limiting in the positional isotope exchange reaction. Kenyon et al. showed that in aqueous solution the barrier to rotation in substituted guanidinium groups such as that of creatine is about 14 kcal/mole(Kenyon, 1976). From a plot of 1/T vs ln k it is possible to extrapolate to an estimated value of 2 x 10^4 sec^{-1} for the rotational rate constant k_{rot} at 25° C. This can be compared to the rate constant of 10^3 sec^{-1} for rotation of the guanidino group of arginine(Kanamori, 1983). However, the enzyme may impart an additional barrier to creatine's rotation on the enzyme. If large enough, this barrier would effectively block the rotation of creatine at the creatine kinase active site.

In unpublished results, Raushel has shown that there is effectively no barrier to rotation for arginine at the active site of argininosuccinate lyase (Raushel, 1986). The PIX experiment showed that there is enough rotational freedom for arginine at the active site of argininosuccinate lyase to prevent complete suppression of

positional isotope exchange. In another isotope exchange experiment (DIXE) the rate of partitioning of the ternary complex at saturating fumarate concentration was shown to be the same as the PIX rate. However, there are important differences between the structures of creatine and arginine that could lead to a difference in the apparent barriers to rotation in the ternary product complexes.

The most obvious difference is the presence of the relatively large alkyl linkage between the guanidino and amino acid portions of arginine (**Figure 9**). Creatine has far fewer degrees of freedom. It is possible that there are strong binding interactions between the amino acid portion of arginine and the enzyme. If so, arginine might remain bound to argininosuccinate lyase while the guanidino group migrated to a relatively distant region via the relatively long, flexible alkyl linkage. The new environment in this region might be more conducive to conformational equilibration of the two guanidino nitrogens. With creatine the guanidino group cannot migrate to a region far from the active site while the carboxyl group remains stationary. Also, it is less likely that creatine would remain enzyme–bound by interaction with the carboxyl group alone. While these arguments are purely speculative, they demonstrate one way that the apparent barrier to rotation for enzyme–bound creatine could be larger than that for enzyme–bound arginine. Thus, a high rotational barrier for creatine at the active site of creatine kinase coupled with complete regiospecificity in the enzyme–catalyzed reaction may explain the failure to observe PIX.

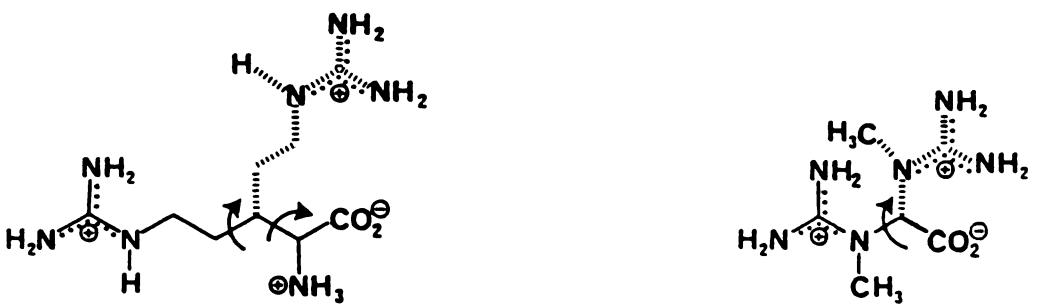


Figure 9 Degrees of rotational freedom of arginine and of creatine.

However, kinetic properties of the enzymatic reaction could also lead to an inability to observe PIX. If the ratio of rate constants (k_r/k_f) is too low, no PIX will be detected. This will be true regardless of whether either regioselectivity or an enzyme-induced barrier to rotation in creatine is a factor in the enzyme-catalyzed reaction. In designing the experiments, we attempted to maximize the relative rate of the reverse reaction as described earlier. Nevertheless, it is possible that even complete inhibition of the bottom pathway in **Scheme II** would fail to increase k_r/k_f enough to produce a discernable amount of PIX. It is known that at pH 7.0 phosphocreatine is "sticky", i.e., EAB partitions to creatine much faster than back to phosphocreatine(Cook, 1981). At this pH kinetic factors alone might be expected to prevent PIX via the enzyme-bound ligand. However, at pH 8.0 phosphocreatine is no longer sticky because catalysis is slower, but the release of phosphocreatine is not. It is also known that at pH 8.0 the association and dissociation steps in the mechanism of creatine kinase are more rapid than the chemical step(Engelborghs, 1973). Thus, k_r/k_f at saturating [MgATP] can be expected to be less than one. However, it is not known how much less than one it is. The uncertainty in the value of k_r/k_f for creatine kinase was estimated by propagation of experimental uncertainties in the measurements V_r and V_f. Thus, the maximum value of k_r/k_f (where k_r reflects positional isotope exchange) is 0.15. The range of exchange to turnover values thus calculated for the PIXE experiment, 0-0.15, may be compared with the results of isotope partitioning experiments reported for two other kinases, hexokinase

(HK) and phosphofructokinase (PFK) (Rose, 1974)(Cleland, 1976). The exchange to turnover ratio for glucose from the ternary complex (HK•glucose•MgATP) was practically zero. In contrast, the exchange to turnover ratio for fructose from the ternary complex, PFK•fructose•MgATP was 1.2. However, it is possible that the true ratio of partitioning rates for the ternary complex EPQ is greater than 0.15.

Clearly, an additional experiment is required to determine whether the creatine kinase reaction is regiospecific with respect to the normal substrate and an enzyme-induced barrier to the rotation of enzyme-bound creatine exists. The required experiment is a DIXE experiment and its purpose is to determine the true value of the relative partitioning of EPQ to phosphocreatine at saturating MgATP. The theory behind this experiment is presented in the **Theory** section of this chapter. In principle, it will provide conclusive evidence of the regiospecificity of creatine kinase and, at the least, will allow the calcuation of a lower limit to the value of k_{13}, the dissociation of creatine form the ternary complex, in the catalytic mechanism outlined in **Scheme II**. In contrast, the PIX experiment reported here measures only the relative partitioning of EPQ to phosphocreatine via a rotation about the C-N_{Me} bond.

In this DIXE experiment, the dissociation of creatine can be made irreversible by coupling away free creatine with creatinase. However, instead of using [2-^{15}N]phosphocreatine, the reaction is run with [^{32}P]-phosphocreatine as substrate. As the concentration of added unlabeled MgATP is raised from zero to saturation, the

rate of exchange of the unlabeled MgATP into the [^{32}P]phosphocreatine pool will increase from zero to a plateau level. By measuring this ratio (k_r/k_f) at saturating MgATP concentration, it should be possible to determine a lower limit to the value of k_{13}, the rate of release of creatine from the enzyme•creatine•MgATP complex. The plateau level of k_r/k_f in this experiment is also a measure of the ratio of the rates of partitioning of EPQ in the forward (EPQ→P) and reverse (EPQ→A) directions. If there is free rotation about the C–N_{Me} bond, the plateau value of (k_r/k_f) under these conditions will be identical to that obtained by the positional isotope exchange method. If, however, there is a large barrier to rotation and the reaction proceeds with stereoselectivity, the rate of this exchange will eventually exceed the rate of observed PIX as the concentration of MgATP is increased to saturation. If the PIX rate at saturating [MgATP] is smaller than the rate of simple exchange of unlabeled MgATP into a pool of phosphocreatine under the same conditions, the presence of a rotational barrier and degree of regioselectivity in the enzymatic reaction will be demonstrated. If, however, the two rates are equal at saturating [MgATP] there is no barrier to rotation. Thus, this experiment will in principle determine whether the true value of k_r/k_f is simply too low for PIX to be detected.

The second isotope exchange experiment (DIXE) was conducted using the highest possible concentration of creatine to inhibit the breakdown of EPQ via the pathways involving release of creatine. The kinetic data are listed in Table I. The

phosphocreatine remaining after partial reaction was converted to creatinine by acid hydrolysis and cyclization(Edgar, 1932). The creatinine was then analyzed by GC-MS as the di-trifluoroacetate derivative. The relative amounts of unlabeled creatinine were calculated from the relative MID responses at m/e 294 and m/e 296. The ion at m/e 294 is due to the fragment formed by loss of a CF_3-group from the molecular ion of unlabeled creatinine. The ion at m/e 296 is the result of the M+1 natural isotopic abundance for the analogous fragment in the labeled creatinine derivative. The intensity of the MID response for the ion at m/e 296 is 12.4% of the fraction of the MID response at m/e 295 due to the labeled creatinine derivative. The ^{15}N isotopic enrichment is decreased to 89 ± 5% from 99 ±0.5% (Chapter 1). This corresponds to a reverse rate of V_r = 6.4 ± 2.9 μmoles/ hr. **Figure 9** shows the ^{31}P NMR spectrum of ^{14}N- and ^{15}N-labeled phosphocreatine recovered from the incubation with MgADP, unlabeled creatine, creatine kinase, and hexokinase. The relative intensity of the central peak, corresponding to phosphocreatine with ^{14}N at N-3, has increased to 15 ± 3.0%. By comparing ^{31}P NMR and GC-MS data one can calculate that 96 ± 5% of the labeled creatine bears ^{15}N at N-2 and 4 ± 5% bears ^{15}N at N-3. As expected, there was no PIX of the labeled phosphocreatine during this experiment. Since V_f = 32.6 ±4.5 μmoles/hr, k_r/k_f = 0.20 ± 0.11. The estimated lower limit for k_7 (V_f/e_t), k_{off} for the dissociation of MgATP from EPQ , is therefore 1.6 ± 0.2 sec^{-1}.

These results contrast sharply with the results of isotope trapping experiments with PFK where the exchange to turnover

Table II

GC-MS Analysis of Partially ^{15}N Labeled Creatinine

Run	Ion Current Intensity 0.124(m/e= 294)	(m/e= 296)	%[^{14}N] Crn	%[^{15}N] Crn
5	2624	12994	16.8	83.2
8	16004	120816	11.7	88.3
9	91852	1310370	6.6	93.4
12	116386	142416	7.5	92.5
13	39778	210479	15.9	84.1
14	74	830	8.2	91.8
15	36377	450511	7.5	92.5
20	131178	1666434	7.3	92.7
21	45671	726238	5.9	94.1
22	21306	356330	5.6	94.8
24	12075	219188	5.2	94.8
25	71707	1059645	6.3	93.7

ratio for ATP from the ternary complex, PFK•fructose•MgATP, was very large. (essentially 100% exchange occurs)(Cleland, 1976). No estimate was made of the value of k_{off} for ATP from this ternary complex, but the value was obviously much larger than that for the reaction to form products. With hexokinase, the calculated rate of dissociation of glucose from the ternary complex was 10 sec^{-1} (Rose, 1974). The estimated lower limit to ATP dissociation from the ternary product complex of creatine kinase measured by the isotope exchange experiment in this thesis is within an order of magnitude of this value (i.e. 1.6 sec^{-1}). However, the calculation of k_7 is based on the assumption that all of the enzyme exists as the ternary product complex under steady state conditions (e_t = EPQ). The true k_{off} is higher, i.e., (1.6 sec^{-1})($e_t/[EPQ]_{ss}$). It will be useful to compare this value with the corresponding rate constants (k_{off} from the ternary complexes in **Scheme II**) for the other substrates in the CPK reaction as they become available in attempting to draw an energy diagram for the course of this reaction.

Summary

We have employed [^{15}N]phosphocreatine specifically labeled at N-2 to investigate the mechanism of creatine kinase. We found that [2-^{15}N]phosphocreatine undergoes complete ^{15}N/^{14}N PIX when the creatine kinase reaction is allowed to come to equilibrium as expected. However, this PIX does not occur from the enzyme-bound form of creatine at pH 8.0 even under conditions where the amount of exchange should be maximized. This may mean that the reaction proceeds with regioselectivity and that there is a

significant barrier to rotation of the guanidino group of creatine at the enzyme's active site. Alternatively, it may simply indicate that the rate of partitioning of the ternary product complex creatine kinase•creatine•MgATP to phosphocreatine is less than 0.15 times the rate of partitioning of this complex to form creatine. Another isotope exchange experiment is proposed that will indicate which of these explanations is correct. [2-^{15}N]Phosphocreatine was also used in an isotope exchange experiment (Raushel, personal communication) to measure the exchange to turnover ratio of the ternary product complex creatine kinase•creatine•MgATP. We found that in this case the ratio of partitioning rates is indeed quite small (0.20±0.11). The results of these isotope exchange experiments will eventually be useful in formulating an energy diagram of the enzyme-catalyzed reaction course.

Acknowledgments

We express our appreciation to Dr. Vladimir Basus, Dr. Max Keniry, Mr. Thomas Marschner and Dr. Michael Moseley for assistance with the NMR measurements at UCSF. We are also grateful to Dr. Thomas Meek and Professors D.V. Santi and Frank Raushel for helpful discussions and to Prof. W.W. Cleland for reading portions of the development of equations. We also thank Mr. Vincent Powers for obtaining GC-MS data on the MS-25 mass spectrometer.

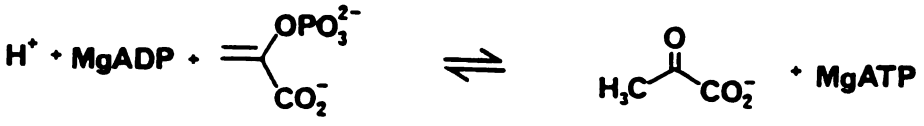
This research was supported by a grant from the NIH AM 17323 to GLK. RER is grateful to the American Foundation for Pharmaceutical Education and the Johnson & Johnson Foundation

for support of graduate studies. Construction of the UCSF wide-bore 240-MHz spectrometer was funded by NIH Grant GM 27387.

Part II

Historical Background–Pyruvate Kinase

Pyruvate kinase (PK) catalyzes the phosphoryl transfer from phosphoenolpyruvate (PEP) to ADP in the glycolytic pathway:



The metabolic conversion of PEP to pyruvate was reported in 1934 by Meyerhof and Lohmann (Meyerhof, 1934). Later workers showed that ATP and ADP were the other reactants (Lehmann, 1935) (Litwak-Mann, 1935) (Boyer, 1942) (Boyer, 1943). Pyruvate kinase was first isolated from rat muscle (Bucher, 1955) . However, the most commonly studied isozyme is that initially crystallized from rabbit muscle by Tietz and Ochoa (Tietz, 1958). The rabbit muscle form of pyruvate kinase is thus far the best characterized of all such isozymes (Boyer, 1962) (Kayne, 1973) (Hall, 1978) (Munday, 1980). This highly exergonic reaction ($\Delta G = -7.5$ kcal/mole) is quite important both to the utilization of glucose and to the regulation of cellular ATP and glucose supplies. The reaction is an essentially irreversible step in the glycolytic pathway. Gluconeogenesis is therefore achieved by a completely different pathway. Thus, PK is an important point in metabolic control.

There are several types of PK including M-type from muscle, brain and heart, L-type from liver and kidney, and K-type from fetal tissues and some adult tissues(Chalkley, 1976). The M-type enzyme has a molecular weight of 237,000 and is composed of 4 identical subunits, whereas the L-type has a molecular weight of only 208,000. The L-type is stimulated by fructose-1,6-diphosphate, however the muscle form is not affected. Therefore, in liver, where L-type PK is the predominant form, high concentrations of glucose lead to its selective metabolism. One important role of insulin is to increase the biosynthesis of PK in the liver to facilitate the removal of glucose from the bloodstream(Lehninger, 1975). Down control of PK by cAMP-dependent phosphorylation of the enzyme may facilitate both gluconeogenesis and fatty acid synthesis via glycogen(Munday, 1980). Although the total amount of PK in muscle tissue is high, the relative activity exhibited *in vivo* is much lower. Several mechanisms may be responsible for this. The normal physiological concentration of the substrates (ADP and PEP) are of the same order of magnitude as the K_m's whereas product levels (ATP) are high compared to the K_m. In addition, phosphocreatine has been shown to inhibit PK at physiological concentrations(Munday, 1980).

The X-ray crystallographic structure of the cat muscle isoenzyme has been determined to a resolution of 2.6 Å(Stuart, 1979). In this way the relative positions of the α-carbons in the peptide backbone have been established. The binding sites for the substrates and a second ADP binding site far from the active site have also been determined by this work. Both PK and

triosephosphate isomerase (TIM) possess a similar α,β-barrel motif at the sites for pyruvate and triose phosphate binding, respectively (Levine, 1978). It is believed that these similarities are related to similarities in mechanism of the enolization and isomerization reactions catalyzed by these two enzymes. In TIM, Glu 165 has been identified as the base involved in pulling off the proton in the isomerization mechanism (Muirhead, 1981). In spite of the lack of sequence information, Muirhead et al. have been able to determine that a glutamate residue is near the binding site of pyruvate. This residue may be in the proper orientation to be the acid-base catalyst in the enolization reaction catalyzed by PK. To date, no common link between the 3 dimensional structure of PK and those of the other kinases has been established. However, this may be largely because the X-ray data available thus far for most kinases only outline the α-carbon backbone structures.

Partial sequence information is available for PK from rabbit, rat, cat, bovine M-types and of the rat and porcine L-types. Comparison of 8-20 amino acid residue sequences from the phosphorylation site of the rat and porcine liver enzyme (Edlund, 1975) (Hjelmqvist, 1974) showed some homology with the corresponding section of the yeast gene (Burke, 1983). A 34 amino acid sequence surrounding the essential lysine at the ADP binding site of bovine muscle pyruvate kinase is 77-91% homologous with the yeast enzyme (Johnson, 1979) (Burke, 1983). The L-type PK from rat liver has been cloned (Simon, 1983) (Noguchi, 1983). However, a complete amino acid sequence is available only for PK from *Saccharomyces cerevisiae* (Burke, 1983) (Lonburg, 1985).

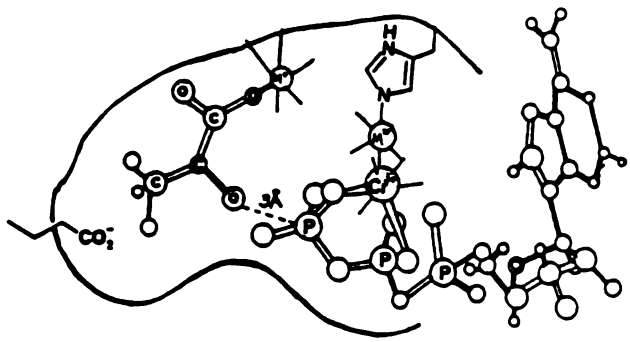
The stereochemistry of the transfer of the phosphoryl group from ATP to pyruvate in the PK reaction is essentially the same as for creatine kinase. All available evidence points toward a mechanism in which direct phosphoryl transfer occurs in a single associative step(Hassett, 1982)(Blattler, 1979). From early kinetic studies it was known that PEP and ATP bind competitively to the active site. Therefore, the sites are at least adjacent as required for direct phosphoryl transfer. More recently, it has been shown that the phosphoryl group is not transferred from ATP in the absence of a phosphoryl acceptor at the active site and the transfer occurs with complete retention of configuration(Blattler, 1979). These studies rule out both mechanisms involving formation of a metaphosphate intermediate and mechanisms involving a two-step phosphoryl transfer via a phosphorylated enzyme intermediate.

Although PK is a kinase, the reaction catalyzed is actually two chemical reactions. One step is phosphoryl transfer and the other is the enolization of pyruvate (Rose, 1960). Certain stereochemical aspects of the enolization reaction have been well established by the use of isotopically labeled pyruvate, PEP and enolpyruvate analogs. The proton transferred comes from the 2-*si*-3-*re* face of enolpyruvate(Rose, 1970)(Stubbe, 1971)(Adlersberg, 1970).

Covalent modification studies with methanethiosulfonate have shown that the Cys at the ATP binding site of PK is essential to catalytic activity. Methanethiolation of all 4 Cys residues per PK monomer completely inactivates the enzyme. In contrast, selective protection of only one Cys by MgATP does not inactivate

the enzyme (Chalkley, 1976)(Bloxham, 1978). Both an arginyl residue and a lysyl residue have been shown to be present at the ADP binding site (Berghauser, 1977). In addition, a histidyl residue on the enzyme is bound to the second divalent metal cation which is not a part of the metal-nucleotide complex(Mildvan, 1966)(Meshitsuka, 1981).

In spite of the lack of an overall view of the three dimensional structure of the enzyme, a picture of the three dimensional characteristics of the active site of the central complexes has emerged as a composite of many studies. A review of the pertinent studies and a model of the active site is presented in an article by A.S. Mildvan(Mildvan, 1981).



The enzyme requires one equivalent of monovalent cation and 2 equivalents of divalent metal cation for full activity. K^+ is believed to coordinate to the PEP/pyruvate carboxyl group and not the enol oxygen as previously hypothesized(Suelter, 1970). One divalent metal is bound to the nucleotide substrate and the other binds directly to the enzyme via a His residue (Meshitsuka, 1980)(Gupta, 1976). The use of bidentate and tridentate isomers of

CrATP and bidentate isomers of CrADP(Dunaway-Mariano, 1980)(Dunaway-Mariano, 1979) and of phosphorothioate analogs (Jaffe, 1978) led the the conclusion that the active conformation of MgATP is analogous to that of Δ bidentate β,γ-CrATP. The spatial relationships between the metals and the substrates at the active site have been partially established through the use of paramagnetic probes(Sloan, 1975)(Melamud, 1975)(Fung, 1973)(Gupta, 1976)(Gupta, 1978)(Mildvan, 1976).

Initial kinetic studies of the enzyme-catalyzed reaction were consistent with a rapid equilibrium, random, bimolecular mechanism(Reynard, 1961). Subsequent studies have confirmed that the mechanism is random. However, studies of the rate of PK catalyzed detritiation of pyruvate by Robinson and Rose show that pyruvate release might be rate limiting(Robinson, 1972). Recently, the PEP⇌pyruvate equilibrium isotope exchange rates were found to be the slower than the other exchanges measured. This supports the conclusion of Robinson and Rose that binding and release of pyruvate contributes towards the rate determining step(Giles, 1976). In addition, PEP binding was found to be rate-limiting when it binds first (Dann, 1978) . The pH rate profile shows two breaks at pH 6.63 and 9.00. These pK_a's are consistent with a mechanism where the β-phosphorus of ADP is the nucleophile and a lysine ε-amino group is involved as a proton donor(Gregory, 1981)

The binding site of the non-nucleotide substrates has been probed by the use of both inhibitors and alternate substrates. Several inhibitors of the PK reaction have been found. Oxalate,

an analog of *enol*-pyruvate, inhibits pyruvate kinase competitively with a K_i value of 4.7 μM (Reed, 1974). but it inhibits a variety of other enzymes as well. 1-Hydroxycyclopropane carboxylic acid phosphate, phosphoglycolate, and phospholactate have K_i values of 2 mM, 2 mM, and 7.2 mM, respectively. These phosphorylated analogs also inhibit a variety of other PEP utilizing enzymes (O'Leary, 1981). An alternate substrate analog, 2-hydroxy-3-nitropropionate, was shown to inactivate the enzyme in an ATP-dependent way (Porter, 1983). However the inactivation was linked to non-enzymatic decomposition of the phosphorylation product and was not specific for the active site of pyruvate kinase. Thus, no specific strongly competitive or irreversible inhibitors have been found for this enzymatic reaction.

Pyruvate kinase has long been known to catalyze the ATP-dependent phosphorylation of hydroxylamine and fluoride ion. (Kupiecki, 1960) (Tietz, 1958) Stubbe and Kenyon have investigated this specificity using several synthetic analogs of PEP (Stubbe, 1971) (Stubbe, 1972). Recently, it has been shown that a number of α-hydroxy acids are substrate for pyruvate kinase (Ash, 1983) (Porter, 1983). Part of the purpose of this thesis work is to probe the active site further by studying the interaction of the enzyme with other analogs of PEP and pyruvate in this series.

Chapter 3

Substrate Specificity in Pyruvate Kinase

Introduction

As discussed in the historical background section pyruvate kinase catalyzes the phosphoryl transfer from phosphoenolpyruvate (PEP) to ADP in the glycolytic pathway. The net two ATPs per glucose formed directly in glycolysis come from this step. Most of the ATP derived from glucose comes from oxidative phosphorylation. Pyruvate kinase is of critical importance to the bloodstream form of *Trypanosoma brucei*. This organism, which causes a sleeping sickness in cattle in parts of Africa, derives 100% of the ATP needed to perform its cellular functions from the glycolytic pathway(Opperdoes, 1981). As a result, inhibition of this enzyme might be expected to lead to the death of such parasites present in the bloodstream(Opperdoes, 1981). Thus, inhibitors of pyruvate kinase may be useful as antiparasitics. Several inhibitors of PK are known, but to date no specific strongly competitive or irreversible inhibitors have been found for rabbit muscle pyruvate kinase.

A rare deficiency of erythrocyte PK has been linked to a form of hemolytic anemia. However, recent work in this field suggests that the clinical manifestations may be more related to the kinetic and allosteric properties of the enzyme produced than by diminished PK activity in the cells(Lakomek., 1983). Preliminary results for cases of acquired PK deficiency in leukemia patients are consistent with these findings. Thus, inhibition of host erythrocyte

PK would not necessarily be an unacceptable side effect in the use of PK inhibitors as antiparasitic agents.

Since the isolation of *T. brucei* PK in quantities needed for our preliminary experiments was not economically feasible (Flynn, 1980), we began our study using the pyruvate kinase isolated from rabbit muscle as a model. The X-ray crystallographic structure of the cat muscle isoenzyme has been determined to a resolution of 2.6 Å (Muirhead, 1981). This has made it possible to tentatively identify some of the amino acid residues on the α-carbon chain, but a detailed three dimensional model of the nature and orientation of the individual side chains is still unavailable. Magnetic resonance studies using paramagnetic probes have led to a model of the spatial arrangement of the monovalent and divalent cations, pyruvate and the metal-nucleotide complex at the active site (Mildvan, 1981). The gene sequence of the pyruvate kinase isozyme from *Saccharomyces cerevisiae* has only recently been determined (Burke, 1983)(Lonberg, 1985). Thus, in spite of all that is known about pyruvate kinase, detailed knowledge about the interactions between enzyme and substrates is not available. One logical first step in the search for inhibitors in such a case is the study of enzyme specificity. This information can be used in conjunction with other available information to design useful inhibitors of pyruvate kinase.

Pyruvate kinase catalyzes the ATP-dependent phosphorylation of hydroxylamine and fluoride ion. (Kupiecki, 1960)(Tietz, 1958) Stubbe and Kenyon have investigated this specificity using several synthetic analogs of PEP(Stubbe, 1971)(Stubbe, 1972). Recently, it has

Table I. Compounds Tested as Alternate Substrates for Pyruvate Kinase.

Number	Name of Compound	Structural Formula	Substrate Activity
1	vinylglycolate	$CH_2{=}CHCH(OH)CO_2^-$	+
2	isoserine	$H_2NCH_2CH(OH)CO_2^-$	+
3a	*erythro*-β-hydroxy-aspartate	$HO_2CCH(NH_2)CH(OH)CO_2^-$	-
3b	*threo*-β-hydroxy-aspartate	$HO_2CCH(NH_2)CH(OH)CO_2^-$	-
4	α-hydroxy-isovalerate	$(CH_3)_2CHCH(OH)CO_2^-$	-
5	α-hydroxy-isocaproate	$(CH_3)_2CHCH_2CH(OH)CO_2^-$	-
6	α-hydroxy-β-methyl-valerate	$CH_3CH_2CH(CH_3)CH(OH)CO_2^-$	-
7	α-hydroxy-α-methyl-butyrate	$CH_3CH_2C(CH_3)(OH)CO_2^-$	-
8	α-hydroxy-α-ethyl-butyrate	$CH_3CH_2C(CH_3CH_2)(OH)CO_2^-$	-
9	oxalomalate	$HO_2CCHCOCO_2^-$	-
10	isocitrate	$HO_2CCH_2CH(CO_2^-)CH(OH)CO_2^-$	-

Table I. Compounds Tested as Alternate Substrates for Pyruvate Kinase.

Number	Name of Compound	Structural Formula	Substrate Activity
11	oxalate	$HO_2CCO_2^-$	-
12	citrate	$HO_2CCH_2C(CO_2^-)(OH)CO_2^-$	-
13	ketomalonic hydrate	$HO_2CC(OH)_2CO_2^-$	-
14	argininate	$H_2N-C(=NH)NH(CH_2)_3CH(OH)CO_2^-$	-
15	tartrate	$HO_2C(CHOH)CO_2^-$	-
16	D-mandelate	$\phi-CH(OH)CO_2^-$	-
17	p-hydroxy-mandelate	$p-OH-\phi-(OH)CO_2^-$	-
18	m-hydroxy-mandelate	$m-OH-\phi-(OH)CO_2^-$	-
19	p-hydroxy-phenyllactate	$p-OH-\phi-CH_2(OH)CO_2^-$	-
20	m-hydroxy-phenyllactate	$m-OH-\phi-CH_2(OH)CO_2^-$	-

Table I. Compounds Tested as Alternate Substrates for Pyruvate Kinase.

Number	Name of Compound	Structural Formula	Substrate Activity
21	4-hydroxy-3-methoxy-phenyllactate	p-OH-m-MeO-ϕ-CH_2(OH)CO_2^-	-
22	4-hydroxy-3-methoxymandelate	p-OH-m-MeO-ϕ-(OH)CO_2^-	-

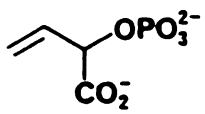
been shown that a number of α-hydroxy acids are substrate for pyruvate kinase in the following general reaction(Ash, 1983)(Porter, 1983):

$$MgATP + R\text{-}CHOHCO_2^- \rightarrow MgADP + R\text{-}CH(OPO_3^{2-})CO_2^- \quad \text{Eq.2}$$

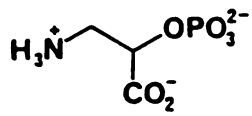
We sought to find new alternate substrates in this series to probe the active site of pyruvate kinase. We hoped this study would assist in the design of inhibitors of pyruvate kinase potentially useful as either antiparasitic agents or as further probes of the enzyme's active site. Thus, the α-hydroxy acids and α-ketoacids in **Table I** were examined for substrate activity.

With two of the compounds, vinylglycolate **(1)** and isoserine **(2)**, it has been possible to characterize the phosphorylated product **Figure 1** by ^{31}P NMR spectroscopy. However, detailed kinetic analyses of the enzymatic syntheses of these products were not possible. This was so because prohibitively large amounts of enzyme would have been needed to produce the data for such kinetic analyses.

The results of the attempts to monitor the initial rates of phosphorylation of vinylglycolate **(1)** suggested that phosphovinylglycolate **(23)** was a strong product inhibitor. In addition, it was noted that the presence of a phosphoryl group at the C-2 oxygen would be expected to activate phosphovinylglycolate as a Michael acceptor. If a nucleophile on the enzyme attacked at C-4, the enzyme might be irreversibly inactivated **(Figure 2)**. Therefore, phosphovinylglycolate was isolated and tested as an



23



24

Figure 1 Structures of phosphovinylglycolate and phosphoisoserine.

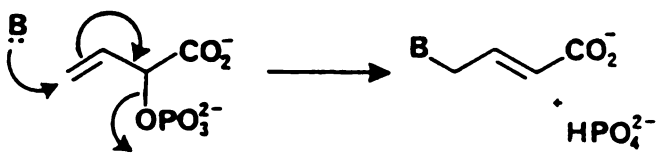
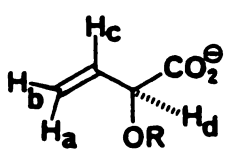


Figure 2 Michael attack of a nucleophile at C-4 of phosphovinylglycolate.



R = H, PO_3^{2-}

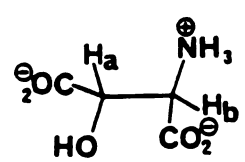


Figure 3 Protons assigned by ^1H NMR.

inhibitor. However, it was found to be neither an irreversible inactivator of pyruvate kinase nor a potent competitive inhibitor of the pyruvate kinase-catalyzed phosphoryl transfer between PEP and MgADP.

In the course of this study it was noticed that two of the compounds prepared for this study had not previously been characterized by ^1H NMR. Interpretations of the proton NMR spectra for vinylglycolate, phosphovinylglycolate and the erythro- and threo- diastereomers of β-hydroxyaspartate are reported here for the first time. By using the Karplus relation it is possible to comment on the probable conformations of the two diastereomers of β-hydroxyaspartate.

Materials and Methods

Pyruvate kinase was prepared from rabbit muscle from Pel-Freeze Biologicals by the method of Tietz and Ochoa. Adenylate kinase was removed by passage of the enzyme through Sephacryl S-200 in 0.1 M tetramethylammonium/HCl, 0.05 M HEPES pH 7.5 (3.5 cmx 1 m). Phosphoenolpyruvate(cyclohexylammonium salt), ADP, ATP and NADH were purchased from Sigma. Lactate dehydrogenase (pyruvate kinase-free) was purchased from Boeringer Mannheim. All substrate analogs except vinylglycolate and the *erythro-* and *threo-* diastereomers of β-hydroxyaspartate were purchased from Sigma.

Organic Syntheses

Vinylglycolate was synthesized by literature preparation(Glattfeld, 1935). The *erythro-* and *threo-* diastereomers of β-hydroxyaspartate were also synthesized and

separated according to literature preparations (Korngluth, 1960) (Benoiton, 1959).

Enzymatic Reactions

A. Screening Experiments

Two 1-mL solutions of 20 mM analog, 7 mM ATP, 5 mM $MnCl_2$, 100 mM KCl ,and 50 mM HEPES (pH 8.0) were made for each of the 26 compounds in **Table I**. In addition, one control solution was made to contain all of the components except for the substrate analog. One to two mg/ml pyruvate kinase was added to the first of each pair of solutions. The second of each pair was incubated in the absence of enzyme as a control for nonenzymatic reaction. After incubation for 20-24 hours at 25 °C, each reaction mixture was analyzed for formation of ADP by PEI-UV cellulose TLC as described by Rowley et al. (Rowley, 1974).

B. Preparation of Phosphorylation Products for ^{31}P NMR Characterization

Two mL solutions of 50 mM analog, 20 mM ATP, 30 mM $MgCl_2$, 100 mM KCl ,and 50 mM HEPES (pH 8.0) were made for each of the following compounds: tartrate, vinylglycolate , isoserine ,oxalomalic acid , p-hydroxyphenyllactic acid, and *erythro*- and *threo*-β-hydroxyaspartic acid. Each was incubated with 1.5-2.0 mg/ml pyruvate kinase at 25 °C. After incubation for 20-24 hours the reaction mixtures were made 20% in D_2O and analyzed by ^{31}P NMR spectroscopy.

C. Kinetic Analysis of Enzymatic Reactions

The rates of the reaction of isoserine and vinylglycolate with pyruvate kinase in the presence of Mn^{2+} and ATP at pH 8.0 were

examined by pH stat. Each incubation mixture contained 2 mM ATP, 30 mM $MnCl_2$, 100 mM KCl ,and up to 10 mM of either vinylglycolate or isoserine. Reactions were initiated by the addition of enzyme.

D. Enzymatic Synthesis and Isolation of Phosphovinylglycolate

A 50 mL solution of 50 mM vinylglycolate, 20 mM ATP, 5 mM $MnCl_2$, 100 mM KCl and 50 mM HEPES/KOH, pH 8.0 was incubated for over 20 hours at room temperature with 2 mg/ml rabbit muscle pyruvate kinase. Analysis of the reaction mixture on PEI-cellulose TLC with 1.2 N LiCl showed that the ATP had been completely consumed. Protein was removed by ultrafiltration using a CX-30 Immersible Ultrafilter from the Millipore Corporation. The product was purified by anion-exchange chromatography at 4°C on DEAE-Sephadex A-25 (HCO_3^-) by using a 4 L linear gradient of 0-0.7 M TEA/HCO_3^- , pH 7.8. The product co-eluted with ATP and ADP in a single broad peak as determined by absorbance at 260 nm and ^{31}P-NMR spectral analyses. The nucleotides were removed by stirring a 50-ml solution of the crude product with an equal volume of 25%(w/v) acid washed charcoal in TEA/HCO_3^- buffer. This suspension was filtered through Celite(Switzer, 1978). The filtrate was concentrated to 8 mL and re-chromatographed on DEAE-Sephadex A-25 (HCO_3^-) under the same conditions as before. Fractions were assayed for acid-labile phosphates according to the procedure of Ames(Ames, 1966). The pooled fractions were concentrated under vacuum at below 30°C to remove water and buffer. The product was stored at 0°C (750 µmol; 30% yield). ^{31}P-NMR : d, 2.2 ppm, J_{HP}= 8.8

Hz. The ^{1}H-NMR (m, 5.6 δ; m, 5.4 δ; m, 5.3 δ; m, 4.9 δ) was similar the that of the unphosphorylated precursor, vinylglycolate.

NMR Measurements

^{31}P NMR spectra were obtained at 41.503 MHz on the UCSF XL-100 NMR spectrometer. Both proton coupled and decoupled spectra were recorded. A spectral width of 2000 Hz and 8192 data points were used to acquire the free induction decay. Both a 30° tip angle and no delay between pulses were used in most cases. An exponential line-broadening factor of 0.5 Hz was applied to the total free induction decay prior to Fourier transformation. Normally, 500 aquisitions were collected. Chemical shifts were measured relative to an 85% solution of H_3PO_4 (in D_2O) as an external standard.

Routine ^{1}H NMR spectra were obtained at 80 MHz. High resolution proton spectra were obtained at 240 MHz on the UCSF wide-bore 240 MHz spectrometer. Simulated proton spectra were obtained using the program NTC-SIM by supplying chemical shifts and coupling constants derived from the interpretation of proton spectra.

Results and Discussion

In the course of this study it was noticed that none of the compounds synthesized for this study had previously been characterized by ^{1}H NMR spectroscopy. Therefore, all proton NMR resonances and proton-proton coupling constants for vinylglycolate, phosphovinylglycolate, *erythro*-β-hydroxyaspartic acid and *threo*-β-hydroxyaspartic acid were assigned for the non-

exchanging protons depicted in **Figure 3**. These data are tabulated in **Table II**. The spectra for vinylglycolate and phosphovinylglycolate, shown in **Figures 4** and **5**, respectively, were quite similar. The only qualitative difference between the splitting patterns was the additional splitting of the H_D resonance expected from vicinal coupling to ^{31}P. All coupling constants were consistent with those between similar vinyl and allyl protons. The assignments for phosphovinylglycolate were confirmed by producing a simulated spectrum using the program NCT-SIM on the Nicolet computer **(Figure 6)**.

The spectra for the *erythro-* and *threo-* diastereomers of β-hydroxyaspartate are shown in **Figures 7** and **8**, respectively. The structures of these diastereomers were analyzed using the Karplus relation:

$$10 \cos \theta = J_{HH'} \qquad 0\text{–}90°$$

$$16 \cos \theta = J_{HH'} \qquad 0\text{–}180°$$

Table II

^{1}H NMR Assignments

Compound	Chemical Shift (ppm)				Coupling Constant (Hz)						
	A	B	C	D	J_{ab}	J_{ac}	J_{ad}	J_{bc}	J_{bd}	J_{cd}	J_d
vinyl-glycolate	5.4	5.3	6.0	4.8	1.5	17.1	1.5	10.0	1.5	5.6	-.-
phospho-vinylglycolate	5.4	5.3	6.0	*4.9	1.1	17.2	1.2	10.4	0.0	6.1	•8.8
erythro-β-hydroxy aspartate	4.1	3.5	-.-	-.-	4.2	-.-	-.-	-.-	-.-	-.-	-.-
threo-β-hydroxy-aspartate	4.4	3.6	-.-	-.-	2.2	-.-	-.-	-.-	-.-	-.-	-.-

*Partially obscured by HOD peak.
•Inferred from ^{31}P NMR spectrum.

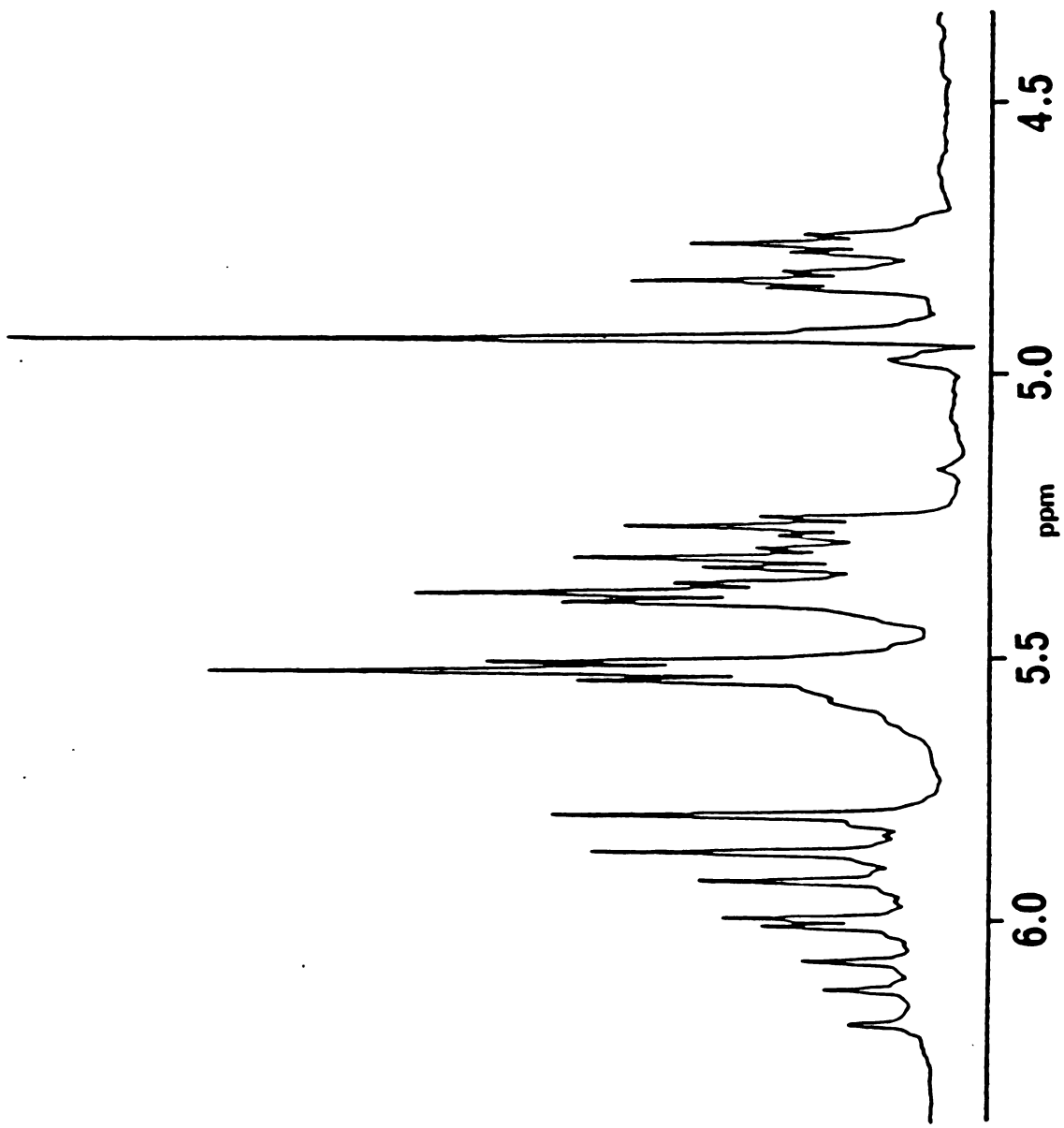


Figure 4 ^{1}H NMR spectrum of vinylglycolate (80 MHz).

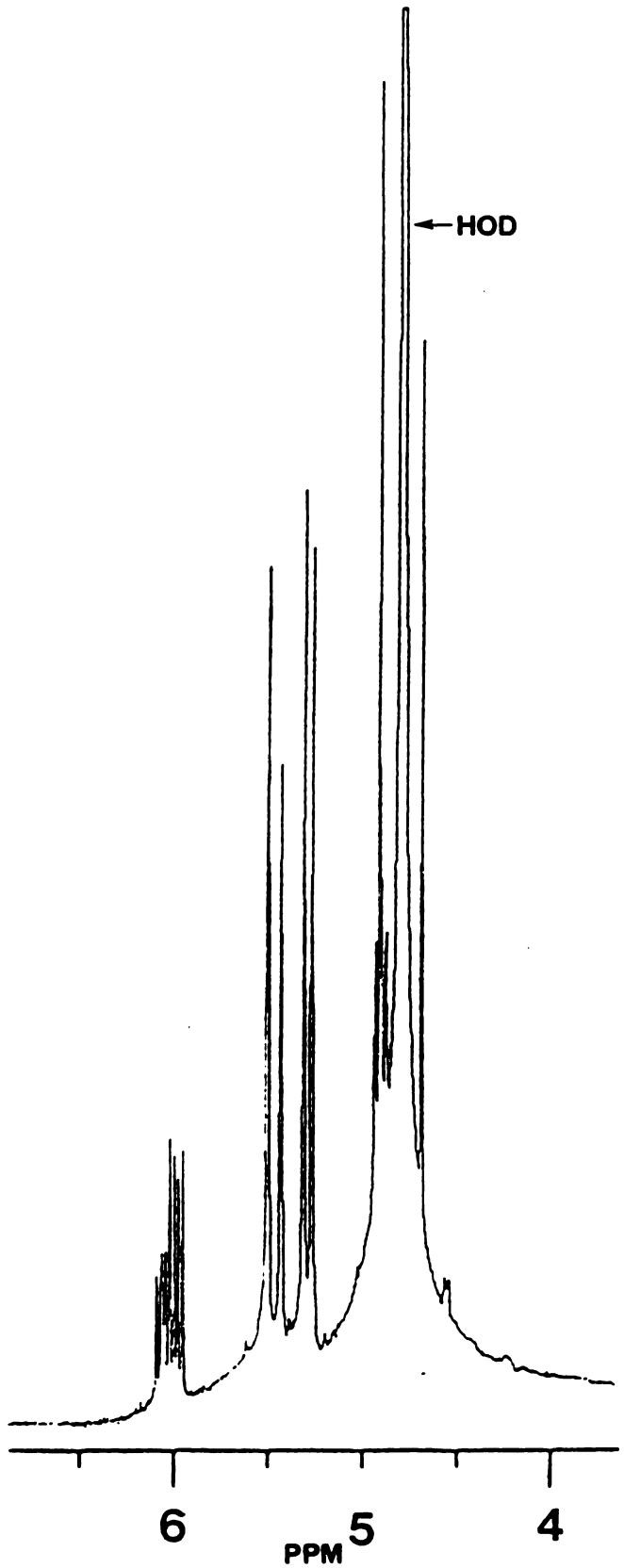


Figure 5 ^{1}H NMR spectrum of phosphovinylglycolate (240 MHz).

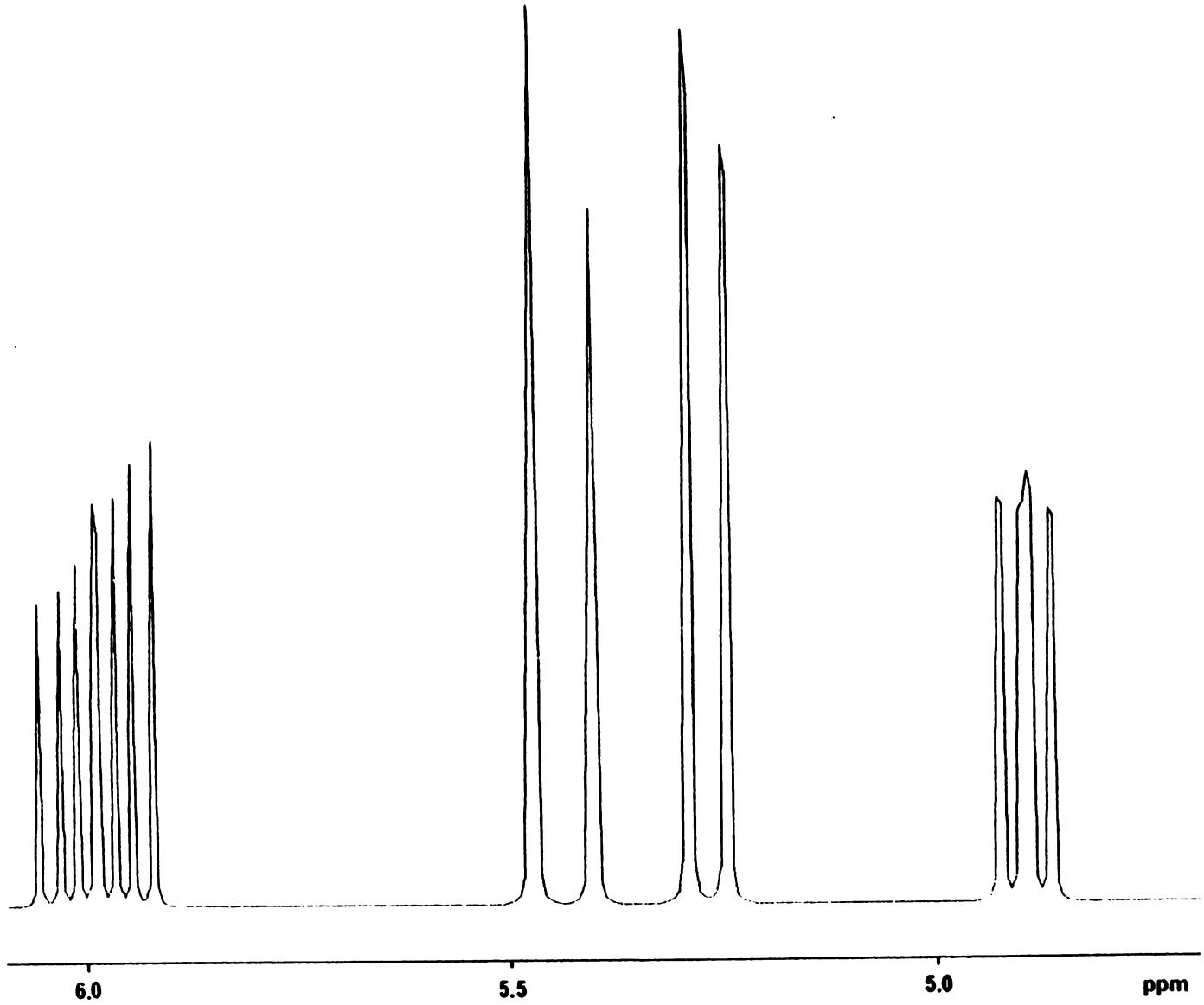


Figure 6 Simulated ^{1}H NMR spectrum of phosphovinylglycolate.

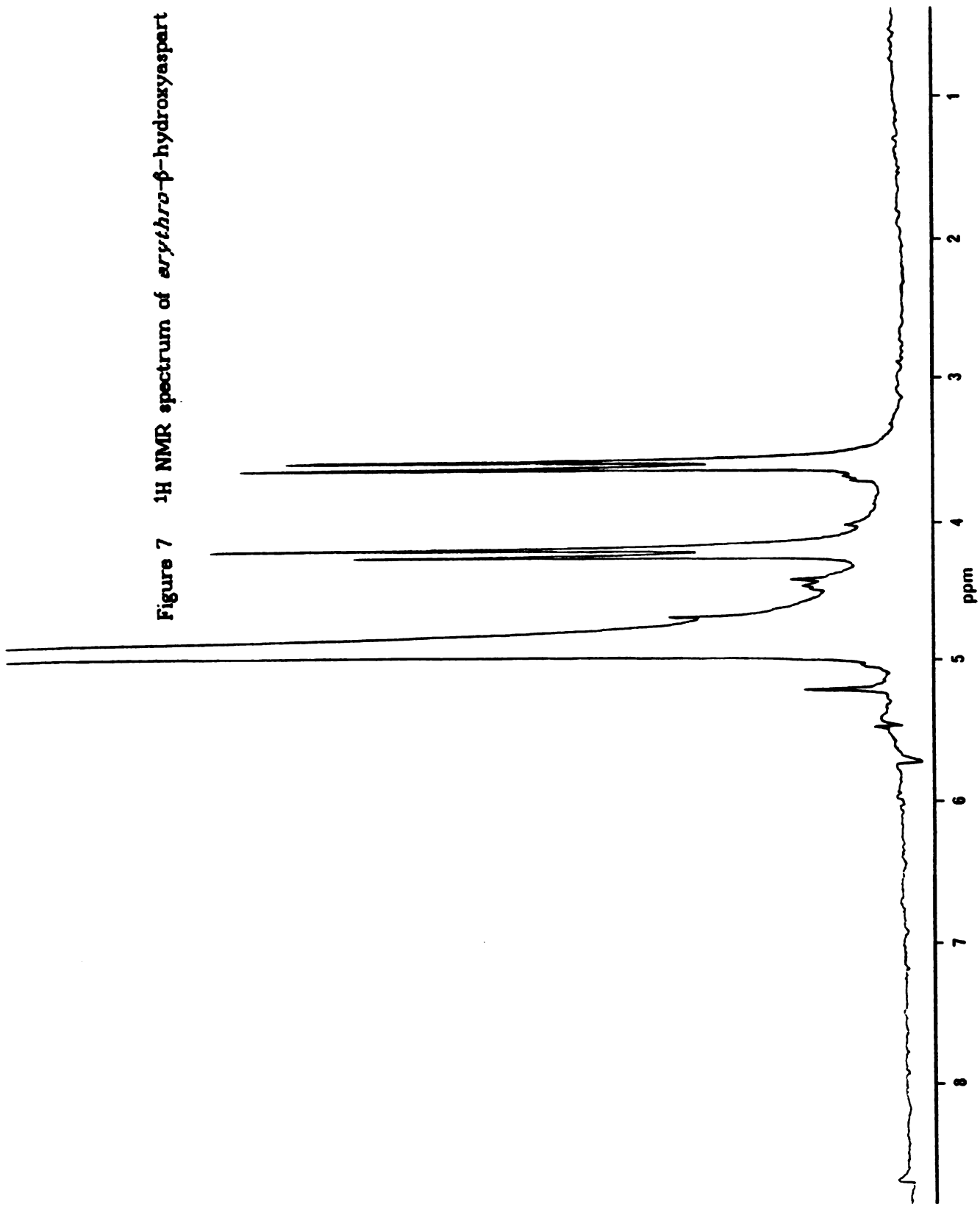


Figure 7 ^{1}H NMR spectrum of *erythro*-β-hydroxyaspart

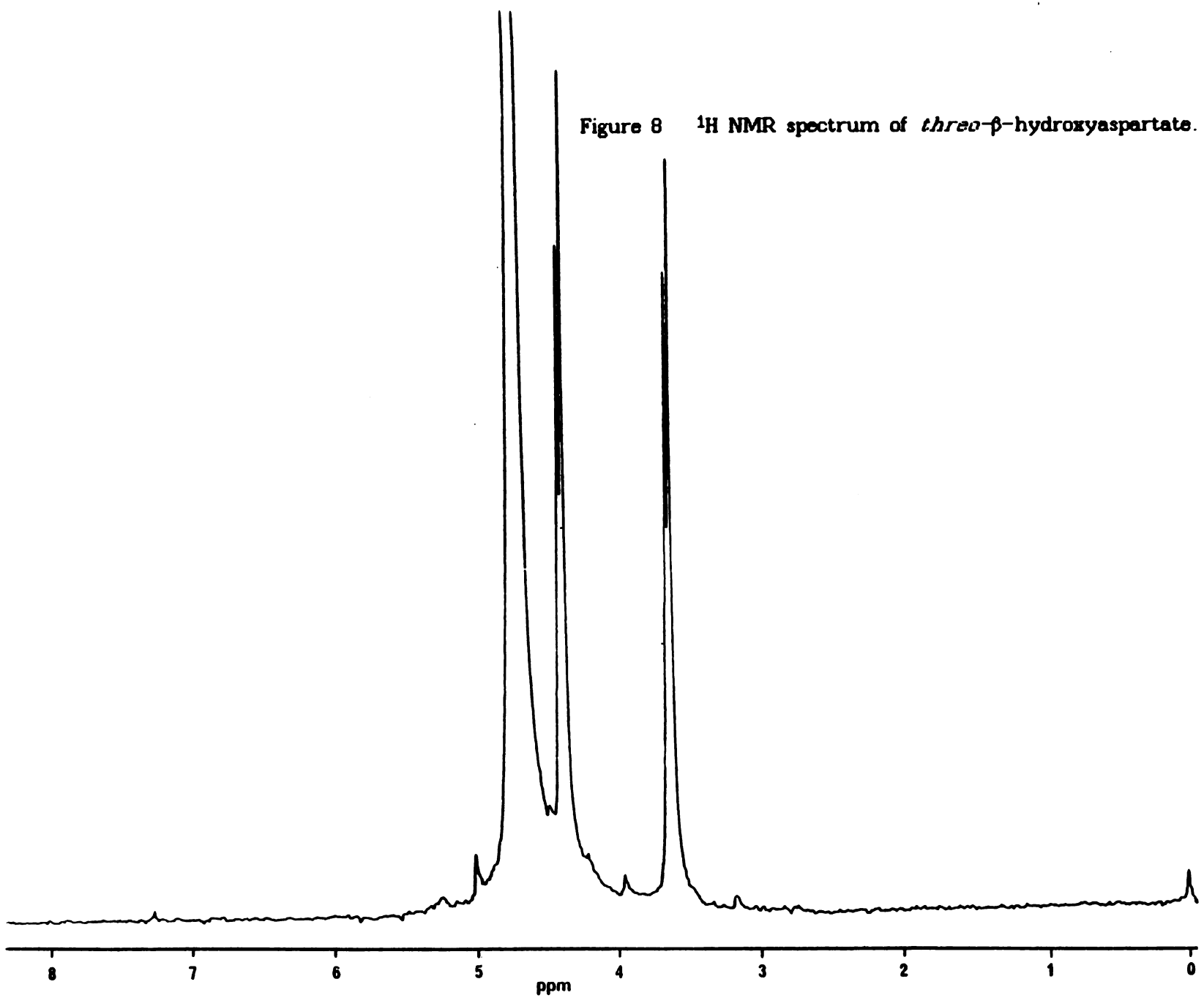


Figure 8 ^{1}H NMR spectrum of *threo*-β-hydroxyaspartate.

Based on this relation, there are two possible solutions to the equation for the average dihedral angle between adjacent protons for each value of $J_{HH'}$ between 0 and 10 Hz. In addition, two distinct conformations of each diastereomer have the same dihedral angle. Newman projections for all four conformations satisfying the Karplus relation for the *erythro*- and *threo*- diastereomers of β-hydroxyaspartate are drawn in **Figures 9** and **10**, respectively. One form of the *threo*- diastereomer shown in **Figure 9B** minimizes unfavorable interactions between the negatively charged α- and β-carboxyl groups while maximizing favorable charge-charge interactions between the β-carboxyl and α-amino groups. However, the groups are nearly eclipsed in this form and there is no precedent for eclipsed conformation in acyclic systems. Of the staggered forms, **Figure 9C**, is somewhat less favorable with respect to charge interactions, but sterically it might be favored over **9D**. It is, therefore, difficult to say which structure most accurately reflects the average conformation of the *threo* diastereomer. With the *erythro* diastereomer the answer is clearer. In the form shown in **Figure 10C** the angle between the two carboxyl groups is nearly 180°. Also, the α-amino and β-carboxyl groups are close, but they do not eclipse one another. Thus, the favored conformation is almost certainly the one shown in **Figure 10.C**.

Each compound in **Table I** was incubated with MnATP in the presence of pyruvate kinase as outlined in the experimental section. Parallel incubations were done in the absence of the enzyme as a control for non-enzymatic reactions. In addition,

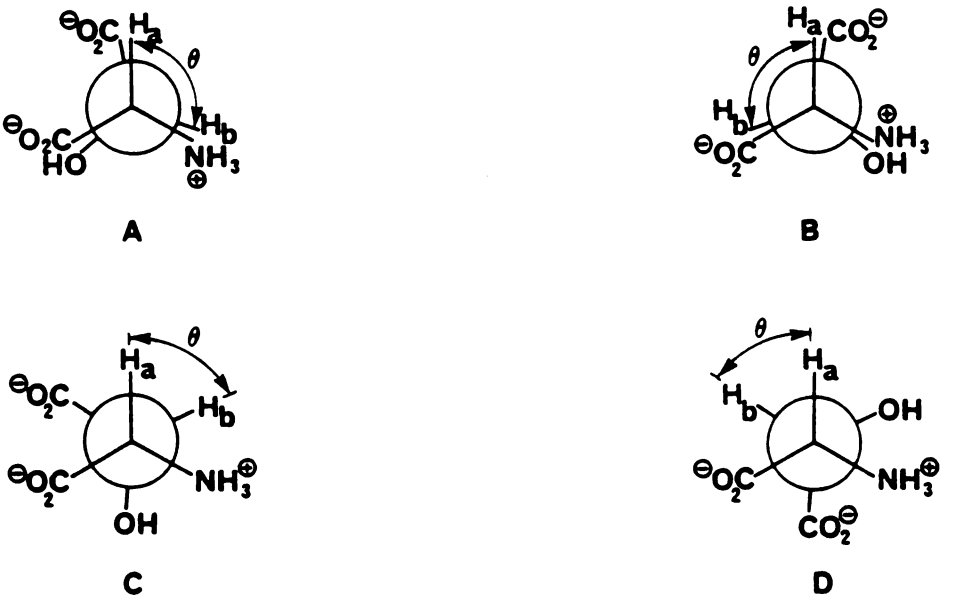


Figure 9 Newman projections of the conformations of *threo*-β-hydroxyaspartate which satisfy the Karplus relation for $J_{HH'}$ = 2.2 Hz.

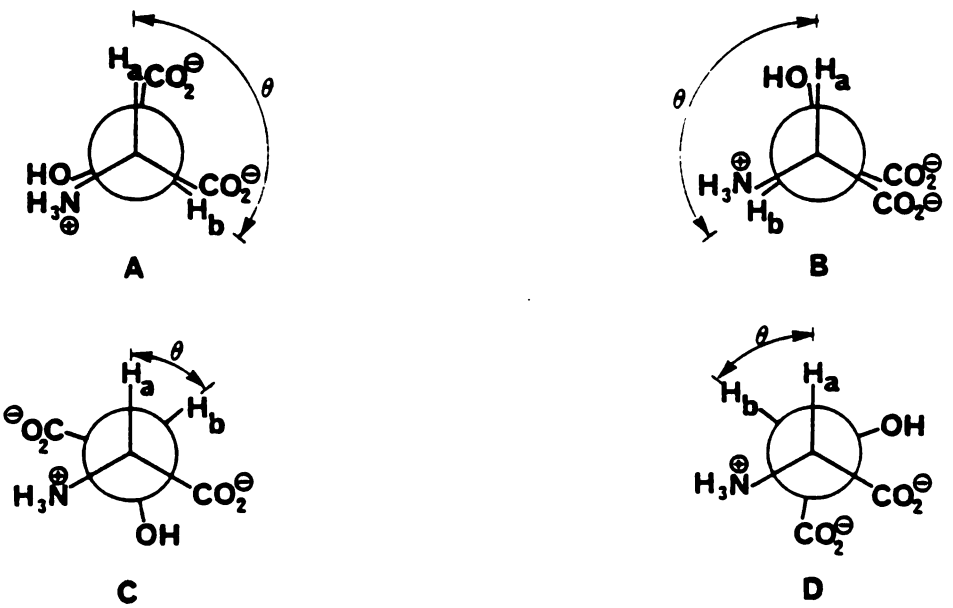


Figure 10 Newman projections of the conformations of *erythro*-β-hydroxyaspartate which satisfy the Karplus relation for $J_{HH'}$ = 4.2 Hz.

one incubation mixture contained all components except for the analog to control for either possible ATPase or adenylate kinase activity. PEI-UV cellulose TLC analysis of mixtures containing vinylglycolate (1), isoserine (2), *erythro*-ß-hydroxyaspartate (3a) or *threo*-ß-hydroxyaspartate (3b), oxalomalate (9), tartrate (15), p-hydroxyphenyllactate (19) showed an increased production of ADP as compared to the no analog or no enzyme controls.

Each of these seven compounds was incubated with MgATP in the presence of pyruvate kinase as outlined in the experimental section to characterize the products by ^{31}P NMR spectroscopy. A control incubation contained no analog. Mg^{2+} was substituted for Mn^{2+} in these enzymatic reactions because Mn^{2+} is paramagnetic and therefore would broaden the resonances of the ^{31}P nuclei. Only for two compounds, vinylglycolate (1) and isoserine (2) was there evidence that the compound was phosphorylated. Proton decoupled and proton coupled ^{31}P NMR spectra of these two products, phosphovinylglycolate and phosphoisoserine and of the no analog control are shown in Figures 11-15, respectively. The ^{31}P NMR data are tabulated in Table III.

It was apparent from the ^{31}P NMR analysis that the phosphorylation of vinylglycolate was nearly complete after 24 hours incubation, i.e., very little ATP remained. This result is consistent with the fact that the equilibrium constant for the reaction with the α-hydroxy acid, glycolate, is ≥ 50 (Rao, 1979). Lineweaver-Burke analysis of the reaction with vinylglycolate gave a value of 0.0015 sec^{-1} for V_{max}/K_m (Figure 16). This is

Table III.

^{31}P NMR Assignments

Compound	Decoupler	Chemical Shift (ppm)	^{1}H–^{31}P Coupling Constant (Hz)
phosphovinyl-glycolate	on	s, 2.1	---
phosphovinyl-glycolate	off	d, 2.2	8.8
phosphoiso-serine	on	s, 3.1	---
phosphoiso-serine	off	d, 3.2	8.8

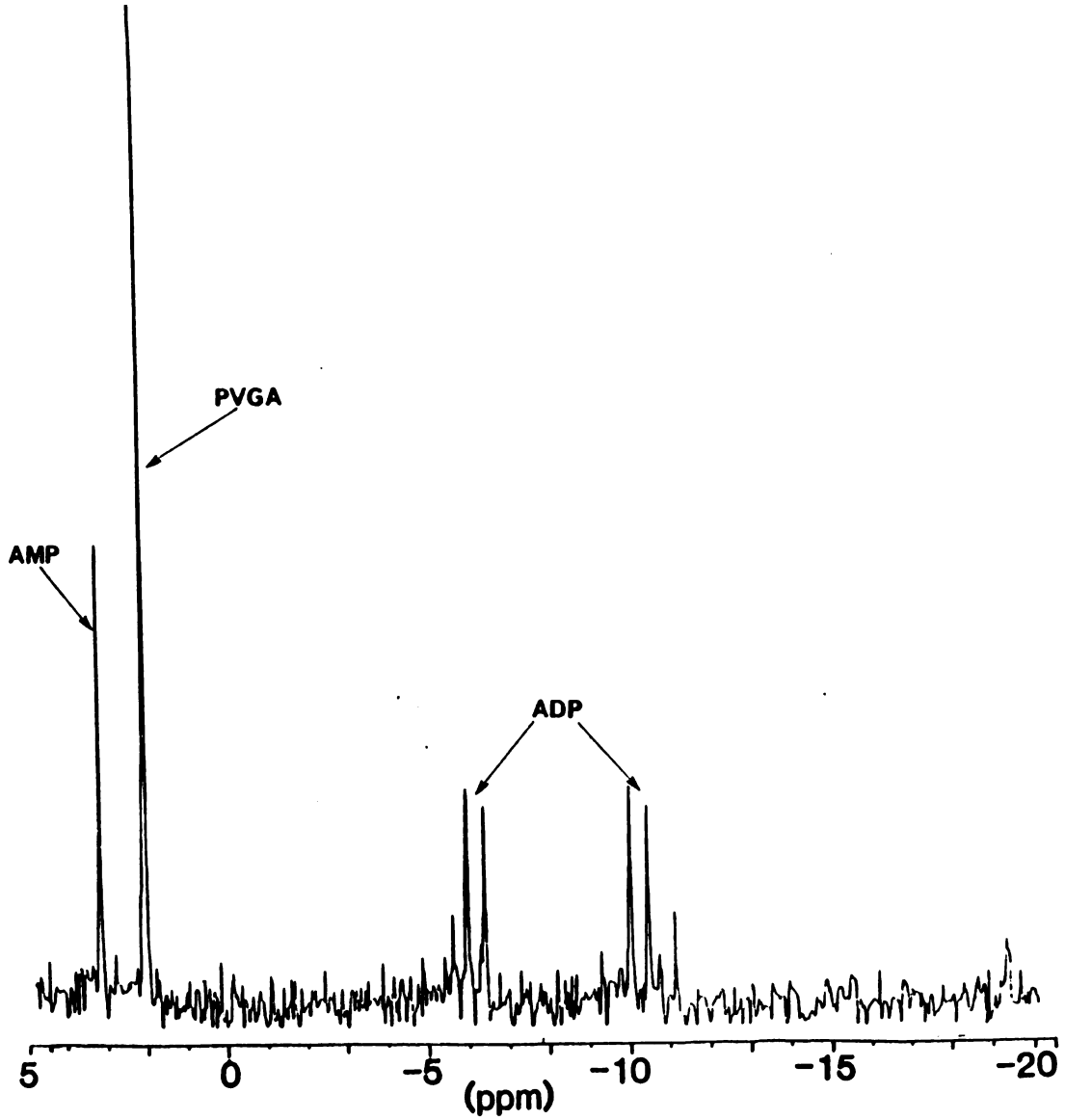


Figure 11 Proton decoupled ^{31}P NMR spectrum of reaction mixture containing vinylglycolate.

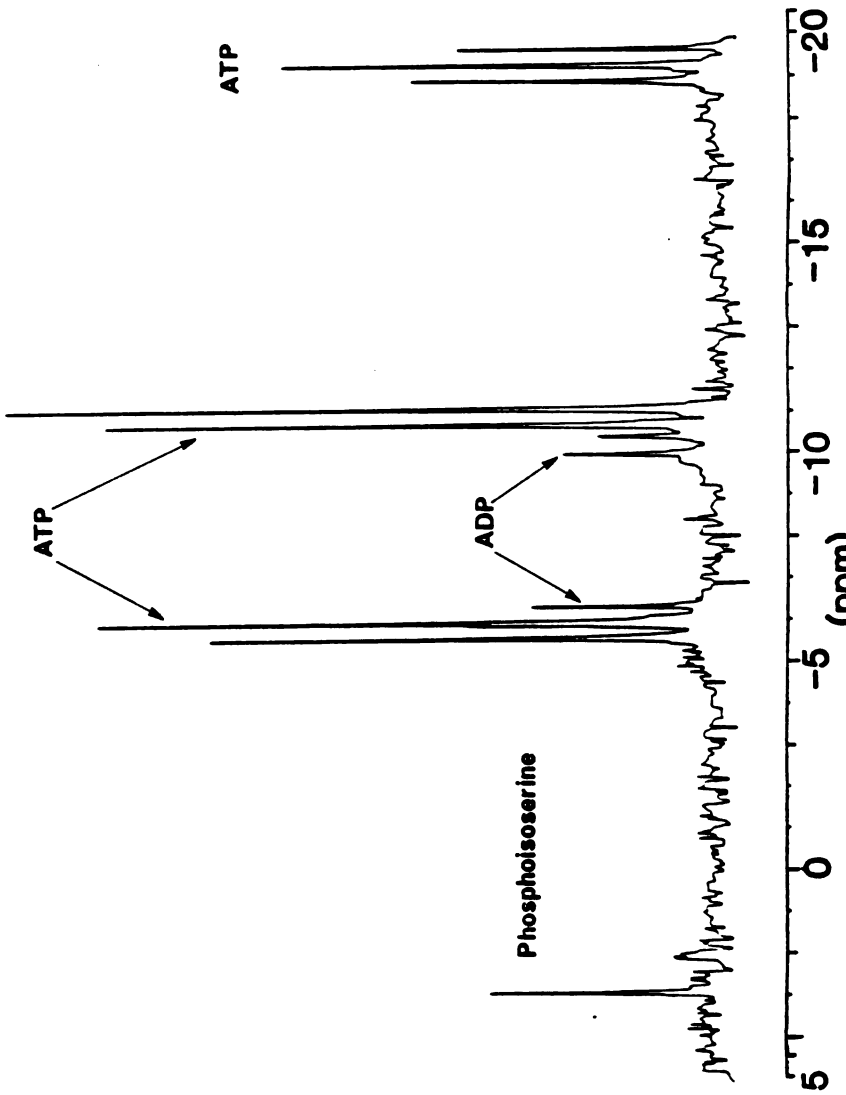


Figure 12 Proton decoupled ^{31}P NMR spectrum of reaction mixture containing isoserine.

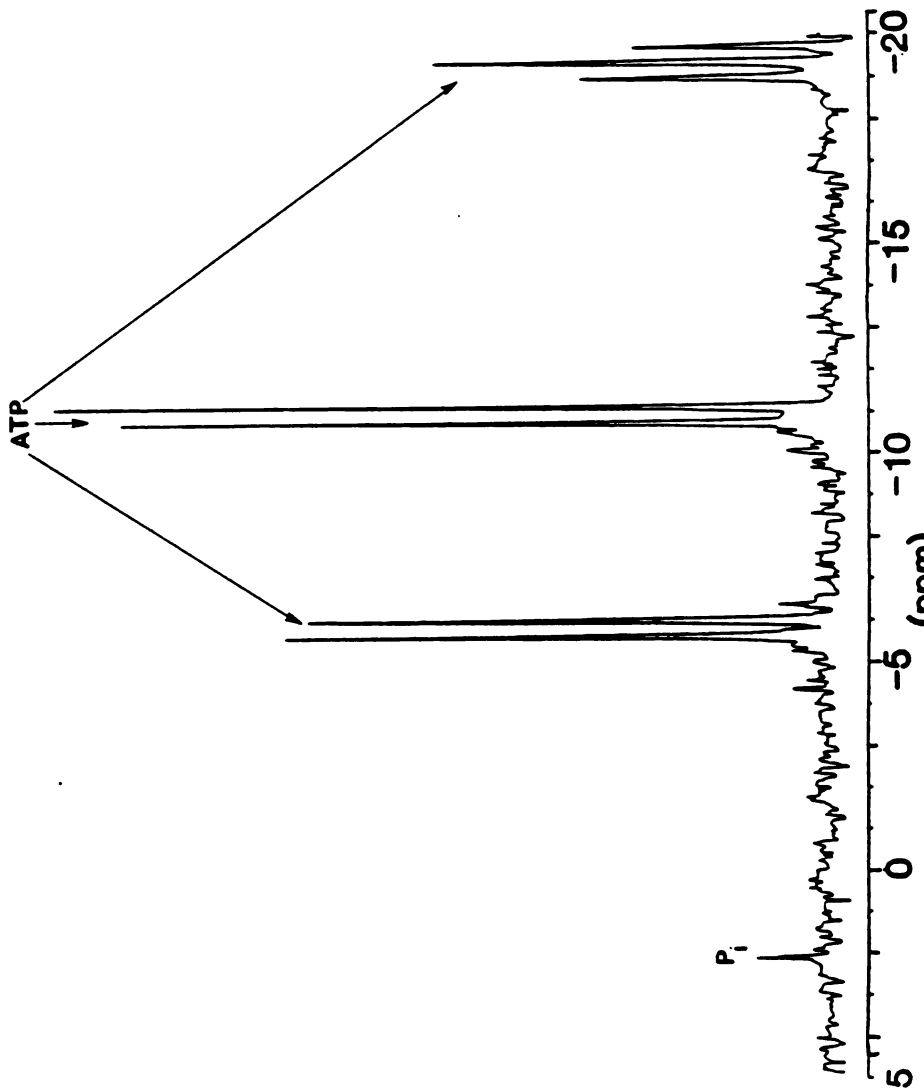


Figure 13 Proton decoupled ^{31}P NMR spectrum of reaction mixture containing no analog (control).

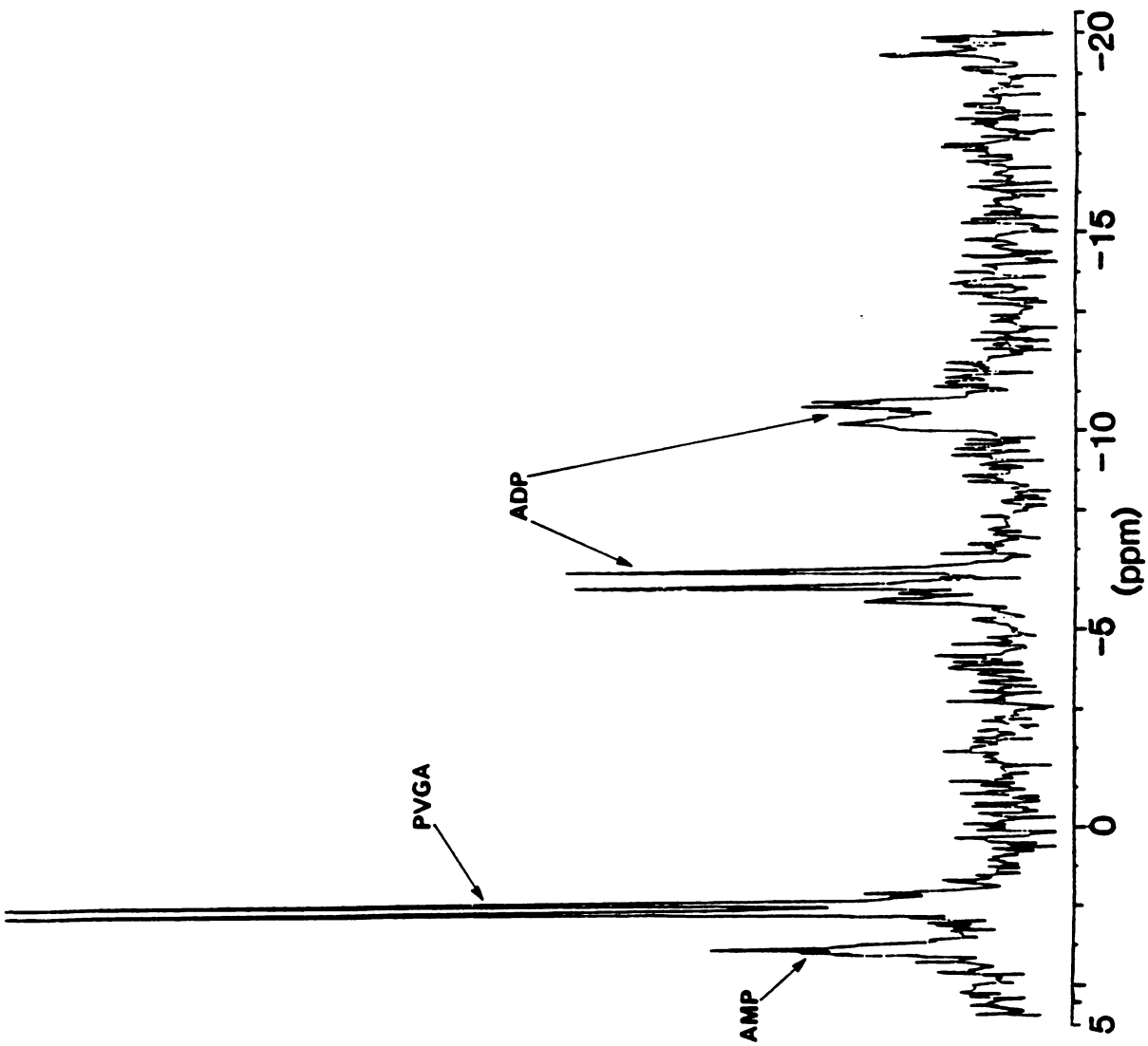


Figure 14 Proton coupled ^{31}P NMR spectrum of reaction mixture containing vinylglycolate.

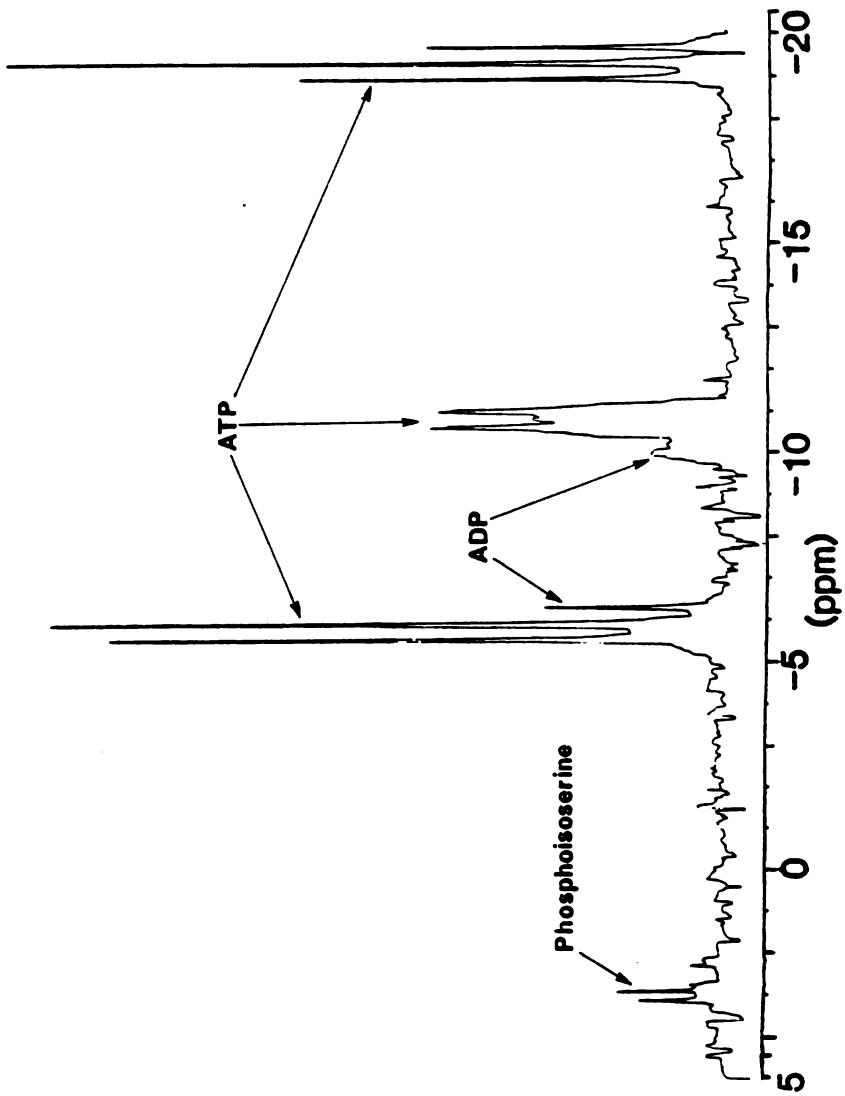


Figure 15 Proton coupled ^{31}P NMR spectrum of reaction mixture containing isoserine.

consistent with the observations of Ash et al.(Ash, 1983) for the reactions of other substrates in a homologous series of straight chain α-hydroxy acids. They reported V_{max}/K_m values of 1.79 sec^{-1} and 0.122 sec^{-1} for glycolate and lactate, respectively, but found that with 4 and 5 carbon homologs the rates were much slower.

In contrast to the reaction with vinylglycolate, the pyruvate kinase-catalyzed phosphorylation of isoserine was still incomplete even after 24 hours. From the ^{31}P NMR spectrum in **Figures 12** and **15** it can be seen that more than half of the initial amount of ATP remained even though isoserine was present in a 2.5-fold excess in the initial reaction mixture. Measurement of V_{max} and K_m for the phosphorylation of isoserine was precluded because no observable rate was detected using the pH-stat method even at high concentrations of substrate and enzyme. There is no reason to believe that the equilibrium constant is significantly different from that for other α-hydroxy acids. Instead, it is more likely that either isoserine is only a poor substrate or the product, phosphoisoserine, is a particularly effective product inhibitor.

Although the rates with vinylglycolate were appreciable in the very initial stages of reaction, they decreased dramatically to extremely low levels as product was formed. Ash et al. reported a similar observation with other hydroxy acid analogs and attributed it to strong product inhibition (Ash, 1983). We decided to to investigate the inhibition properties of phosphovinylglycolate further. The rate of reaction was measured at points along reaction timecourses corresponding to product concentrations of 0,

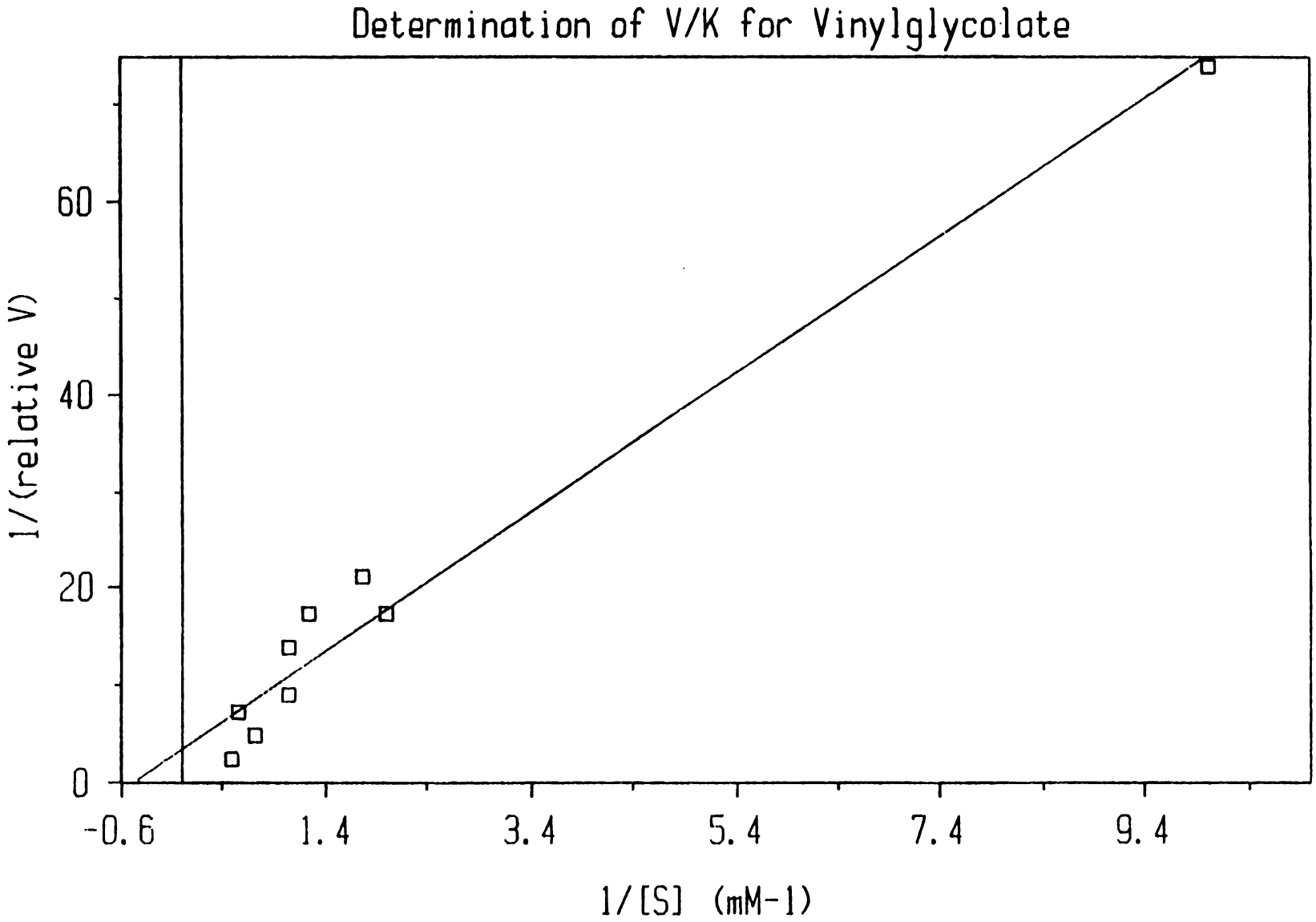


Figure 16 Lineweaver-Burke plot for vinylglycolate.

8, 16 and 24 μM. From these data (not shown) it was estimated that the K_i value was on the order of 10 μM.

As noted in the introduction, phosphovinylglycolate is a potential candidate for irreversible inactivation of pyruvate kinase by the mechanism shown in **Figure 2**. Irreversible inactivation of the enzyme would explain the dramatic fall-off in the rate of reaction to form phosphovinylglycolate. If phosphovinylglycolate were an irreversible inhibitor, the specific activity of enzyme catalyzing the formation of phosphovinylglycolate should decrease as the concentration of this product built up in solution. However, there was no change in the enzyme's specific activity even many turnovers of vinylglycolate to form product.

To investigate the apparent product inhibition further, phosphovinylglycolate was isolated from an enzymatic reaction mixture (**Figure 17**) and found to be a simple competitive inhibitor with a K_i value of 2.5 ± 0.3 mM. Dixon plots and replots of initial rate data are shown in **Figures 18-19**, respectively. K_i values derived for related hydroxyacids such as 1-hydroxycyclopropanecarboxylic acid phosphate, phosphglycolate and phospholactate are in the mM range as well (O'Leary, 1981).

The failure of phosphovinylglycolate to inactivate the enzyme irreversibly may mean that the phosphoryl group is not a good enough leaving group at pH 7-8. It may also mean that there is no nucleophile oriented properly for attack at C-4 of phosphovinylglycolate. However, the fact that vinylglycolate is a substrate and the product, phosphovinylglycolate, is a competive inhibitor indicates that these compounds do bind to the active site

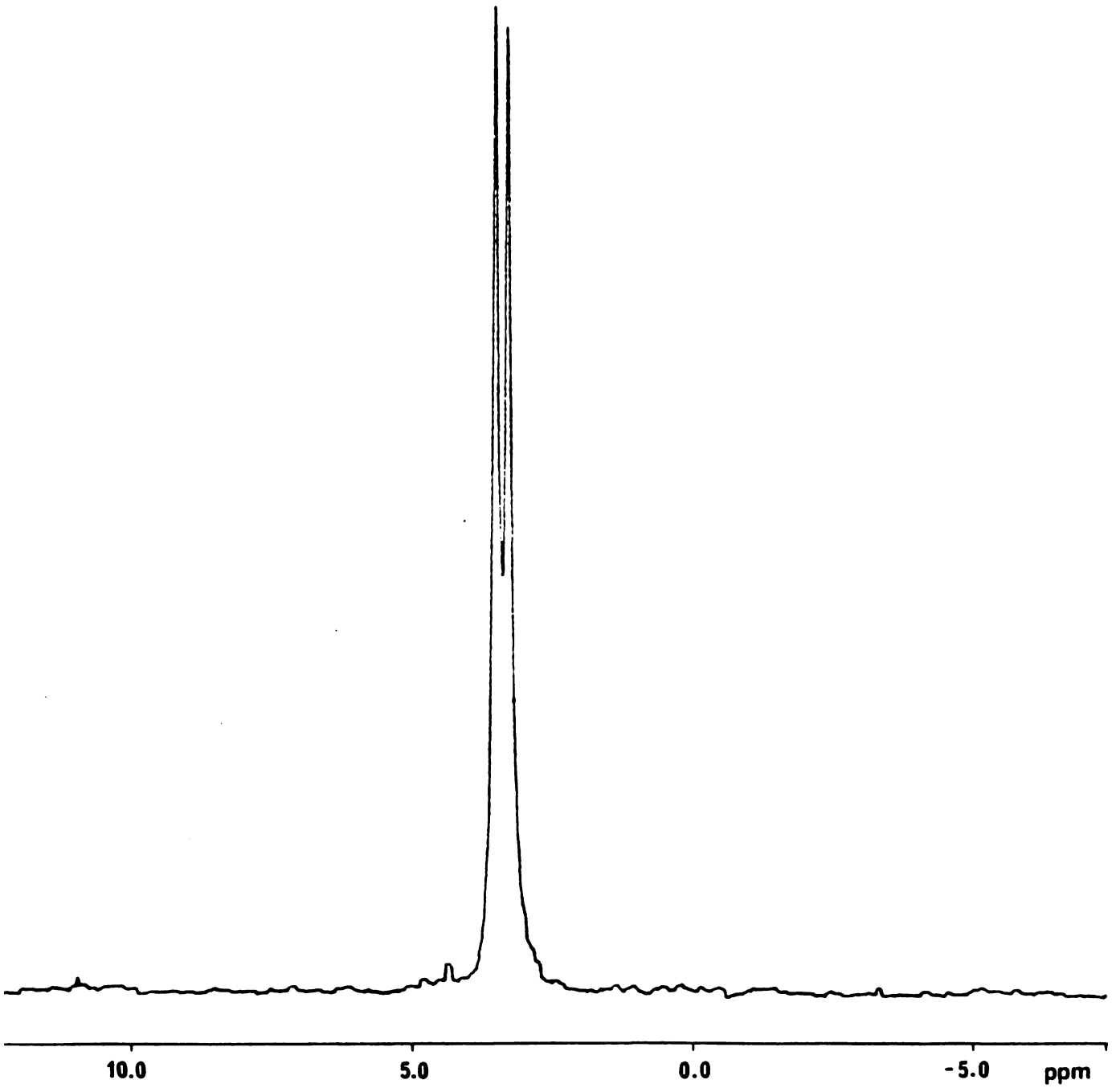


Figure 17 ^{31}P NMR spectrum of phosphovinylglycolate isolated from the enzymatic reaction mixture.

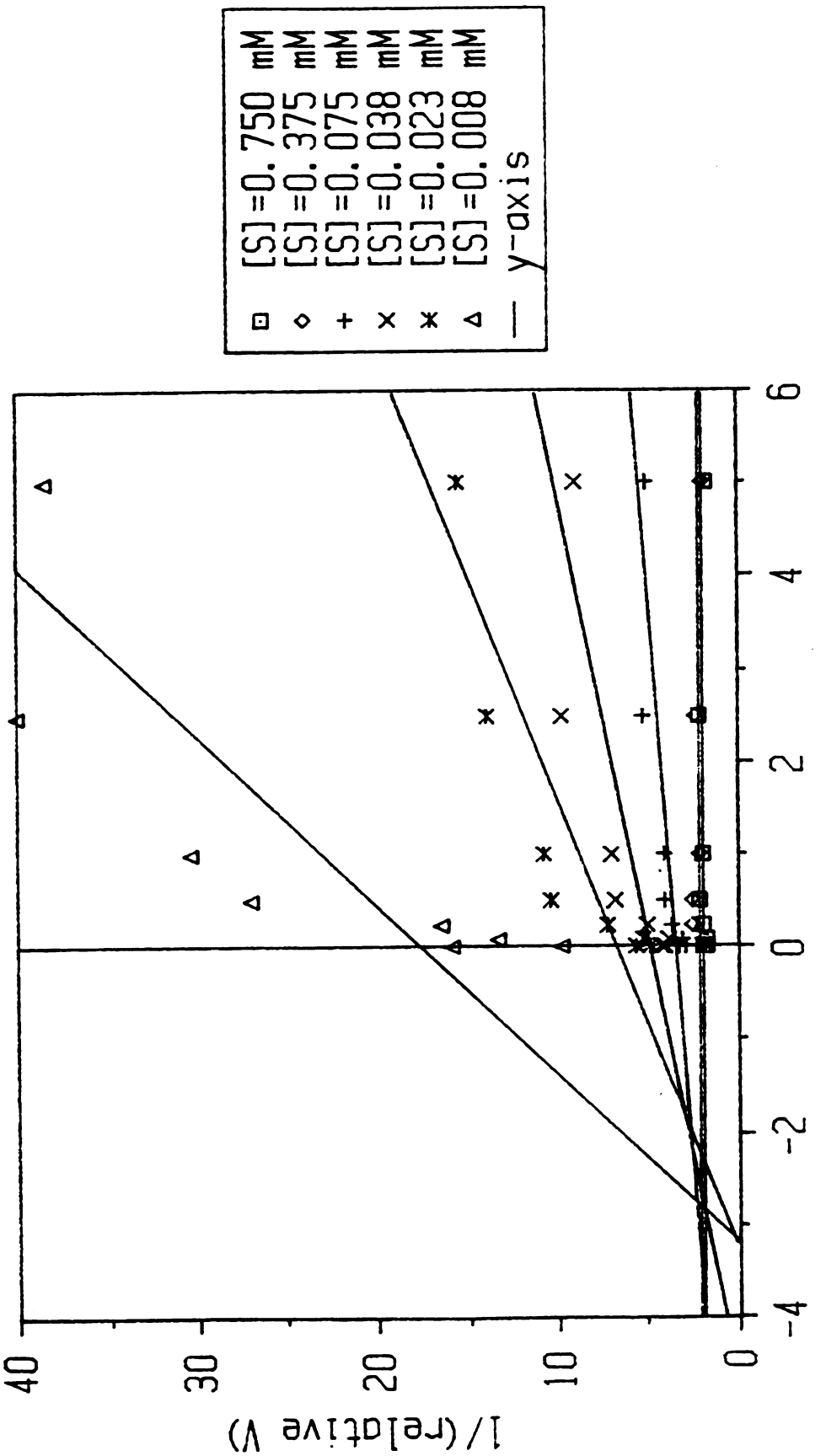
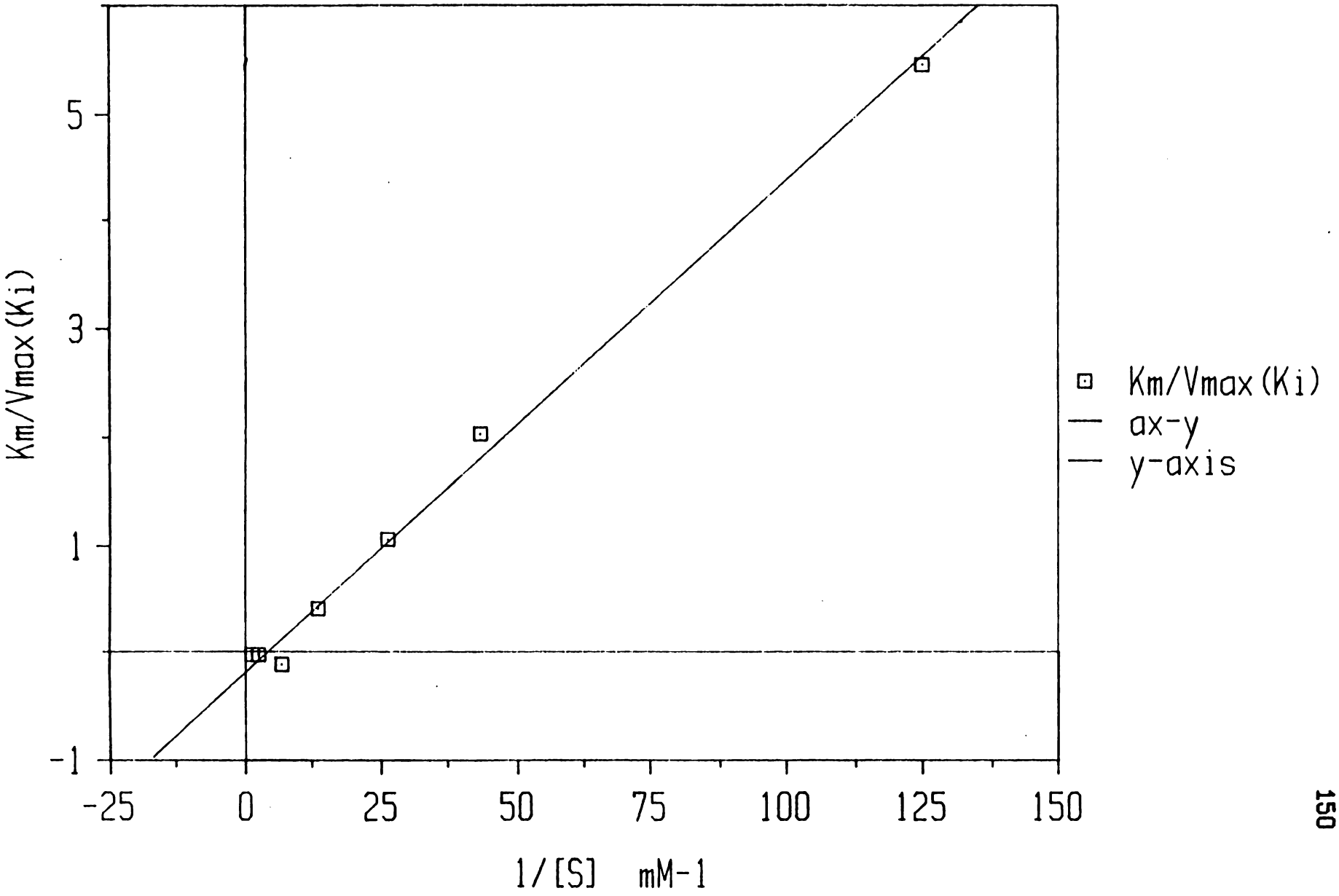


Figure 18 Dixon plots of phosphovinylglycolate.

Figure 19 Slope replot of Dixon plot.



of pyruvate kinase. There is some X-ray crystallographic evidence that a glutamyl carboxyl group from the enzyme is positioned so that it could participate in the protonation and deprotonation of C-3 of the physiological substrate, pyruvate (Muirhead, 1981). It has been suggested that such a group acts as a base in the enzyme-catalyzed enolization of pyruvate (Robinson, 1972)(Ash, 1983). Therefore, 3,4-epoxyvinylglycolate may prove to be useful as an affinity label. It is structurally related to the substrate vinylglycolate and attack at either C-3 or C-4 by a nucleophile on the enzyme would, in principle, lead to covalent modification **(Figure 20)**. A similar mechanism involving attack of the carboxyl group of Glu or Asp on an epoxide substrate analog has been proposed to account for the affinity labeling of creatine kinase by epoxycreatine (Marletta, 1979). Thus, this compound may be useful as an affinity label for further investigation of pyruvate kinase.

There is a difference of 2-3 orders of magnitude between the K_i measured for isolated phosphovinylglycolate and that estimated from the time dependent decrease in its rate of formation. The reason for this is not known. The equilibrium constant for the reaction of vinylglycolate with ATP lies far to the right as discussed before. Thus, we expected the K_i of phosphovinylglycolate as an inhibitor of the vinylglycolate reaction to be the same as the K_i of phosphovinyglycolate as an inhibitor of the PEP reaction. It thus appears that the inhibitor is not the same in each case. One explanation is that the initial product formed on the enzyme may

Figure 20 A. Base at active site of pyruvate kinase proposed by Rose et al. B. Proposed inhibitor of pyruvate kinase.

be different from the product observed by ^{31}P NMR of the reaction mixture and subsequently isolated from the reaction mixture.

It is generally believed that water is excluded from the active site of kinases so that it does not compete as a nucleophile in phosphoryl transfer. Pyruvate kinase may also have a mechanism to prevent interference from the carboxyl groups of pyruvate and PEP in the phosphoryl transfer reaction. This may be the reason for the requirement of the monovalent cation that coordinates the carboxyl group of the substrates at the active site. If so, the efficiency of the mechanism would probably be optimal for the geometry of the natural substrate, PEP. The hybridization of C-2 in PEP and enolpyruvate is sp^2 whereas that in phosphovinylglycolate and vinylglycolate is sp^3. Thus, K^+ may be less efficient in preventing interference by the carboxyl group at the active site. The carboxyl group might interfere by attacking the phosphate group attached to the product, phosphovinyglycolate. Isotopic labeling studies have provided evidence that an analogous reaction may occur in aqueous solutions of PEP at low pH (O'Neal, 1983). A trigonal bipyramidal intermediate thus formed would somewhat resemble the transition state expected for a direct, associative phosphoryl transfer. Such an intermediate could be the source of the strong inhibition observed for the reaction of vinylglycolate. Since the breakdown of this intermediate would be expected to be essentially irreversible, it would not be observed except during the reaction of vinyglycolate.

Summary

The ^1H NMR spectra of three compounds prepared for this study, vinylglycolate, and *erythro- and threo*-β-hydroxy aspartate, were interpreted for the first time. The ^1H NMR data were used in conjunction with Newman projections of the structure of β-hydroxyaspartate to comment on the probable conformations of the individual diastereomers. It was concluded that the most favorable conformation of the *erythro*-β-hydroxyaspartate was that drawn in **Figure 10C** and that of the *threo*-β-hydroxyaspartate was either that in **Figure 9C** or **9D**.

Twenty-two α-hydroxy acids and α-keto acids were screened as possible alternate substrates for the enzymatic reaction catalyzed by pyruvate kinase. Only two of the compounds, vinylglycolate and isoserine, were found to be alternate substrates. The products were characterized by ^{31}P NMR analysis. The pyruvate kinase-catalyzed reaction with isoserine did not proceed to completion even though isoserine was present in excess. In addition, this reaction was too slow to measure using the pH-stat method. For the reaction of vinylglycolate a value of 0.0015 sec^{-1} was determined for k_{cat}.

The product of the reaction with vinylglycolate, phosphovinylglycolate, was found to be only a weakly competitive inhibitor with a K_i value of 2.5 m<u>M</u>. This contrasts sharply with a K_i value on the order of 10 μ<u>M</u> necessary to account for the observed inhibition of the reaction to form the product. Thus, phosphovinylglycolate is not the inhibiting species in the pyruvate kinase catalyzed phosphorylation of vinylglycolate.

This study led to the proposal that 3,4-epoxyvinylglycolate may be an affinity label of pyruvate kinase. The fact that vinylglycolate is a substrate for the reaction suggests that the epoxide may also be a substrate and thus bind at the active site. Recent X-ray crystallographic studies suggest that a certain glutamate residue present at the active site may be positioned favorably for attack at C-3 or C-4 of the proposed epoxide.

Adlersburg, M; Dayan, J.; Bondinell, W.E.; Sprinson, D.B. *1970 40* 1464.

Ames, B.N. *Methods in Enzymology* **1966** *8* 115.

Anderson, P.J.; Randall, R.F. *Biochem. J.* **1975** *145* 575.

Ash, D.E.; Goodhart, P.J; Reed, G.H. *Arch. Bioch. Biophys.* **1983** *228* 31.

Barman, T.E. *PNAS* **1985** *82* 7495.

Benedict, S.R.; Behre, J.A. *J. Biol. Chem.* **1936** *114* 515.

Benfield, P.; Zivin, R.A.; Miller, L.S.; Sowder, R.; Smythers, G.W.; Henderson, L; Oroszlan, S.; Pearson, M.L. *J. Biol. Chem.* **1984** *259* 14979.

Benoiton, L.; Winitz, M.; Colman, R.F.; Birnaum, S.M.; Greenstein, J.B. *J. Am. Chem. Soc.* **1959** *81* 1726.

Berghauser, J. *Hoppe-Seyler's Z. Physiol.* **1977** *358* 1565.

Bjorkhem, I.; Blomstrand, R.; Ohman, G. *Clin. Chem.* **1977** *23* 2114.

Blattler, W.A.; Knowles, J.R. *Biochem.* **1979** *18* 3927.

Bloxham, D.P.; Coghlin, S.J.; Sharma, R.P. *BBA* **1978** *525* 61.

Blum, H.E.; Deus, B.; Gerok, Wolfgang *J. Biochem.* **1983** *94* 1247.

Boyer, P.D in "The Enzymes" (Boyer, P.D.; Lardy, H.; Myerback, K.; eds.) 2nd Ed., **1962** *6* 95. Acad. Press.

Boyer, P.D.; Lardy, H.A.; Phillips, P.H. *J.Biol Chem.* **1942** *146* 673.

Boyer, P.D.; Lardy, H.A.; Phillips, P.H. *J.Biol Chem.* **1943** *149* 529.

Brindle, K.M.; Radda, G.K. *Bioch. Biophys. Acta* **1985** *829* 188.

Brindle, K.M.; Porteous, R.; Radda, G.K. *Biochem. Biophys. Acta* **1984** *786* 18.

Bucher, R.; Pfleiderer, G. *Meth. Enz.* **1955** *1* 435.

Burgers, P.M.J.; Eckstein, F. *J. Biol. Chem.* **1980** *255* 8229.

Burke, R.L.; Tekampol, P.; Najarian, R.; *J. Biol. Chem.* **1983** *258* 2193.

Cain, A.H.; Sullivan, G.R.; Roberts, J.D.; *J. Am. Chem. Soc.* **1977** *99* 6423.

Chalkley, R.A.; Bloxham, D.P.; *Biochem. J.* **1976** *159* 213.

Clark, V.M.; Kirby, A.J. *J. Am. Chem. Soc.* **1963** *85* 3705.

Cleland, W.W. *Biochemistry* **1975** *14* 3220.

Cohn, M. *Ann. Rev. Biophys. Bioeng.* "^{18}O and ^{17}O Effects on ^{31}P–NMR as Probes of Enzymatic Reactions of Phosphate Compounds"

Cook, P.K.; Kenyon, G.L.; Cleland, W.W. *Biochemistry* **1981** *20* 1204.

Dann, L.G.; Britton, H.G. *Biochem. J.* **1978** *169* 39.

Dawson, M.J.; Gadian, D.G.; Wilkie, D.R. *J. Physiol.* **1977** *267* 703.

Dietrich, R.F.; Kenyon, G.L.; Douglas, J.E.; Kollman, P.A. *J. Chem. Soc. Perkin Trans. II* **1980** 1592.

Dietrich, R.F., Miller, R.B., Kenyon, G.L., Leyh, T.S., Reed, G.H. *Biochemistry* **1980** *19* 3180.

Dietrich, R.F.; Kenyon, G.L. *Bioorganic Chem.* **1984** *12* 221.

Dunaway–Mariano, D.; Cleland, W.W. *Biochem.* **1980** *19* 1506.

Dunaway–Mariano, D.;Benovic, J.L.; Cleland, W.W.; Gupta, R.K.; Mildvan, A.S. *Biochem.* **1979** *18* 4347.

Edgar, G.; Hinegardner, W.S. *Org. Synth.* **1932** *I* 172.

Endlund, B;.; Andersson, J.; Titanji, V.; Dahlqvist, U.; Ekman, P.; Zetterqvist, O.; Engstrom, L. *Bioch. Biophys. Res. Comm.* **1975** *67* 1516.

Engleborghs, Y.; Marsh, A.; Gutfreund, H. *Bioch. J.* **1975** *151* 47.

Flynn, I.W,.; Bowman, I.B.R. *Arch. Bioch. Biophys.* **1980** *200* 401.

Fung, C.H.; Mildvan, A.S.; Allerhand, A.; Komoroski, R.; Scrutton, M.C. *Biochemistry, Wass.* **1973** *12* 620.

Gercken, G.;, Doring, V. *FEBS Letters* **1974** *46* 87.

Giles, I.G.; Poat, P.C.; Munday, K.A. *Biochem. J.* **1978** *157* 577.

Giraudat, J.; Devillers–Thiery, A.; Peruard, J.C.; Changeux, J.P. *PNAS* **1984** *81* 7313.

Glattfeld, J.W.E.; Hoen, R.E. *J. Am. Chem. Soc.* **1935** *57* 1405.

Gonnella, N.C.; Nakanishi, H.; Holtwick, J.B.; Horowitz, D.S.; Kanamori, K.;Leonard, N.J.; Roberts, J.D. *J. Am. Chem. Soc.* **1983** *105* 2050.

Gregory, R.B.; Ainsworth, S. *Bioch. J.* **1981** *195* 745.

Gupta, R.K.; Benovic,J.L. *J. Biol. Chem.* **1978** *253* 8878.

Gupta, R.K.; Oesterling, R.M.; Mildvan, A.S. *Biochemistry* **1976** *15* 2881.

Gupta, R.K.; Fung, C.H.; Mildvan, A.S. *J. Biol. Chem.* **1976** *251* 2421.

Gupta, R.K.; Benovic, J.L. *J. Biol. Chem.* **1978** *253* 8878.

Hansen, D.E., Knowles, J.R. *J. Biol. Chem* **1981** *246* 5967.

Hall, E.; Cottam, G.L. *Int. J. Bioch.* **1978** *9* 735.

Hassett, A.; Blattler, W.A.; Knowles, J.R. *Biochem.* **1982** *21* 6340.

Herriott, J.R.; Love, W. E. *Acta Cryst.* **1968** *B 24* 1014.

Hjelmqvist, G.; Andersson, J.; Edlund, B.; Engstrom, L. *Bioch. Biophys. Res. Comm.* **1974** *67* 559.

Hollenberg, P.F.; Flashner, M.; Coon, M.J.; *J. Biol. Chem.* **1971** *246* 946.

Hore, P.J. *J. Magnetic Resonance* **1983** *55* 283.

Hoult, D.I.; Busby, S.J.W.; Gadian, D.G.; Richards, R.E.; Seeley, P.J. *Nature* **1974** *252* 285.

Ingwall, J.S. *Am. J. Physiol.* **1982** *242* H729.

Iyengar, M.R.; Coleman, D.W.; Butler, T.M. *J. Biol. Chem.* **1985** *260* 7562.

Jaffe, E.;Cohn, M. *J. Biol. Chem.* **1978** *253* 4823.

James, T.L. *J. Biol. Chem* **1974** *249* 2599.

James, T.L. *Biochemistry* **1976** *15* 4724.

Johnson, S.C.; Bailey, T.; Becker, R.R.; Cardenas, J.M. *Bioch. Biophys. Res. Comm.* **1979** *90* 525.

Kaltwasser, H.;Schlegel, H.G. *Anal. Biochem.* **1966** *16* 132.

Kanamori, K.; Roberts, J.D. *J. Am. Chem. Soc.* **1983** *105* 4698.

Kayne, F.J. in "The Enzymes" (Boyer, .D. ed.) 3rd Ed., **1973** *6* 353. Acad. Press.

Kenyon, G.L.; Reed, G.H. *Adv. Enzymol.* **1983** *54* 367.

Kenyon, G.L.; Struve, G.E.; Kollman, P.A.; Moder, T.I. *J. Am. Chem. Soc.* **1976** *98* 3695.

Kenyon, G.L. **1986** *Seminar.*

Kupiecki, F.P.; Coon, M.J. *J. Biol. Chem.* **1959** *234* 2428.

Koons, S.J.; Eckert, B.S.; Zobel, C.R. *Exp. Cell Res.* **1983.**

Kornguth, M.L.; Sallach, H.J. *Arch. Bioch. Biophys.* **1960** *91* 39.

Kuby, S.A.; Noda, L.; Lardy, H.A. *J. Biol. Chem.* **1954** *209* 191.

Kuby, S.A.; Noda, L.; Lardy, H.A. *J. Biol. Chem.* **1954** *210* 65.

Kupiecki, F.P.; Coon, M.J. *J. Biol. Chem.* **1960** *235* 1944.

Kwiatkowski, R.W.; Schweinfest, C.W.; Dottin, R.P. *Nucl. Acids Res.* **1984** *12* 6925.

Lonberg, N.; Gilbert, W. *Cell* **1985** *40* 81.

Lawson, J.W.R.; Veech, R.L. *J. Biol. Chem.* **1979** *254* 6528.

Leyh, T.S.; Goodhart, P.J.; Nguyen, A.C.; Kenyon, G.L.; Reed, G.H. *Biochemistry* **1985** *24* 308.

Lowe, G.; Sproat, B.S. *J.Biol.Chem.* **1980** *255* 3944.

Lakomek, M; Tillmann, W.; Scharnetzky, M.; Schister, W.; Winkler, H. *Enzyme* **1983** *29* 189.

Lawson, J.W.R.; Veech, R.L. *J. Biol. Chem.* **1979** *254* 6528.

Lawson, J.W.R.; Veech, R.L. *J. Biol. Chem.* **1979** *254* 6528.

Lehman, H. *Biochem. Z.* **1935** *281* 271.

Lehninger, A.L. "Biochemistry", 2nd Ed. **1975**, 847. Worth Publishers, Inc. N.Y., N.Y.

Levine, M., Muirhead, H.; Stammers, D.K.; Stuart, D.I. *Nature* **1978** *271* 626.

Litwak-Mann, C.; Mann, T. *Biochem. Z.* **1935** *281* 140.

Litwin, S.; Wimmer, M.J. *J. Biol. Chem.* **1979** *254* 1859.

Lonberg, N.; Gilbert, W. *Cell* **1985** *40* 81.

Lowe, G.; Sproat, B.S. *J. Biol. Chem.* **1980** *255* 3944.

Maggio, E.T.; Kenyon, G.L.; Markham, G.D.; Reed, G.H. *J. Biol. Chem.* **1977** *252* 1202.

Markowski, V.; Sullivan, G.R.; Roberts, J.D. *J. Am. Chem. Soc.* **1977** *99* 714.

Marletta, M.A.; Kenyon, G.L. *J. Biol. Chem* **1979** *254* 1879.

Martonosi, A. *Biophys. Bioch. Res. Comm.* **1960** *2* 12.

Matsumoto, K.; Rapoport, H. *J. Org. Chem.* **1968** *33* 552.

McLaughlin, A.C.; Cohn, M.; Kenyon, G.L. *J. Biol. Chem.* **1972** *247* 4382.

Mendel. H.; Hodgkin, D.C. *Acta Cryst.* **1954** *7* 443.

Meyer, R.A.; Kushmerick, M.J.; Brown, T.R. *Am. J. Physiol.* **1982** *11*C1.

Melamud, E.; Mildvan, A.S. *J. Biol. Chem.* **1975** *250* 8193.

Meshitsuka, S.; Smith, G.M.; Mildvan, A.S. *J. Biol. Chem.* **1981** *256* 4460.

Meyerhof, O.; Lohmann, K. *Biochem. Z.* **1934**, *273* 60.

Midelfort, C.F.; Rose, I.A. *J. Biol. Chem.* **1976** *251* 5881

Mildvan, A.S. *Phil. Trans. R. Soc. Lond.* **1981** *B293* 65.

Mildvan, A.S.; Cohn, M. *J. Biol. Chem.* **1966** *241* 1178.

Mildvan, A.S.;Sloan, D.L.; Fung, C.H.; Gupta, R.K.; Melamud, E. *J. Biol. Chem.* **1976** *251* 2431.

Milner-White, E.J.; Watts, D.C. *Bioch. J.* **1971** *122* 727.

Morrison, J.F.; James, E. *Biochem. J.* **1965** *97* 37.

Morrison, J.F.; Cleland, W.W. *J. Biol. Chem.* **1966** *241* 673.

Morrison, J.F.; James, E. *Biochem. J.* **1965** *97* 37.

Morrison, J.F.; White, A. *Eur. J. Bioch.* **1967** *3* 145.

Morrison, J.F.; White, A. *Eur. J. Bioch.* **1967** *3* 145.

Morrison, J.F.; Cleland, W.W. *J. Biol. Chem.* **1966** *241* 673.

Muirhead, H.; Grant, J.P.; Lawton, M.A.; Midwinter, C.A.; Norton, J.C.; Stuart, D.I. *Bioch. Soc. Trans.* **1981** *9* 212.

Munday, K.A.; Giles, I.G.; Poat, P.C. *Comp. Bioch. Physiol.* **1980** *67B* 403.

Nageswara Rao, B.D.; Kayne, F.J.; Cohn, M. *J. Biol. Chem.* **1979** *254* 1286.

Nguyen, A.C., **1984** *Thesis.*

Noguchi, T.; Inoue, H.; Chen, H.-L.; Matsubara, K; Tanaka, T. *J. Biol. Chem.* **1983** *24* 15220.

Nunnally, R.L.; Hollis, D.P. *Biochemistry* **1979** *18* 3642.

O'Leary, M.H.; DeGooyer, W.J.; Dougherty, T.M.; Anderson, V. *Bioch. Biophys. Res. Comm.* **1981** *100* 1320.

O'Neal, Jr., C.C.; Bild, G.S.; Smith, L.T. *Biochemistry* **1983** *22* 611.

Opperdoes, F.R. *Molec. Bioch. Parasitol.* **1981** *3* 181.

Opperdoes, F.R.; *Abstract*, Bellagio Meeting, Bellagio, Italy, **Feb. 1981**.

Ordahl, C.P.; Evans, G.L.; Cooper, T.A.; Kunz, G. *J. Biol. Chem.* **1984** *259* 15224.

Phillips, Jr., G.N.; Thomas, Jr., J.W.; Annesley, T.M.; Quiocho, F.A. *J. Am. Chem. Soc.* **1979** *101* 7120.

Phillips, J.P., Jones, J.M., Hitchcock, T., Adams, N., Thomson, R.J. *Br. Med. J.* **1980** *281* 7777.

Porter, D.J.T.; Ash, D.E.; Bright, H.J.; *Arch. Bioch. Biophys.* **1983** *222* 200.

Prellwitz, W. in *"Creatine Kinase Isoenzymes"*, **1979**. Lang, H. Ed., Springer-Verlag New York 176-195.

Putney, S.; Herlihy, W.; Royal, N.; Pang, H.; Aposhian, H.V.; Pickering, L.; Belagage, R.; Biemann, K.; Page, D.; *J. Biol. Chem.* **1984** *259* 14317.

Rao, B.D.N.; Kayne, F.J.; Cohn, M. *J. Biol. Chem.* **1979** *254* 2689.

Raushel, F.M.; Cleland, W.W. *Fed. Proc.* **1976** *35* 1578.

Raushel, F.M.; Garrard, L.J. *Biochemistry* **1984** *23* 1791.

Raushel, F.M. *personal communication* **1986**.

Reddy, S.R.R.; Watts, D.C. *Bioch. Biophys. Acta* **1979** *569* 109.

Reed, G.H.; Barlow, C.H.; Burns, R.A., Jr. *J. Biol. Chem.* **1978** *253* 4135.

Reed, G.H.; Leyh, T.S. *Biochemistry* **1980** *19* 5472.

Reed, G.H.; Morgan, S.D. *Biochemistry* **1974** *13* 3537.

Reynard, A.M.; Hass, L.F.; Jacoben, D.D.; Boyer, P.D. *J. Biol. Chem.* **1961** *236* 2277

Reynolds, M.A.; Gerlt, J.A.; Demou, P.C; Oppenheimer, N.J.; Kenyon, G.L. *J. Am. Chem. Soc.* **1983** *105* 6475.

Reynolds, M.A.; Oppenheimer, N.J.; Kenyon, G.L. *J. Am. Chem. Soc.* **1983** *105* 6666.

Robinson, J.L.; Rose, I.A. *J. Biol. Chem.* **1972** *247* 1096.

Rose, I.A. *J. Biol. Chem.* **1960** *235* 1170.

Rose, I.A. *J. Biol. Chem.* **1970** *245* 6052.

Rose, I.A.; O'Connell, E.L.; Litwin, S.; BarTana, J., *J. Biol. Chem.* **1974** *249* 5163.

Rosevear, P.R.; Desmeules, P.; Kenyon, G.L.; Mildvan, A.S. *Biochemistry* **1981** *20* 6155.

Rowley, G.L.; Greenleaf, A.L.; Kenyon, G.L. *J. Am. Chem. Soc.* **1971** *93* 5542.

Rowley, G.L.; Kenyon, G.L. *Anal. Bioch.* **1974** *58* 525.

Schray, K.J.; Benkovic, S.J. *J. Am. Chem. Soc.* **1971** *93* 2522.

Shoptaugh, N.H.; Buckley, L.J.; Ikawa, M.; Sasner, Jr., J.J. *Toxicon* **1978** *16* 509.

Simon, M.-P.; Besmond, C.; Cottreau, D.; Weber, A.; Chaumet-Riffaud, P.; Dreyfus, J.-C.; Trepat, J.S.; Marie, J.; Kahn, A.; *J. Biol. Chem.* **1983** *23* 14576.

Silver, R.B.; Cole, R.D.; Cande, W.Z. *Eur. J. Cell. Biol.* **1980** *22* 315.

Sjovall, K.; Voight, A. *Nature* **1964** *202* 701.

Sloan, D.L.; Mildvan, A.S. *J. Biol. Chem.* **1976** *251* 2412.

Struve, G.E.; Gazzola, C.; Kenyon, G.L. *J. Org. Chem.* **1977** *42* 4035.

Stuart, D.I.; Levine, M.; Muirhead, H.; Stammers, D.K.; *J. Mol. Biol.* **1979** *134* 109.

Stubbe, J.A.; Kenyon, G.L. *Biochemistry* **1971** *10* 2669.

Stubbe, J.A.; Kenyon, G.L. *Biochemistry* **1972** *11* 338.

Suelter, C.H. *Science* **1970** 789.

Swanson, J.R.; Wilkinson, J.H. *Stand. Meth. Clin. Chem.* **1972** *7* 33.

Switzer, R.L.; and Gibson, K.J. *Methods in Enzymology* **1978** *51* 3.

Tietz, A.; Ochoa, S. *Arch. Bioch. Biophys.* **1958** *78* 477.

Travers, F.; Barman, T.E.; Bertrand, R. *Eur. J. Bioch.* **1979** *100* 149.

Travers, F.; Barman, T.E. *Eur. J. Bioch.* **1980** *110* 405.

Visser, N.; Opperdoes, F.R. *Eur. J. Bioch.* **1980** *103* 623.

West, B.L.; Babbitt, P.C.; Mendez, B.; Baxter, J.D. *Proc. Natl. Acad. Sci.* **1984** *81* 7007.

Witowski, M.; Webb, G.A., Eds., *"Nitrogen NMR"*, Plenum Press, N.Y.,.N.Y. **1973**, p. 227.

Zeile, K.; Meyer, H. *H.-Z. P. Chem.* **1938** *252* 101.