Title
ATP analogs

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ATP ANALOGS: SYNTHESIS AND NMR STUDIES

by

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Abstract

^{15}N- and ^{17}O-enriched samples of imidodiphosphate (PNP), its tetraethyl ester, and adenylyl imidodiphosphate (AMP-PNP) have been prepared. The ^{15}N NMR spectra of both PNP and AMP-PNP reveal the presence of a ^{15}N-^{1}H coupling constant of about 70 Hz, demonstrating an imido tautomeric structure in both cases. The ^{17}O NMR chemical shifts of the resonances associated with the phosphoryl oxygens have been assigned. The effect of pH on these resonances has been determined; upfield chemical shifts indicate that protonation of the tetraanions of PNP and AMP-PNP occur exclusively on the oxygens and γ-phosphoryl oxygens, respectively. Although ^{17}O NMR demonstrates that protonation of the monoanion of PNP occurs predominantly on nitrogen, the corresponding ^{15}N NMR chemical shift change was only 2.5 ppm. ^{15}N NMR spectra of tetraethyl PNP in deuterochloroform indicate that the N-H proton is in fast exchange. An analog of AMP-PNP possessing an N-methyl group at the β,γ-bridge has been prepared in about 70% purity. The effect of pH on the ^{31}P NMR spectrum of this compound is similar to that for AMP-PNP: the β-phosphoryl resonance experiences the largest upfield change in chemical shift upon protonation of the tetraanion.

Pyrophosphate and ATP have been prepared with ^{18}O-enrichment in the bridge and β,γ-bridge, respectively. The ^{18}O-ATP analog has been used to measure β,γ-bridge to β-nonbridge positional oxygen exchange catalyzed by carbamoyl phosphate synthetase from E. coli in the presence of bicarbonate. The β,γ-^{18}O-bridge to β-^{18}O-nonbridge species of ATP could be distinguished by ^{31}P NMR at 97.3 MHz; the ^{18}O-isotope exerts small upfield chemical shifts on the

$^{31}P_{\beta}$-resonances which are different for each species.

Pyrophosphate and ATP possessing ^{17}O-enrichment in the bridge and β,γ-bridge have also been prepared. Due to the line-broadening effect on a ^{31}P-resonance exerted by a directly bound ^{17}O-atom, the positional oxygen exchange described above could be measured with the ^{17}O-ATP analog using ^{31}P NMR at 40.5 MHz by virtue of an increase in the intensity of the P_{γ}-resonances with time.

George L. Kenyon

Acknowledgments

I wish to extend special thanks to Professor George Kenyon for his continual input and support while this work was in progress. His ideas were always a great inspiration to me, and I owe him much for what he has taught me with regard to scientific thinking.

I also wish to express my sincere thanks to Professor Norman Oppenheimer for advising me in this work. He has provided me with a sound appreciation for NMR as a tool in scientific problems, and with a concern for careful experimentation.

Professor John Gerlt was always a phone call away when I needed technical advice; for this, for his contributions with the ^{17}O NMR spectra, and for the knowledge he has shared with me I am truly grateful.

In addition, I am grateful to Vladimir Basus, Alan Koretsky and Joe Murphy for their patient assistance in the acquisition of ^{15}N and ^{31}P NMR spectra.

I want to thank all my friends in the Kenyon and Oppenheimer groups for being a terrific bunch of people to work with. I could have wished for none better.

I am thankful for many other friendships from this department which have added much value to the time I have spent here, and for two special friends in particular: Jeff Blaney and Jim Matthews, who shared with me throughout this ordeal. Jeff, thanks for letting me know the thrill of nearly capsizing on the Hobie Cat at 20 knots plus. Jim, thanks for making me get out and ride those boomers.

Thanks to the American Foundation for Pharmaceutical Education for generous financial assistance.

My family has always been a rock of support from which to draw strength when things get difficult. Thank you all for your love and care.

Finally, I want to dedicate this work and my love to Sandy, who has patiently awaited and supported these efforts which have meant so much to me.

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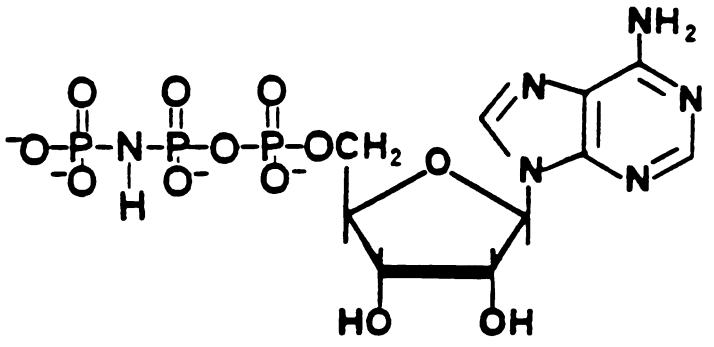
Part 1: Phosphorus-Nitrogen Bridged Analogs of ATP.

I Foreword.

For many years, enzymatic reactions involving the γ-phosphoryl group of ATP have attracted the interests and efforts of numerous investigators involved in the study of enzyme mechanisms. Much attention has been centered on the preparation of ATP analogs that can serve as inhibitors of enzymes such as adenosine triphosphatases and kinases that normally catalyze either the hydrolysis or transfer of the γ-phosphoryl group of ATP. Such enzymatic reactions of ATP almost always require Mg^{2+} as a cofactor. In the work discussed in Part I of this dissertation, we will be concerned with analogs of ATP in which the oxygen atom of the β,γ-bridge has been replaced by nitrogen. The parent compound of this type, adenylyl imidodiphosphate (AMP-PNP, 1), will be discussed in detail, particularly with regard to what new information we have obtained about its structure, sites of protonation, and Mg^{2+} binding properties in solution. The potential for synthesizing N-alkyl derivatives of AMP-PNP will also be discussed, in addition to preliminary studies on the N-methyl analog. It is hoped that compounds of this type will prove useful in future studies of enzymatic reactions in which the β,γ-bridge of ATP is normally cleaved.



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II. Introduction.

A. Adenylyl Imidodiphosphate.

AMP-PNP was first introduced by Yount and co-workers (1971a) as a close structural analog of ATP. Three major reasons can be given for the fact that this compound has seen such widespread use as a competitive inhibitor of enzyme reactions of the type just described. First, the bond angles and distances in the P_{β}-O-P_{γ} region of ATP and the P_{β}-N(H)-P_{γ} region of AMP-PNP are thought to be nearly identical. This is based on the observed similarity in the P-O-P and P-N-P bond angles and distances of crystalline pyrophosphate and imidodiphosphate as obtained from x-ray crystallographic data (see Figure 1, Larson et al., 1969).

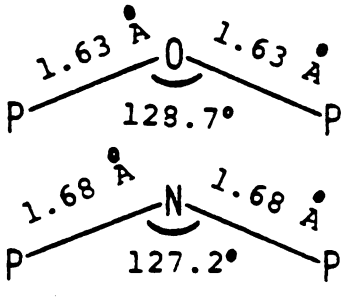


Figure 1. Comparisons of the P-X-P bond angles and P-X bond distances for crystalline pyrophosphate and imidodiphosphate taken from Larsen et al. (1969).

Second, the P-N-P bridge in AMP-PNP was shown to be chemically stable at neutral or alkaline pH for long periods of time (Yount et al., 1971b). Third, it has been generally found that ATP requiring enzymes will not cleave the β,γ P-N-P linkeage of AMP-PNP. In fact, only two enzymes are currently known which will cleave this bond: alkaline phosphatase from E. coli (Yount et al., 1971a) and sarcoplasmic reticulum ATPase from skeletal muscle (Taylor, 1981). In both cases, as in the case of acid-hydrolysis of AMP-PNP in solution, the hydrolysis product is adenyl-5'-yl phosphoramidate ($ADP\text{-}NH_2$).

AMP-PNP is particularly useful in studies where the effects of hydrolysis versus binding of ATP need to be isolated. For example, this analog has proven to be especially useful in replacing ATP in studies with myosin (Yount et al., 1971b; Bagshaw et al., 1974; Harrington and Himmelfarb, 1972; Siedel and Gergely, 1973; and Werber et al., 1972, to name a few early studies). AMP-PNP binds to myosin with nearly equal affinity to ATP and has been shown to dissociate myosin and actin at high ionic strength (Yount et al., 1971b). AMP-PNP appears to affect muscle fiber contraction, although it fails to completely mimic ATP (Chaplin and Frommelt, 1968; dos Remedios et al., 1972; and Marston et al., 1979), demonstrating that relaxation is initiated solely due to the binding of ATP, not its cleavage.

Rodbell and coworkers (1971) have shown that AMP-PNP can act as a substrate for adenylate cyclase. Unlike ATP, however, AMP-PNP is resistant to cleavage by membrane ATPases. Thus, in the presence of Pb^{2+}, AMP-PNP has provided a means for the cytochemical location of adenylate

cyclase on specific membrane surfaces by virtue of the formation of lead imidodiphosphate precipitates (Wagner et al.,1972). Such a study would not have been possible with ATP itself, since the inorganic phosphate which would have been formed by the other ATPases present also precipitates with lead.

Another interesting application of AMP-PNP has been as a nonhydrolyzable competitive inhibitor of ATP cleavage by both the soluble and membrane-bound forms of mitochondrial F_1 ATPase (Penefsky, 1974; Krull and Schuster, 1981). AMP-PNP was also found to inhibit the partial reactions of oxidative phosphorylation catalyzed by phosphorylating submitochondrial particles. Thus, the ATP-dependent reduction of NAD^+ by succinate was inhibited, as was the exchange between Pi and ATP. On the other hand, AMP-PNP did not affect the synthesis of ATP from ADP and Pi linked to the oxidation of succinate. These findings were suggested to imply a high affinity for ADP of the phosphorylating sites or to reflect two catalytic sites on the enzyme which are specialized, respectively, for ATP synthesis and ATP utilization.

Many other examples could be listed where AMP-PNP has been applied to the study of ATP-utilizing enzymes. In fact, since AMP-PNP became commercially available, such studies have proliferated throughout the literature. It is beyond the scope of this dissertation to discuss these studies in detail. It is worth pointing out, however, that many conclusions made from experimental studies with AMP-PNP are based to some extent on the assumption that AMP-PNP and

ATP are binding in a similar manner at the active site of the particular enzyme being investigated. Clearly, in cases where AMP -PNP and ATP appear to behave differently this assumption breaks down.

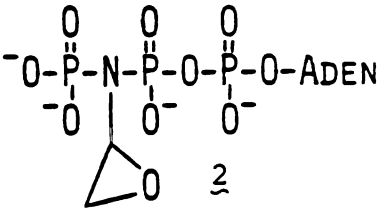
Our interest in the study of AMP-PNP was stimulated by the finding of Bagshaw et al. (1972) that AMP-PNP and ATP differ markedly in their rates of binding to subfragment 1 of myosin. Although the apparent binding constants with the enzyme were found to be similar, AMP-PNP was shown to bind at 1/300th the rate of ATP. It was proposed that this difference in rate could be attributed to differences in the structure of the predominant metal ion complex for AMP-PNP or ATP with Mg^{2+} in solution. It was previously known that AMP-PNP binds Mg^{2+} in solution with an affinity somewhat greater than ATP (Yount et al., 1971a), so the observed differences were clearly not due to an inability of AMP-PNP to bind Mg^{2+}. The slower rate of binding of AMP-PNP could perhaps be understood, however, if it could be shown, for example, that the predominant Mg^{2+}·AMP-PNP complex in solution possesses the wrong stereochemical conformation to fit into the enzyme binding site. If this were the case, the effective concentration of Mg^{2+}·AMP-PNP possessing proper geometry for binding would be less than the absolute concentration in solution.

We realized that the structure of AMP-PNP in solution, in the presence and absence of Mg^{2+}, was a problem worthy of study to help explain the differences observed between AMP-PNP and ATP in the type of enzyme studies just described. The work which we have carried out directed

toward this problem constitutes a major portion of this dissertation, and it will be developed and discussed in the next section.

B. N-Alkyl Derivatives of Adenylyl Imidodiphosphate.

At the time I began work toward this dissertation, we had also proposed to synthesize a series of ATP analogs that contain an epoxide moeity as a potential active-site directed alkylating agent in order to probe the active sites of ATP-requiring enzymes. We realized that N-alkyl derivatives of AMP-PNP could possibly be a family of such compounds in which the alkylating group is directed at the phosphate binding region of such enzymes. The simplest of these analogs (2) has the following structure:



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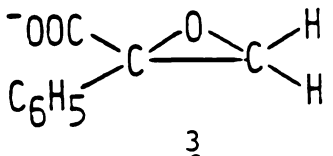
Our choice of the epoxide moiety was based largely on the previous successful employment of this group in a variety of affinity labels, including two from our own laboratory; i.e., α-phenylglycidate (3) was shown to be an effective affinity label for mandelate racemase (Fee et al., 1974) and N-(2,3-epoxypropyl)-N-amidoglycine (epoxycreatine, 4, Marletta and Kenyon, 1979) was shown to be a similarly



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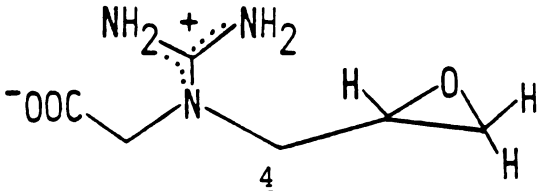

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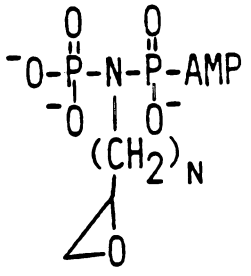
effective affinity label for the creatine binding site of creatine kinase. Both of these affinity labels are quite stable in basic aqueous solution and are highly selective for the active sites of their target enzymes. We thought it reasonable to speculate that the positively charged groups at enzyme's ATP binding sites normally have nearby carboxyl groups as counterions which have been shown in several cases to be alkylated by epoxide groups. Thus, by extending the length of the carbon chain in our proposed N-alkyl group (i.e., 5) we hoped to increase the potential for the epoxide group to reach out and intercept one of



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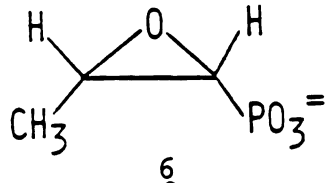
these proposed nucleophiles.

We reasoned that an epoxide group would be compatible with phosphate and phosphoramidate groups based on the known compatability of this group with phosphonate as is demonstrated by the natural product antibiotic fosfomycin (6).



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Although we have succeeded in synthesizing the N-methyl derivative of AMP-PNP, our attempts thus far at preparing derivatives with N-alkyl groups having three or more carbons have been unsuccessful. This work will be summarized in Section III, along with some suggestions for future work in this area.

III. AMP-PNP: Structural Studies in Solution.

A. Preliminary Considerations.

Several possibilities exist which could accentuate steric and chemical differences between AMP-PNP and ATP in solution, both in the presence and absence of Mg^{2+}. For example, if the pKa of the β,γ N-H proton of AMP-PNP is sufficiently low, this proton could reside primarily on oxygen rather than on nitrogen (see Figure 2a). Such a property could be stabilized by the contribution of "imino" structures of AMP-PNP, which in turn could be stabilized by intramolecular hydrogen bonding (see Figure 2b). Alternatively, in the presence of Mg^{2+} the metal could complex directly with either the protonated or unprotonated nitrogen of the P-N-P bridge, as has been suggested by Tran-Dinh and Roux (1977).

The predominance of an "imino" tautomer in a P-N-P bridge system was proposed for the tetramethyl and tetraethyl esters of imidodiphosphoric acid (PNP) using infrared spectroscopy (Kabachnik et al., 1961; Kireev et al., 1970, see Figure 3). In addition, these esters were found to be reasonably acidic. The pKa values for the tetramethyl and tetraethyl esters of PNP have been measured as 2.6 and 3.7, respectively (Riesel et al., 1977a). Considering the acidic nature of these two compounds it seemed likely that the site of protonation is on one of the two phosphoryl oxygens rather than on the bridging nitrogen and that the "imino" tautomers predominate in aqueous

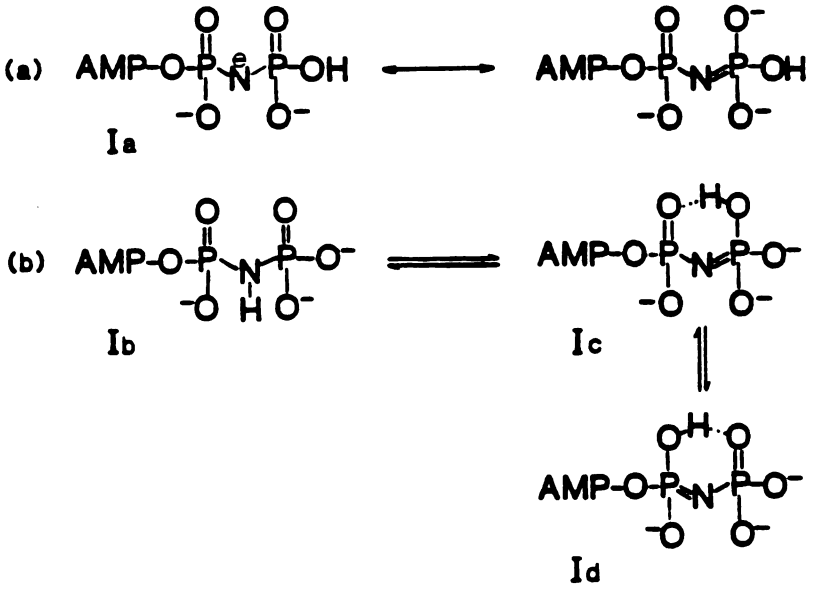


Figure 2: Proposed structures for the tetraanion of AMP-PNP.

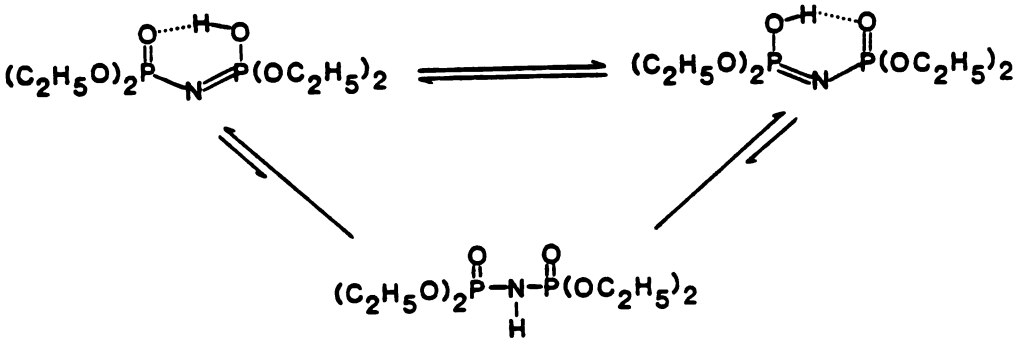


Figure 3: Proposed tautomers for the protonated form of tetraethyl PNP.

solution.

The pKa for the N-H proton in AMP-PNP is expected to be higher than found for the tetraalkyl PNP compounds due to the effects of the negative charged neighboring oxygens. Nevertheless, if this pKa were found to be below 12, for example, it could be inferred that "imino" tautomers of AMP-PNP might be significant in aqueous solutions around physiological pH. We felt that this problem needed to be considered both in the presence and absence of Mg^{2+}, since it was expected that Mg^{2+} could influence the pKa and, correspondingly, influence the thermodynamic stability of "imino" tautomers of AMP-PNP, if present.

B. Previous Studies on AMP-PNP Using ^{31}P NMR.

Previous ^{31}P NMR studies on AMP-PNP as a function of both pH and Mg^{2+} concentration have revealed properties markedly different from those observed for ATP itself (Tran-Dinh et al., 1975, 1977). For example, both in the presence and absence of Mg^{2+}, the resonance associated with β-phosphorus of AMP-PNP experiences a greater (upfield) change in chemical shift than does that for γ-phosphorus upon protonation of the most basic phosphoryl oxygen, whereas with ATP the reverse is true. Furthermore, the resonances of the β- and γ-phosphorus nuclei shift simultaneously, but in opposite directions, over the pH range of 4 to 11.5 when Mg^{2+} is present, while such an effect on chemical shift is only observed for the β-phosphorus of ATP. Such results were originally

interpreted as indicating that AMP-PNP and ATP differ in the site(s) of protonation of their tetrabasic species and in the structure of their Mg^{2+} complexes. Nevertheless, these studies were far from conclusive.

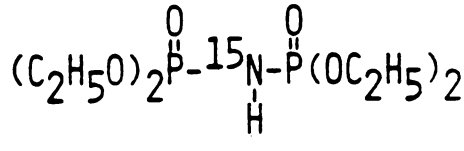
In a more recent study, Jaffe and Cohn (1978) summarized experimental data which led to their conclusion that the chemical shift change of a ^{31}P NMR resonance in a polyphosphate chain cannot be related to the site of protonation. In particular, the ^{31}P NMR titration behavior of thiophosphates was found to be strikingly anomalous when compared to phosphates: protonation of phosphates leads to upfield changes in chemical shift whereas protonation of thiophosphates leads to downfield changes in chemical shift.

It is presently impossible to quantitatively interpret ^{31}P NMR chemical shifts. However, Gorenstein and coworkers (1976a,b) have provided the results of theoretical calculations which show that ^{31}P NMR chemical shifts are sensitive to changes in both O-P-O bond angles and P-O torsional angles. Thus, a large change in ^{31}P NMR chemical shift observed for a particular phosphoryl group in a polyphosphate chain as a function of pH could conceivably result from conformational changes introduced by protonation of a different group in that chain.

C. Employment of ^{15}N and ^{17}O NMR to Determine Protonation Sites.

1. ^{15}N NMR. In recent years, ^{15}N NMR spectroscopy has been employed successfully to determine the site(s) of protonation on nitrogen atoms by virtue of sizable upfield changes in chemical shift. The sensitivity of ^{15}N-resonances to protonation is usually ascribed to changes in the paramagnetic contribution to the nitrogen scree constant. For example, this technique has been used to locate the protonation sites on the purine and pyrimidine-rings of various nucleosides, nucleotides, and thiamine (Markowski et al., 1977; Cain et al., 1977). The magnitude of these shifts ranged approximately between 60-100 ppm. In another study, ^{15}N NMR was used to determine the protonation site on trioxodinitrate ($N_2O_3^{2-}$) (Bonner et al., 1981). In this case, an upfield change in chemical shift of 24 ppm was observed.

These ^{15}N NMR studies encouraged us to prepare ^{15}N enriched analogs of tetraethyl imidodiphosphate (7), for which a pKa of 3.7 had previously been measured, and of AMP-PNP in which the β,γ-bridge nitrogen is enriched with ^{15}N (8).



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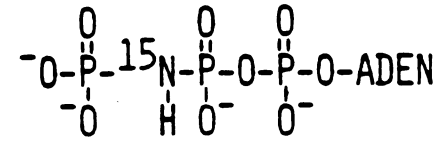

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2. ^{17}O NMR. Until recently, attempts to obtain ^{17}O NMR spectra of phosphoryl oxygens was considered impractical for several reasons: (1) the low natural abundance of ^{17}O (0.037%) necessitates the preparation of ^{17}O-enriched samples; (2) the quadrupolar nature of ^{17}O (I=5/2) may be expected to produce resonances which are too broad to quantitate; and (3) high field spectrometers which are equipped to observe ^{17}O while simultaneously decoupling directly bonded ^{31}P nuclei are not routinely available. This last criterion is important, since the ability to decouple ^{31}P nuclei while observing ^{17}O resonances of directly bound oxygens is expected to provide a means to assign ^{17}O-resonances in addition to providing narrower peaks.

The first application of ^{17}O NMR to the determination of protonation sites on oxygen in phosphorus compounds was achieved by John Gerlt and coworkers (1982) at Yale University. A high field magnet (6.3 T) capable of observing ^{17}O while selective decoupling of ^{31}P nuclei was employed. In addition, Gerlt recognised that ^{17}O NMR linewidths are predicted to decrease at elevated temperatures.

Gerlt's decision to acquire ^{17}O NMR spectra at higher temperatures can be rationalized as follows. Assuming that the predominant relaxation mechanism for ^{17}O is quadrupolar in nature, the linewidth is predicted by theory to be directly related to the nuclear quadrupolar coupling constant, e qQ/h, and to the rotational correlation time of the ^{17}O nucleus, τ_c (Gorenstein et al., 1976b):

$$\text{Linewidth} = \frac{1}{\pi T_2} = \frac{12\pi}{125}\left(1 + \frac{\eta^2}{3}\right)\left(\frac{e^2qQ}{h}\right)\tau_c$$

where eq is the field gradient at the nucleus due to the electrons, eQ is the field gradient due to the nucleus, η is an asymmetry parameter (which can vary from 0 to 1), and T_2 is the spin-lattice relaxation time. For spherical molecules, the Stokes equation predicts that τ_c should be inversely proportional to temperature:

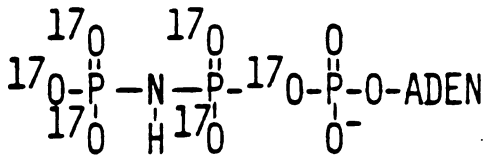
$$\tau_c = \frac{4\pi a^3 \eta'}{3kT}$$

where a is the molecular radius, T is the temperature, and η' is the solvent viscosity. Thus, an increase in the temperature is expected to decrease τ_c and, correspondingly, the linewidth.

Interestingly, Gerlt has found that the upfield shift observed for an ^{17}O resonance associated with protonation of oxygen is remarkably constant for most phosphorus compounds. The upfield shift resulting for a change of a full unit charge on oxygen is about 50 ppm. The actual shift observed is less if this change in unit charge is shared by two or more oxygens. For example, the shift observed upon protonation of the tribasic form of inorganic phosphate was about 13 ppm (roughly 1/4 X 50 ppm, indicating the change of 1 unit charge is shared by all four oxygens).

We were encouraged by this work to prepare a sample of AMP-PNP enriched with ^{17}O in the P_β and P_γ oxygens (9). The ^{17}O chemical shift behavior of this compound as a function of

pH was examined in collaboration with Professor Gerlt at Yale. Similar experiments were later performed on a sample of tetraethyl imidodiphosphate which had been enriched with ^{17}O in one of the phosphoryl oxygens (10).



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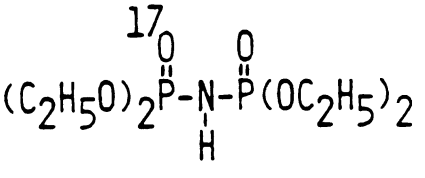

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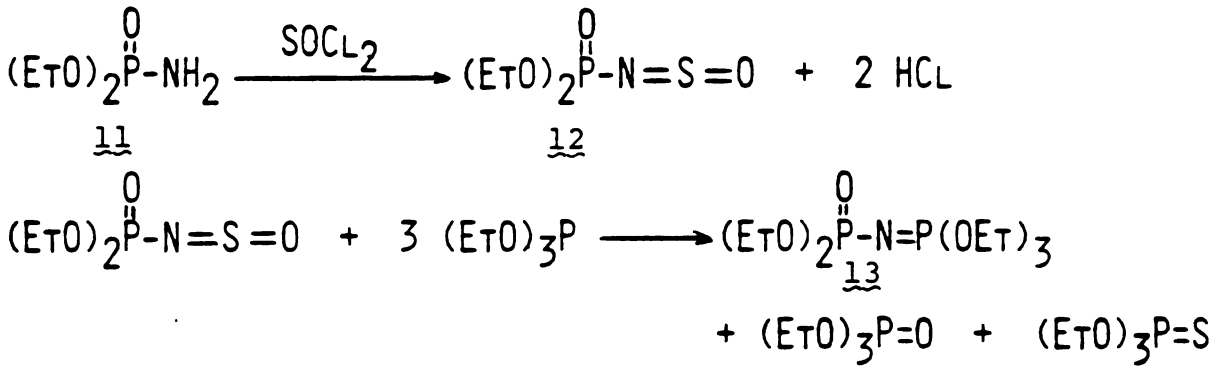
D. Results and Discussion: Structure of Tetraethyl Imidodiphosphate in Solution.

1. Rationale for Synthesis.

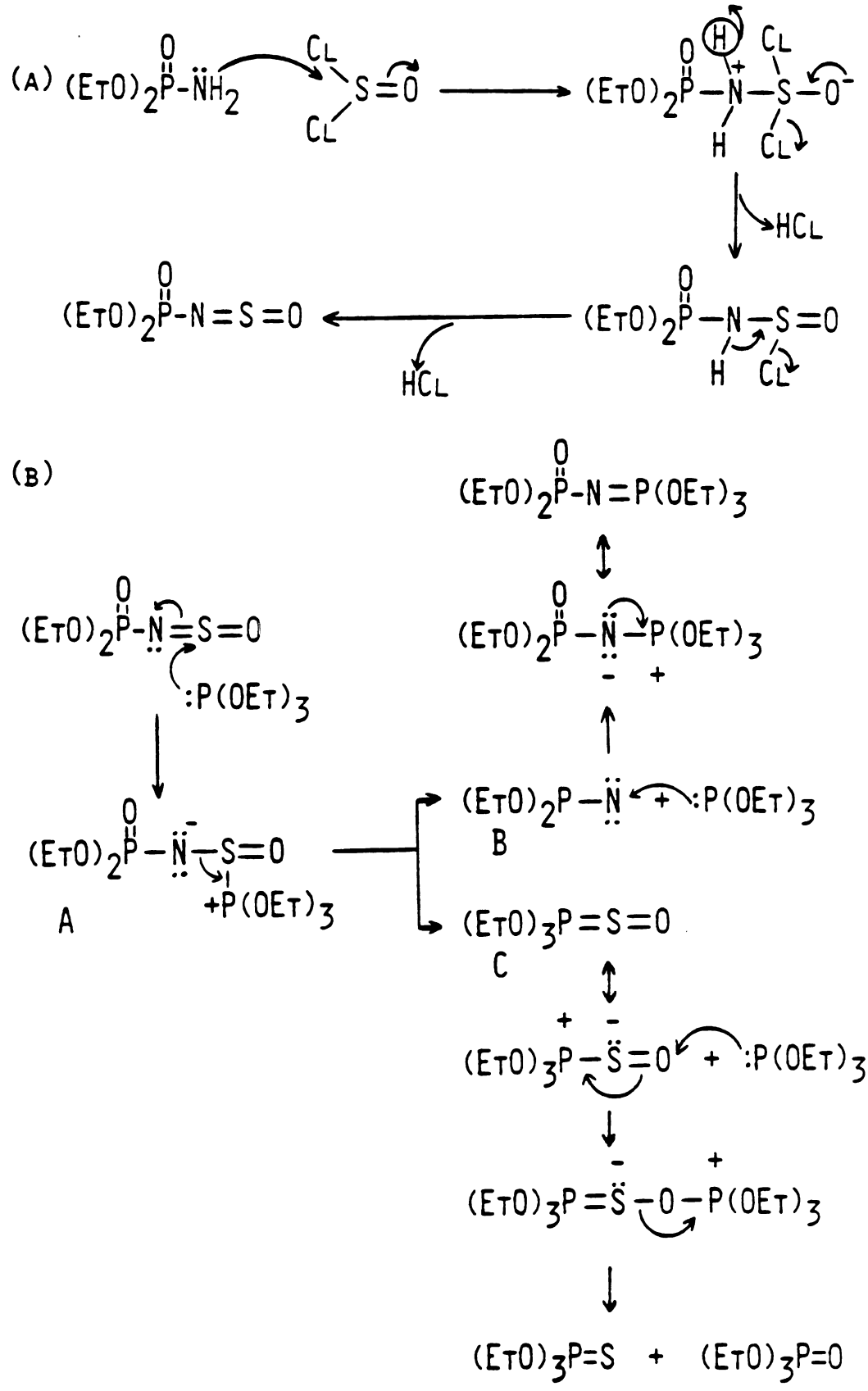
The general procedure for preparing tetraethyl imidodiphosphate begins with diethyl phosphoramidate (11) as outlined in Scheme I. This procedure was adapted from the synthesis of Wieczorkowski (1963, 1965). Thus, reaction of diethyl phosphoramidate with thionyl chloride (1 equiv.) in dry benzene gives diethyl phosphoro(thionylamidate) (12). The mechanism for this reaction may be considered to proceed with initial attack by nitrogen on sulfur followed by the elimination of two molecules of HCl (Scheme IIa).

SCHEME I. PREPARATION OF TETRAETHYL IMIDODIPHOSPHATE FROM DIETHYL PHOSPHORAMIDATE



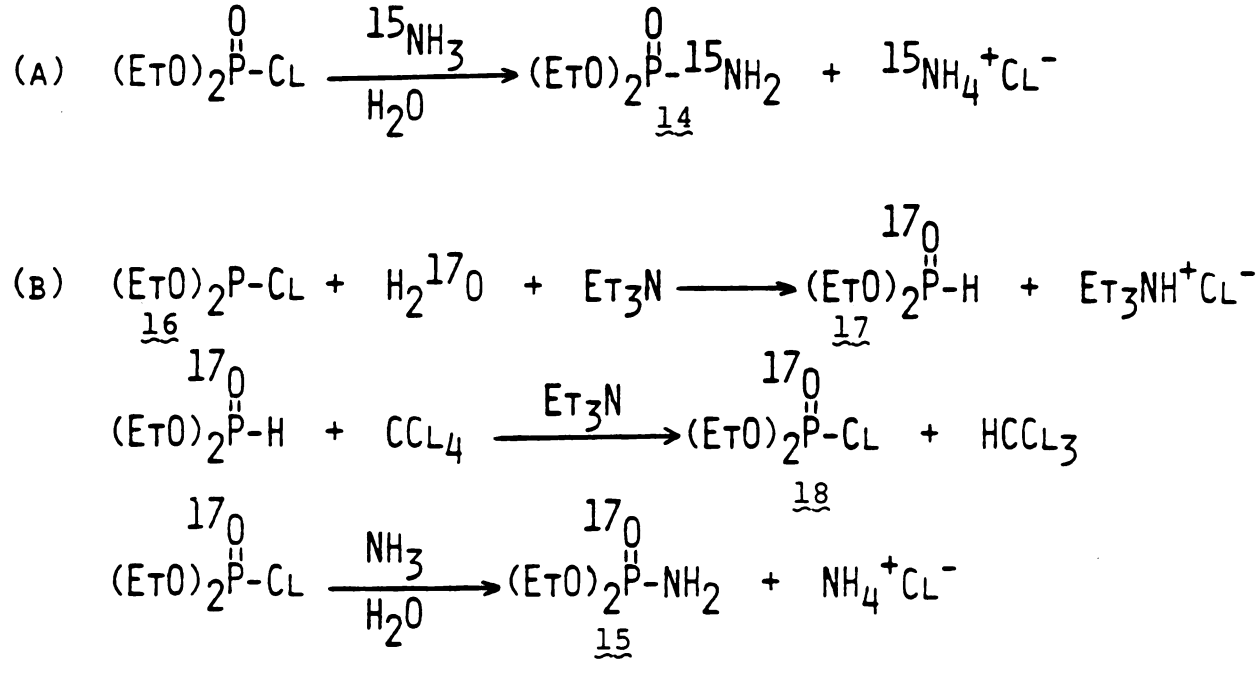
The proposed mechanism for the reaction of diethyl phosphoro(thionylamidate) is a bit more complicated (Sheme IIb). It involves initial nucleophilic attack by a triethyl phosphite molecule at the sulfur atom of diethyl phosphoro(thionylamidate), which is presumed to be the most electrophilic center of this compound, to form an intermediate adduct (A). The adduct (A) subsequently decomposes to form an electron-deficient nitrene intermediate (B), which is expected to combine with a second molecule of triethyl phosphite to give the final phosphoramidate. The second intermediate in this scheme, termed a phorphorothionate S-oxide (C), is expected to be unstable and therefore should react with a third molecule of triethyl phosphite to produce a molecule each of triethyl phosphorothioate and triethyl phosphate.

SCHEME II.

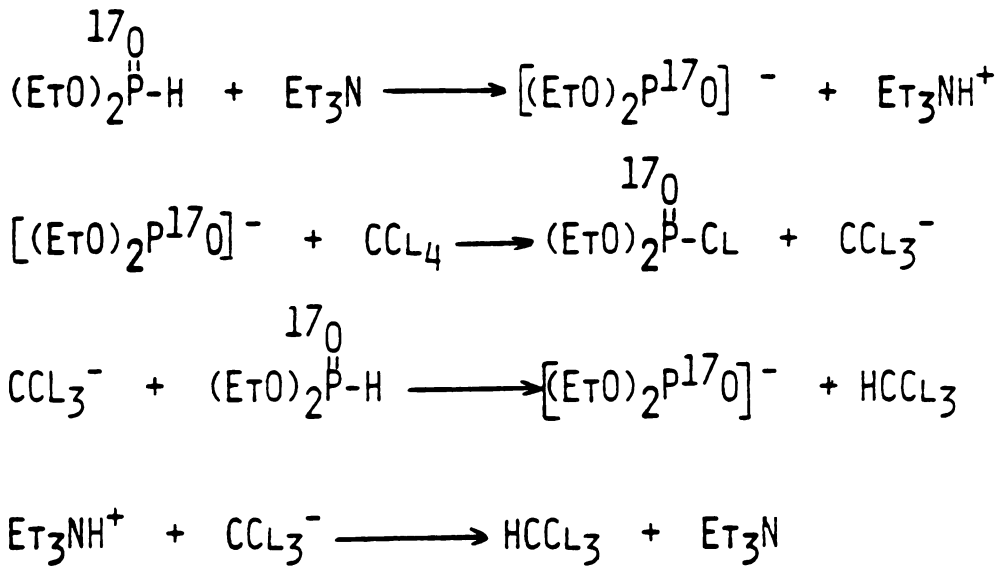

(A)
(EtO)2P-NH2
Cl
S=O
Cl
(EtO)2P-N-S-O
H
Cl
H
Cl
HCl
(EtO)2P-N-S=O
H
Cl
(EtO)2P-N=S=O
HCl
(B)
(EtO)2P-N=P(OEt)3
(EtO)2P-N-P(OEt)3
(EtO)2P-N=S=O
:P(OEt)3
(EtO)2P-N-S=O
+P(OEt)3
A
(EtO)2P-N + :P(OEt)3
B
(EtO)3P=S=O
C
(EtO)3P-S=O + :P(OEt)3
(EtO)3P=S-O-P(OEt)3
(EtO)3P=S + (EtO)3P=O

^{15}N and ^{17}O labeled analogs of tetraethyl imidodiphosphate were prepared according to Scheme I using ^{15}N- and ^{17}O-labeled diethyl phosphoramidate. ^{15}N-Diethyl phosphoramidate was prepared from diethyl chlorophosphate and aqueous ^{15}N-enriched ammonia according to Scheme IIIa. ^{17}O-Diethyl phosphoramidate (15) was prepared from the procedure of Goehring and Niedenzu (1956). ^{17}O-Diethyl phosphite (17) was prepared by reaction of diethyl chlorophosphite (16) with ^{17}O-enriched water and triethylamine according to a procedure developed in our laboratory. The reaction of ^{17}O-diethyl phosphite with carbon tetrachloride in the presence of a catalytic amount of triethylamine to produce ^{17}O-diethyl chlorophosphate (18) was performed according to the procedure of Steinberg (1950). A proposed mechanism for this reaction appears in Scheme IV.

SCHEME III. PREPARATION OF ^{15}N- AND ^{17}O-LABELED DIETHYL-PHOSPHORAMIDATE.



SCHEME IV.


17O
(EtO)2P-H + Et3N
[(EtO)2P17O]- + Et3NH+
[(EtO)2P17O]- + CCl4
17O
(EtO)2P-Cl + CCl3-
CCl3- + (EtO)2P-H
17O
[(EtO)2P17O]- + HCCl3
Et3NH+ + CCl3-
HCCl3 + Et3N

2. ^{31}P and ^{17}O NMR pH Titrations of Tetraethyl PNP.

^{31}P NMR chemical shifts were measured for unlabeled tetraethyl PNP in 20% D_2O at 25°C as a function of pH, and the results are presented in Figure 4. Although the magnitude of the shift observed over this pH range was small (about 1.2 ppm), the data produced a calculated pKa of 3.8 for this compound, in good agreement with the value of 3.7 obtained by potentiometric titration (Riesel et al., 1977a). No noticeable decomposition of tetraethyl PNP was observed in the ^{31}P NMR spectra recorded in this experiment, confirming that the previously reported pKa value for this compound was in fact due to tetraethyl PNP and not one of its hydrolysis products.

The effect of pH on the ^{17}O NMR spectra of ^{17}O-labeled tetraethyl PNP (10) was determined in 20% D_2O at 30°C, and the results are given in Figure 5. The data produced a calculated pKa value of 4.21 $\pm$ 0.04. However, the magnitude of the upfield shift in the ^{17}O NMR spectrum occuring upon protonation was only 5.4 ppm; this is smaller than would be predicted if protonation were occurring exclusively on one of the phosphoryl oxygens of tetraethyl PNP (1/2 X 50 = 25 ppm). In fact, the upfield ^{17}O NMR chemical shift observed could possibly be an indirect measure of protonation occurring on nitrogen instead. We attempted to answer this question by ^{15}N NMR using the ^{15}N-labeled analog of tetraethyl PNP (7).

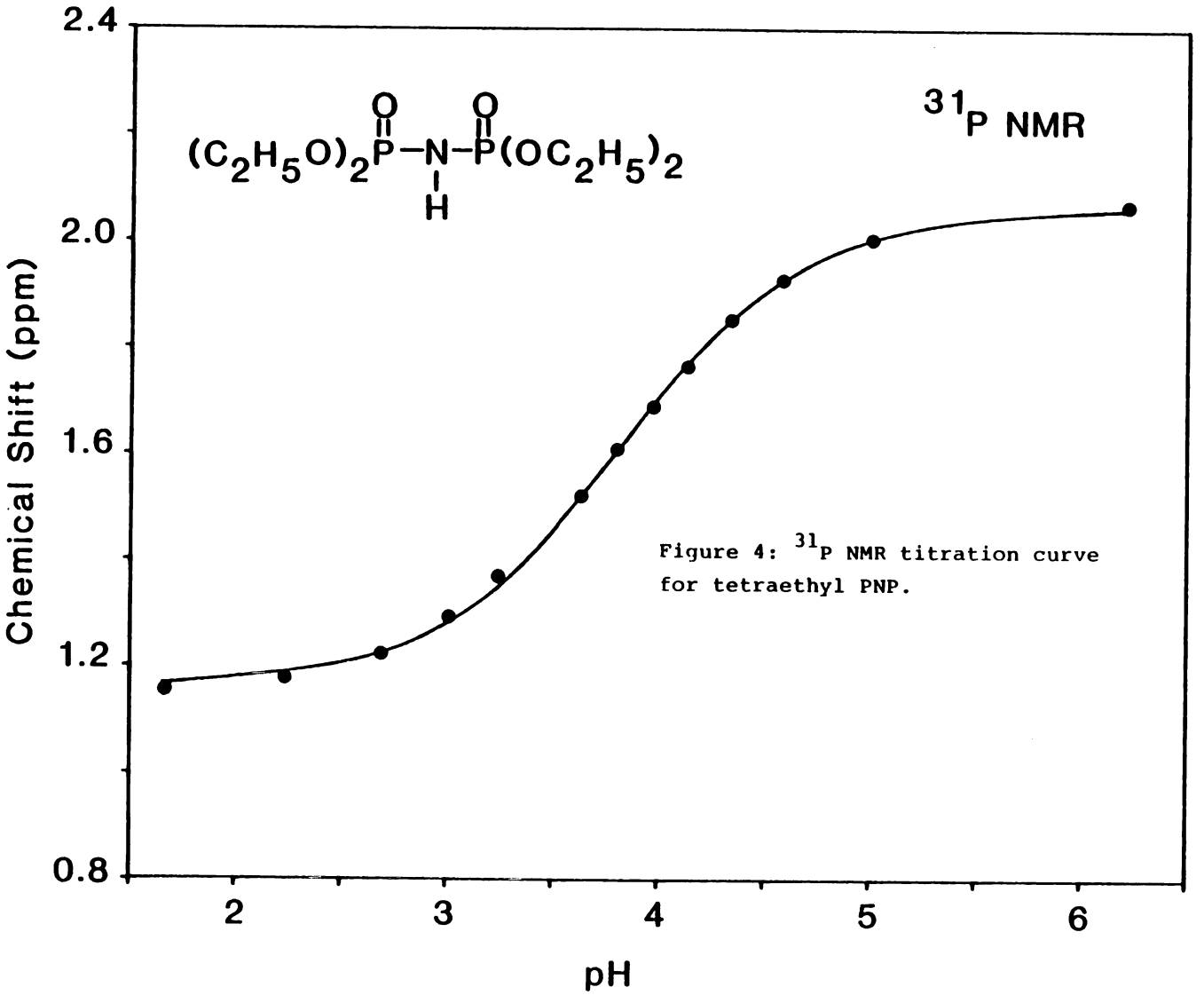


Figure 4: ^{31}P NMR titration curve for tetraethyl PNP.

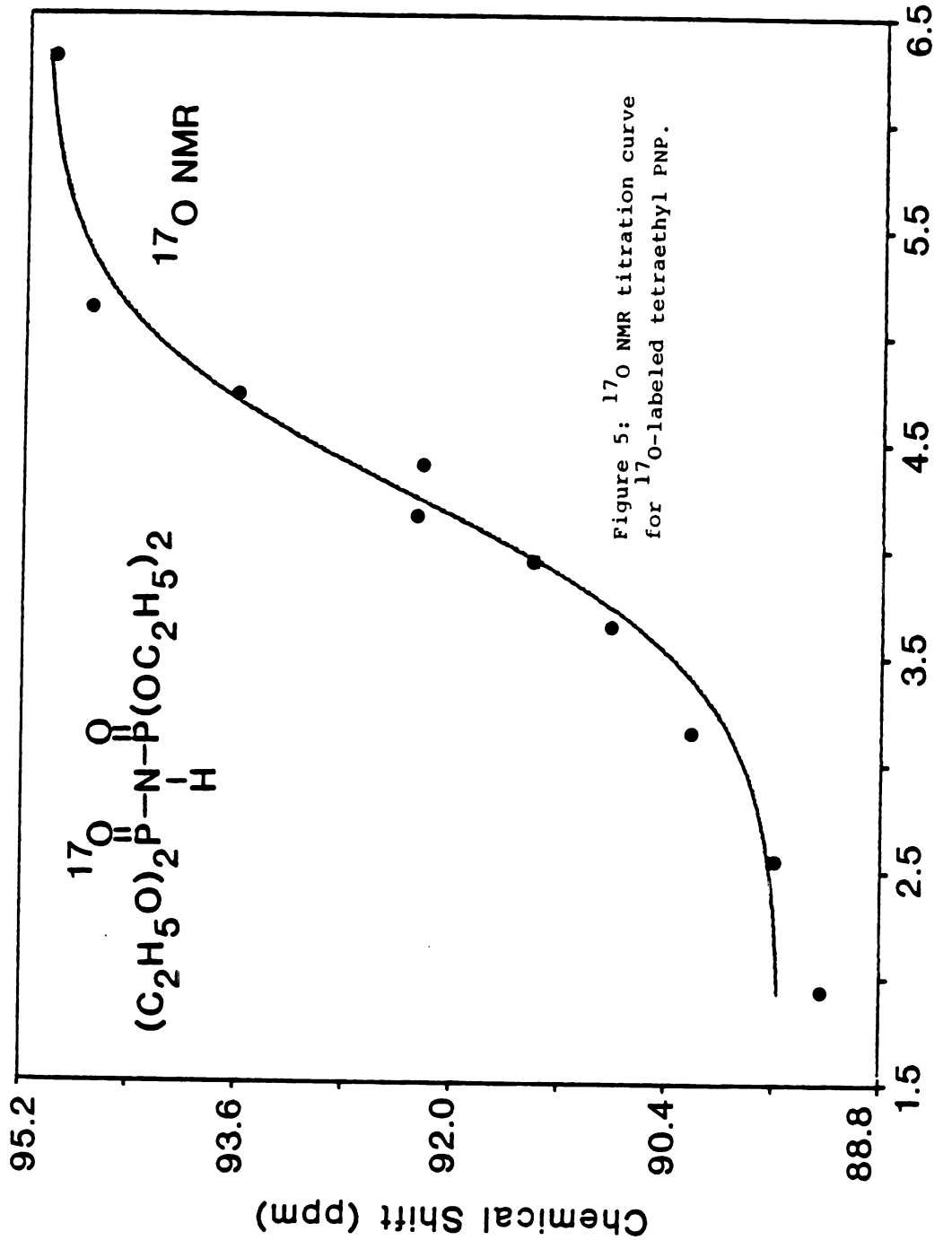


Figure 5: ^{17}O NMR titration curve for ^{17}O-labeled tetraethyl PNP.

3. ^{15}N NMR Chemical Sifts versus pH for ^{15}N-Tetraethyl PNP.

Considering the large upfield shifts typically observed in ^{15}N NMR spectra when protonation occurs on nitrogen, we expected the effect of pH on the ^{15}N NMR chemical shift of N-tetraethyl PNP to be substantial if protonation occurs on nitrogen.

Preliminary attempts at obtaining ^{15}N NMR spectra for ^{15}N tetraethyl PNP in 20% D_2O proved to be difficult; the spin-lattice relaxation time for the ^{15}N nucleus in this compound appears to be long in aqueous solvent. No measurable ^{15}N resonances could be observed for a 100 mM sample of ^{15}N tetraethyl PNP after 6 hours of data accumulation at 24.36 MHz with broad-band ^{1}H decoupling. Therefore, we decided to measure the ^{15}N NMR chemical shift indirectly using narrow-band ^{15}N decoupling while observing the ^{31}P NMR spectrum. Since the proton r.f. synthesizer was devoted to obtaining a proton lock, proton decoupling was not performed, so the peaks were somewhat broad. Figure 6 shows the ^{31}P NMR spectra at 97.3 MHz recorded for this compound at pH 6.22 (25°C) in the presence (Figure 6 b) and absence (Figure 6 a) of narrow-band ^{15}N decoupling when the r.f. synthesizer setting was on resonance. Once an approximate ^{15}N r.f. synthesizer setting was found which decoupled the ^{15}N splitting on the ^{31}P resonances, the setting which would produce the maximum decoupling was calculated as follows. The linewidth of the ^{31}P peak at various ^{15}N r.f. synthesizer settings was calculated using a Lorentzian line fitting routine. Plots were then made of

^{31}P NMR: $(C_2H_5O)_2\overset{O}{\overset{\|}{P}}-\overset{15}{\underset{H}{N}}-\overset{O}{\overset{\|}{P}}(OC_2H_5)_2$

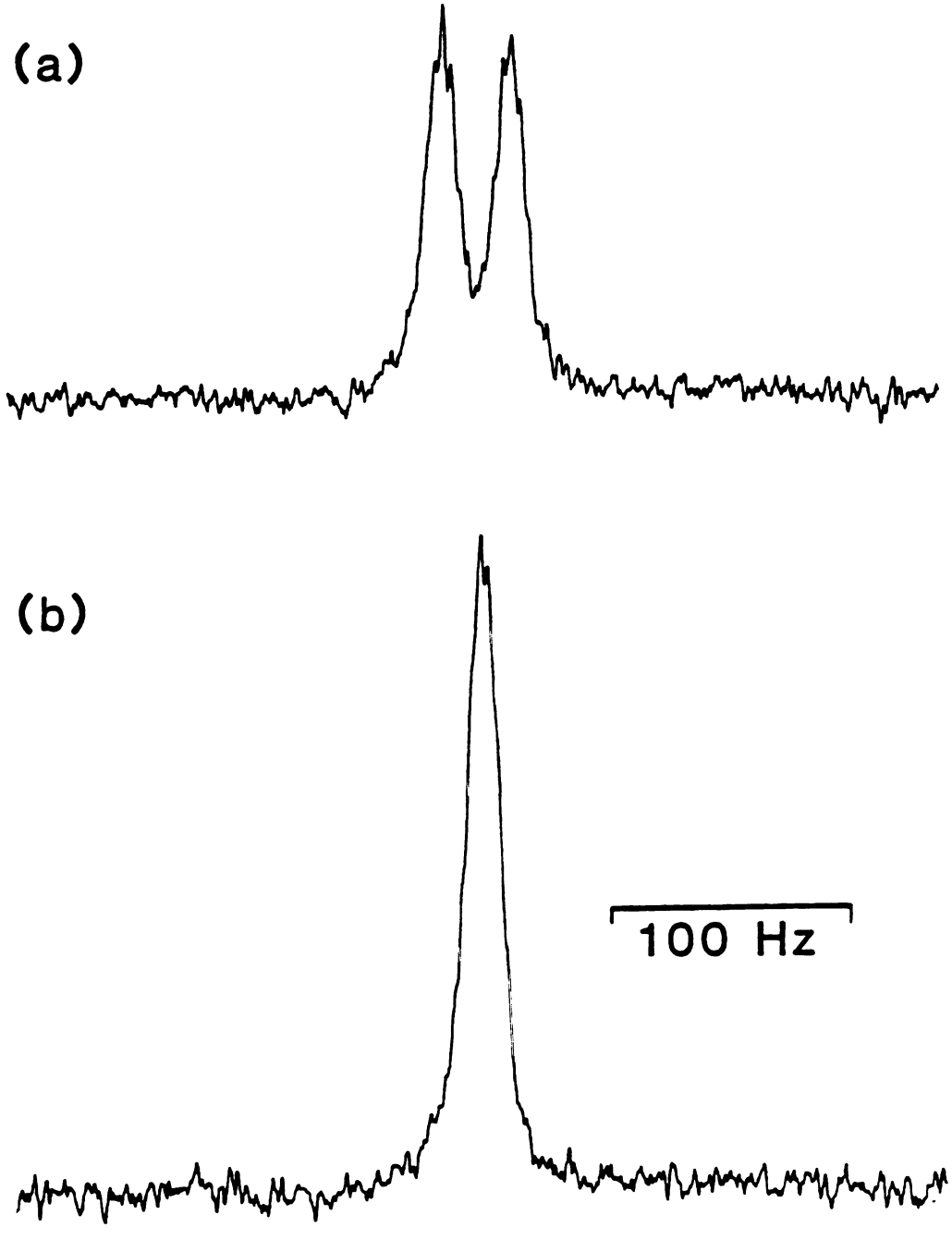


Figure 6: ^{31}P NMR spectra (proton coupled) of ^{15}N-labeled tetraethyl PNP: (a) without and (b) with ^{15}N-decoupling.

^{31}P linewidth versus ^{15}N r.f. synthesizer settings at pHs 6.22 and 1.88 (Figure 7). A smooth curve was drawn through each set of data, and the optimal ^{15}N r.f. synthesizer setting was determined at the minimum of each curve. The ^{15}N r.f. synthesizer was calibrated with respect to chemical shift in a separate experiment where ^{15}N decoupling was performed while observing the ^{31}P NMR spectrum of ^{15}N-labeled PNP. (The ^{15}N NMR spectrum of ^{15}N-labeled PNP in aqueous solvent can be readily observed--see Section E.)

The results from this experiment are summarized in Table 1. The ^{15}N NMR chemical shift for ^{15}N tetraethyl PNP only shifts about 2.5 ppm upfield as the pH is dropped from 6.22 to 1.88.

Table 1. ^{15}N NMR Chemical Shifts for ^{15}N Tetraethyl PNP as a Function of pH.

pH	δ^a (Relative to $(^{15}NH_4)_2SO_4$)
6.22	71.2 ppm
1.88	68.7 ppm

a) Measured in water at 25°C.

We next attempted to determine if the acidic proton of ^{15}N tetraethyl PNP is localized on nitrogen in an organic solvent. In contrast with our findings in aqueous solvent, the ^{15}N NMR spectrum of ^{15}N tetraethyl PNP can be readily obtained in $CDCl_3$. The ^{15}N NMR spectra of this compound in the presence of broadband ^1H decoupling and with broadband ^1H decoupling off during data acquisition are shown in Figure

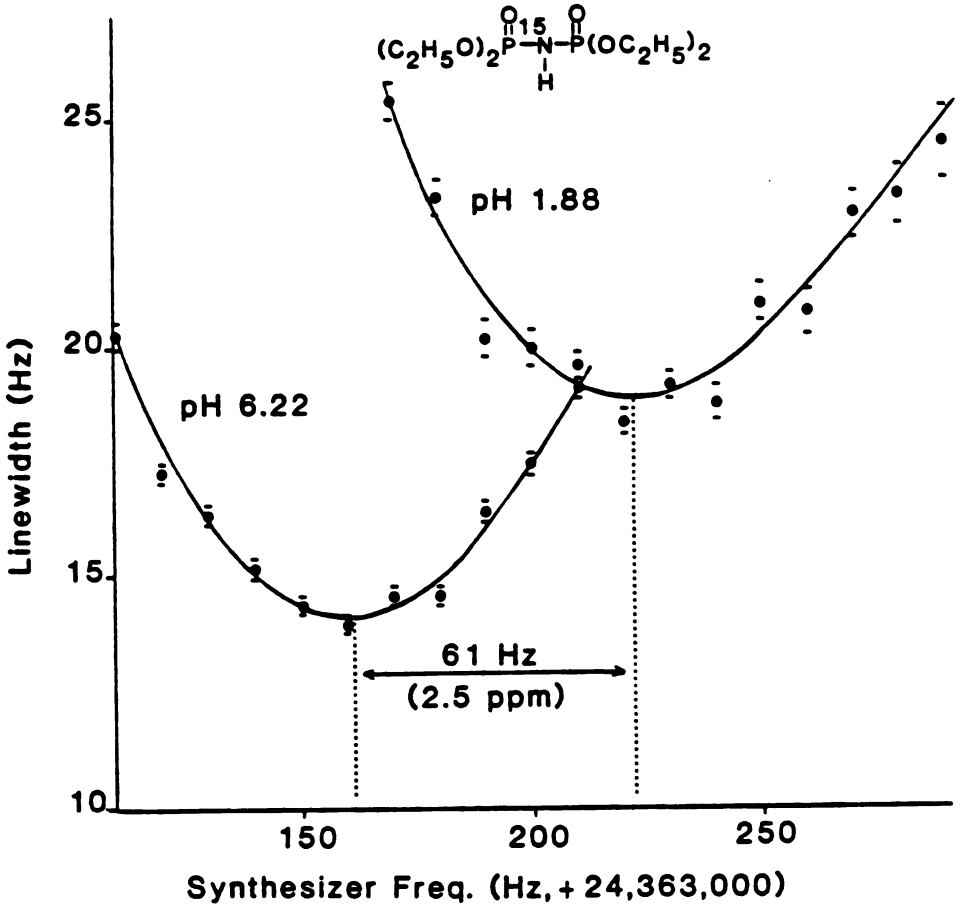


Figure 7: Plots of ^{31}P NMR linewidths for ^{15}N-labeled tetraethyl PNP as a function of ^{15}N-decoupler frequency.

8. These spectra indicate that if the acidic proton does reside on nitrogen it is in fast exchange. (This result is in contrast with the sizable ^{15}N-^{1}H coupling constants we have measured for ^{15}N-labeled PNP and AMP-PNP--see Section E.)

In summary, the ^{17}O and ^{15}N NMR experiments just described are inconclusive with respect to the site of protonation for tetraethyl PNP in aqueous solution. The magnitudes of ^{17}O and ^{15}N NMR chemical shifts for this compound as a function of pH are both much smaller than would be predicted if protonation were occurring exclusively on either oxygen or nitrogen. On the other hand, it is possible that ^{17}O NMR and/or ^{15}N NMR chemical shifts for this compound are somewhat insensitive to changes in pH. Perhaps the most satisfying proposition is that all three tautomers of the protonated form of tetraethyl PNP exist (see Figure 3), with the acidic proton exchanging rapidly between the two phosphoryl oxygens and the nitrogen. This would explain the presence of a P=N bond proposed for this compound from infrared evidence, as well as the fact that little ^{15}N-^{1}H coupling is observed for the ^{15}N-labeled analog in deuterochloroform.

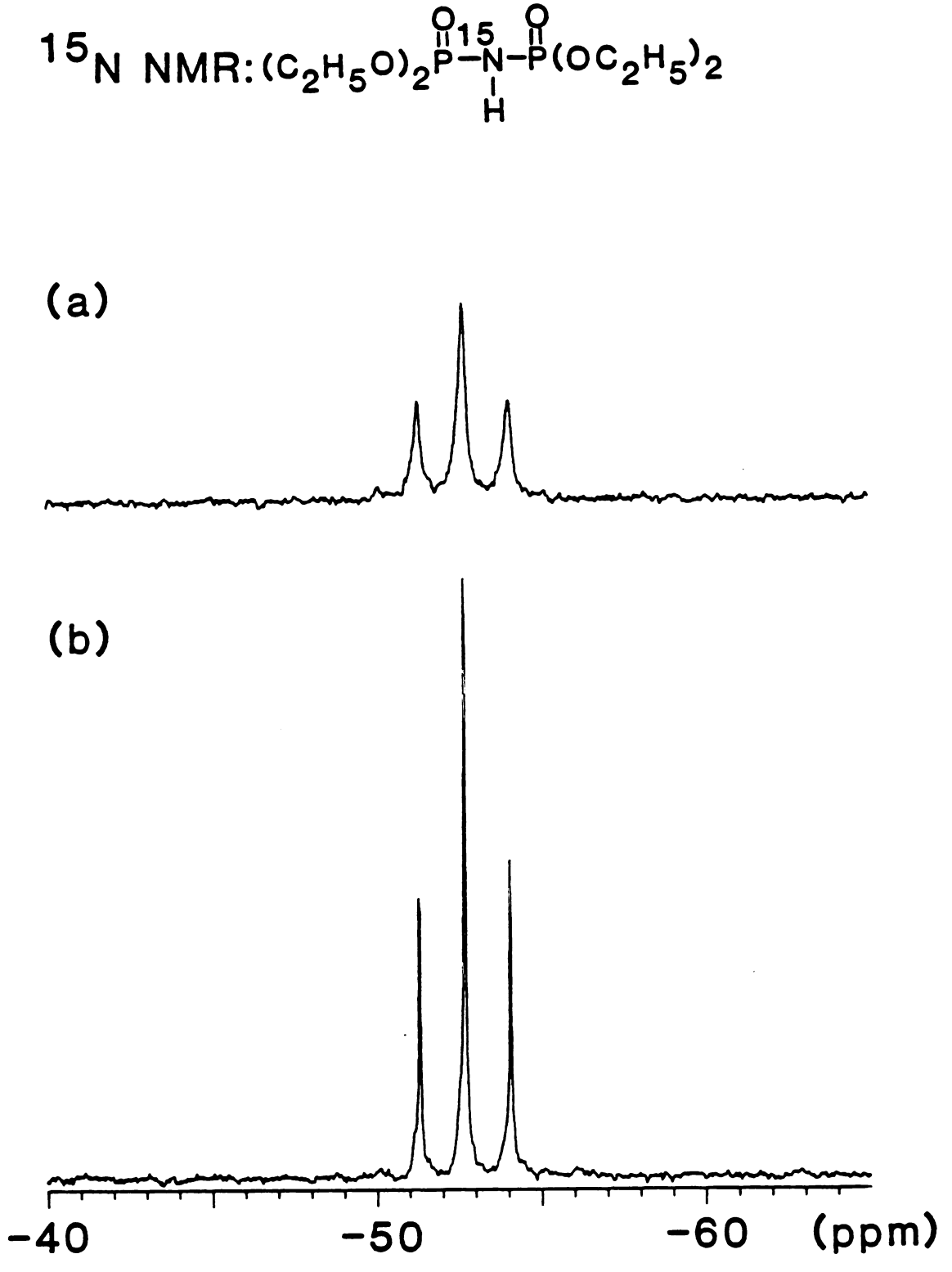


Figure 8: ^{15}N NMR spectra of ^{15}N-labeled tetraethyl PNP in $CDCl_3$: (a) without and (b) with broadband ^{1}H decoupling.

E. Results and Discussion: Structure of AMP-PNP and PNP in Solution.

1. Synthesis of ^{15}N- and ^{17}O-Labeled AMP-PNP.

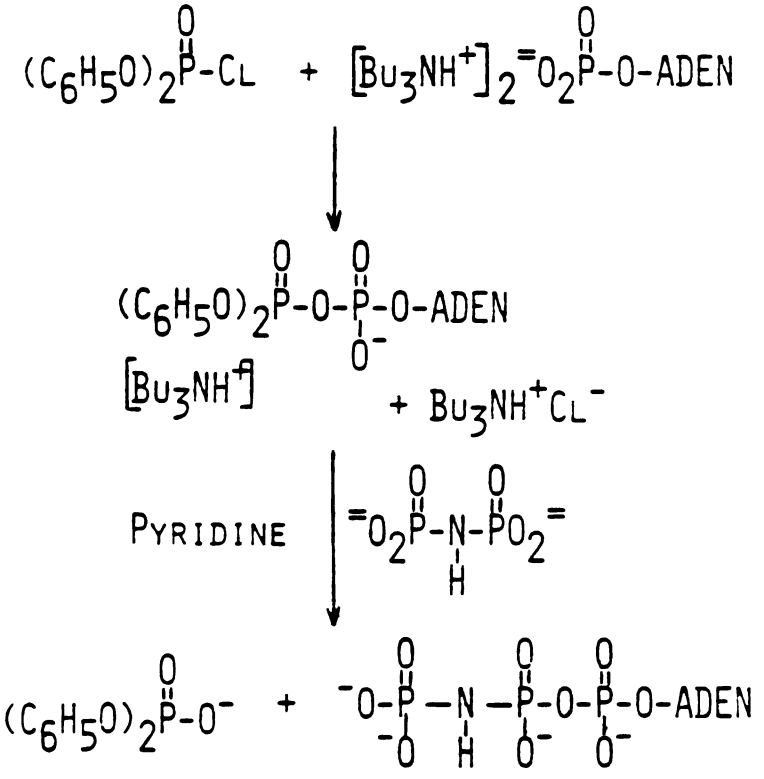
In the synthesis of ^{15}N- and ^{17}O-labeled AMP-PNP, the general procedure of Michelson (1964) was used to couple the suitably labeled PNP analogs with AMP using diphenyl chlorophosphate (Scheme V). In this scheme, AMP is reacted as its tributylammonium salt with diphenyl chlorophosphate to produce the P^1-nucleoside-5' P^2-diphenyl pyrophosphate (A). The diphenyl phoshpate group is readily displaced by imidodiphosphate in the presence of pyridine. With this procedure in hand, the problem of preparing ^{15}N- and ^{17}O-labeled AMP-PNP becomes one of preparing ^{15}N- and ^{17}O-labeled PNP.

2. Synthesis of ^{15}N-Labeled PNP.

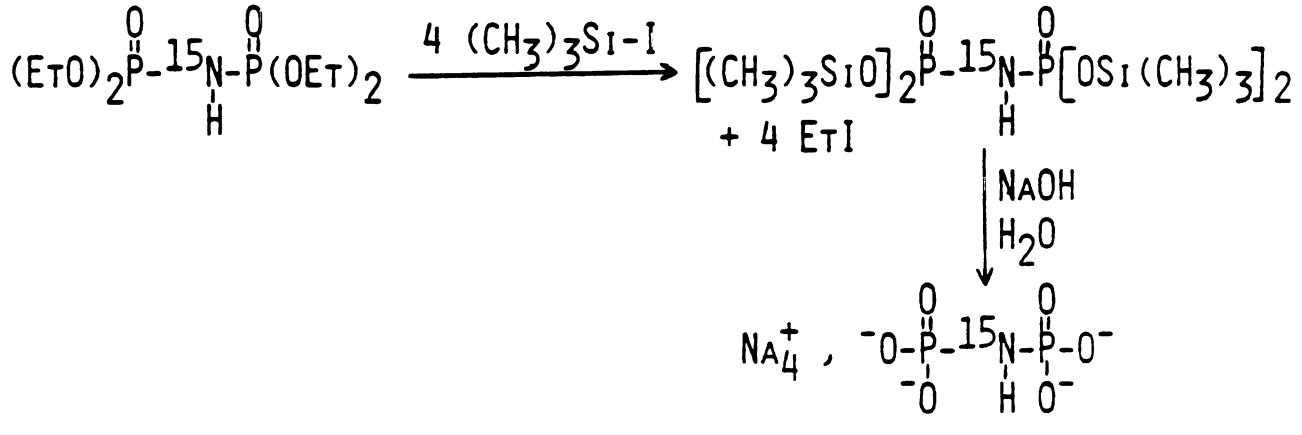
Our first preparation of ^{15}N-labeled PNP (19, see Reynolds et al., 1981) was achieved by way of ^{15}N-labeled tetraethyl PNP (7) (Scheme VI). Removal of the ethyl ester groups in (7) with trimethylsilyl iodide was an adaptation of the method of Chojnowski et al. (1978). The overall yield of ^{15}N-labeled PNP by this procedure from ^{15}N-ammonia was 11.3%.

Subsequent to this work, a more direct route to ^{15}N-PNP was discovered. This procedure is outlined in Scheme VIIa. The synthesis of ^{15}N-dichlorophosphoryl

SCHEME V.



SCHEME VI.

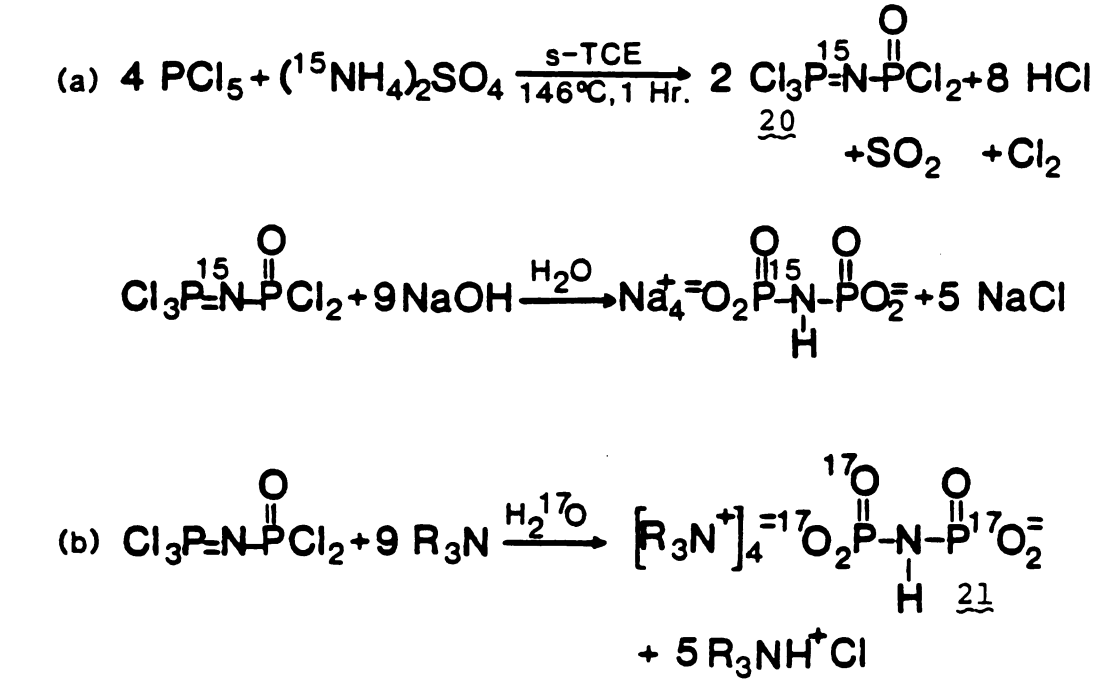


trichloroiminophosphorane (20) was achieved from PCL_5 and $(^{15}NH_4)_2SO_4$ according to the procedure of Emsley et al. (1971). Scheme VIII shows a proposed mechanism for this reaction. Thus, two molecules of PCl_5 are known to be in equilibrium with the charged species PCl_4^+ and PCL_6^-. It is assumed that the electrophilic PCl_4^+ species is favored for attack by nucleophiles. If the attacking nucleophile is SO_4^{2-}, the resulting intermediates are $POCl_3$ and SO_3Cl^-. The nucleophile SO_3Cl^- is capable of attacking a second molecule of PCL_5 (again presumed as PCl_4^+) to produce a second molecule of $POCl_3$ along with the volatile gases SO_2 and Cl_2. If, on ther other hand, the attacking nucleophile is NH_3, the resulting intermediate is Cl_3PNH. When the reaction is run at the reflux temperature of s-tetrachloroethane (146°C),

the rates of production of $POCl_3$ and Cl_3PNH are so balanced that the reaction terminates with the formation of the desired dichlorophosphoryl trichloroiminophosphorane.

When ^{15}N-PNP was prepared according to Scheme VIIa, the overall yield was 59% based on $(^{15}NH_4)_2SO_4$. This was a substantial improvement compared with the yield obtained by our original synthetic route.

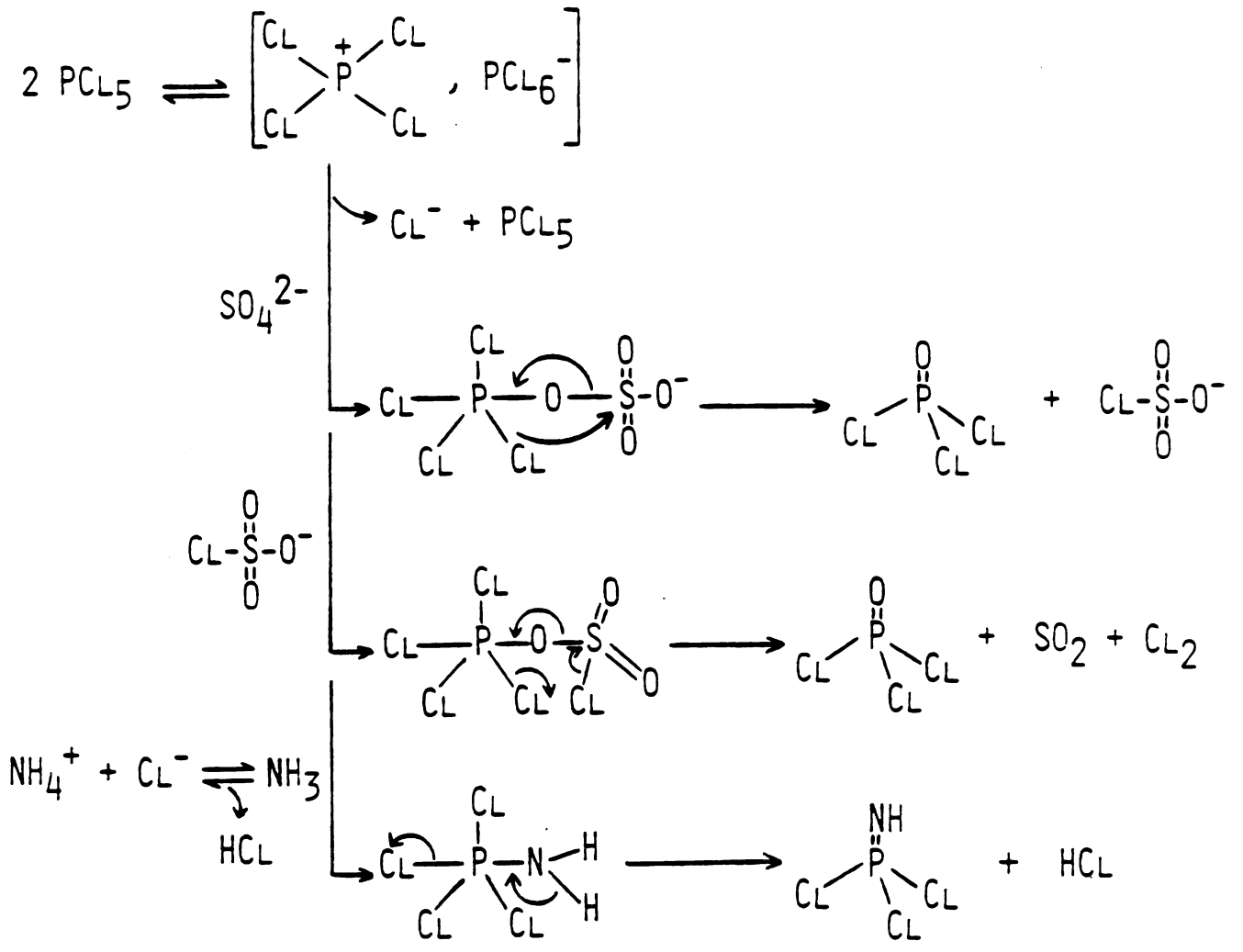
SCHEME VII.



s-TCE = s-Tetrachloroethane

R = C_2H_5

SCHEME VIII.

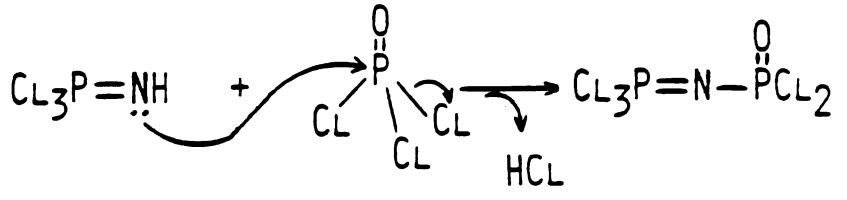


NET PARTIAL REACTIONS:

$$PCl_5 + NH_4^+ \longrightarrow Cl_3P{=}NH + H^+ + 2\ HCl$$

$$2\ PCl_5 + SO_4^{2-} \longrightarrow 2\ POCl_3 + 2\ Cl^- + SO_2 + Cl_2$$

COUPLING REACTION:



3. Synthesis of ^{17}O-Labeled PNP.

After preparing ^{15}N-labeled PNP by Scheme VIIa, we recognized that ^{17}O-labeled PNP (21) could be prepared by the trivial hydrolysis of unlabeled dichlorophosphoryl trichloroiminophosphorane in the presence of $H_2{}^{17}O$ and triethylamine (Scheme VIIb).

4. ^{31}P and ^{17}O NMR pH Titrations of PNP.

In Section B we discussed the anomalous findings of several ^{31}P NMR studies which contribute to the conclusion that ^{31}P NMR can not be used to reliably quantitate the degree of charge neutralization of phosphoryl oxygens. Therefore, we decided to compare the results of ^{31}P and ^{17}O NMR titration experiments with PNP and ^{17}O-labeled PNP.

Results of ^{31}P NMR chemical shift measurements for PNP as a function of pH are given in Figure 9. Protonation of both the tetra- and trianionic species results in upfield changes in the ^{31}P NMR resonances, but the magnitudes of the shift changes are significantly different (0.21 and 1.86 ppm, respectively). The pKas derived from these data are 10.26 ± 0.24 and 7.42 ± 0.03; these are in excellent agreement with those obtained by potentiometric titration (10.22 and 7.32, see Irani and Callis, 1961). Although the pKa's measured by ^{31}P NMR are consistent with those obtained by conventional means, nothing can be said about the absolute magnitudes of the chemical shift changes induced by protonation given the possibility for contribution by the imino tautomer (refer to Figure 2).

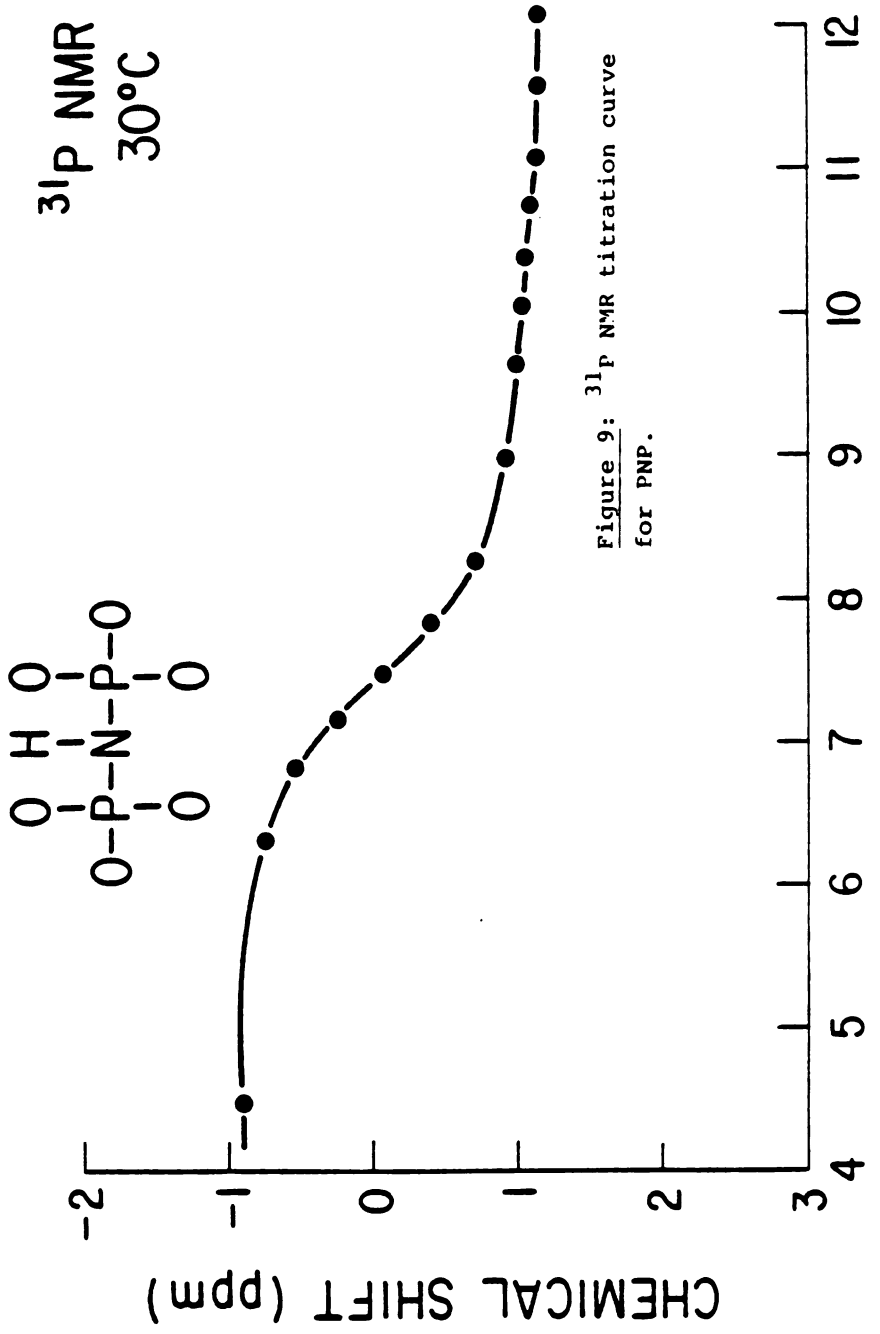


Figure 9: ^{31}P NMR titration curve for PNP.

The ^{17}O NMR pH titration curve of ^{17}O-PNP was measured at 30°C, and the data are shown in Figure 10. Equal upfield chemical shift changes are found for protonation of both the tetra and trianionic species (9.6 and 8.8 ppm, respectively). Assuming that charge neutralization occurs exclusively on the phosphoryl oxygens, the magnitudes of the upfield shifts per charge neutralized are 58 and 53 ppm, respectively; these values are in good agreement with those determined for a variety of phosphates (Gerlt et al., 1982) and phosphate analogs (Gerlt et al., submitted), thereby confirming the assumption that protonation occurs on the oxygens. The pKa values obtained from this titration curve, 10.37 ± 0.12 and 7.41 ± 0.10, are in good agreement with those obtained by ^{31}P NMR. Therefore, the ^{17}O NMR data indicate that the ^{31}P NMR pH titration behavior of PNP must be considered anomalous, thereby providing yet another example of the inability of ^{31}P NMR to quantitate charge neutralization of phosphoryl oxygens.

5. ^{15}N NMR pH Titration of ^{15}N-Labeled PNP.

When ^{15}N NMR spectra of ^{15}N-labeled PNP were taken as a function of pH, only a small change in chemical shift was observed. The ^{15}N resonance was shifted only 2.2 ppm upfield in going from pH 11.0 (56.9 ppm) to pH 6.65 (54.7 ppm). This seemed to indicate that there is no change in the protonation state on nitrogen over this pH range.

^{15}N NMR spectra of ^{15}N-labeled PNP were taken at pH 11.0 in 10% D_2O with continuous broad band ^1H decoupling and

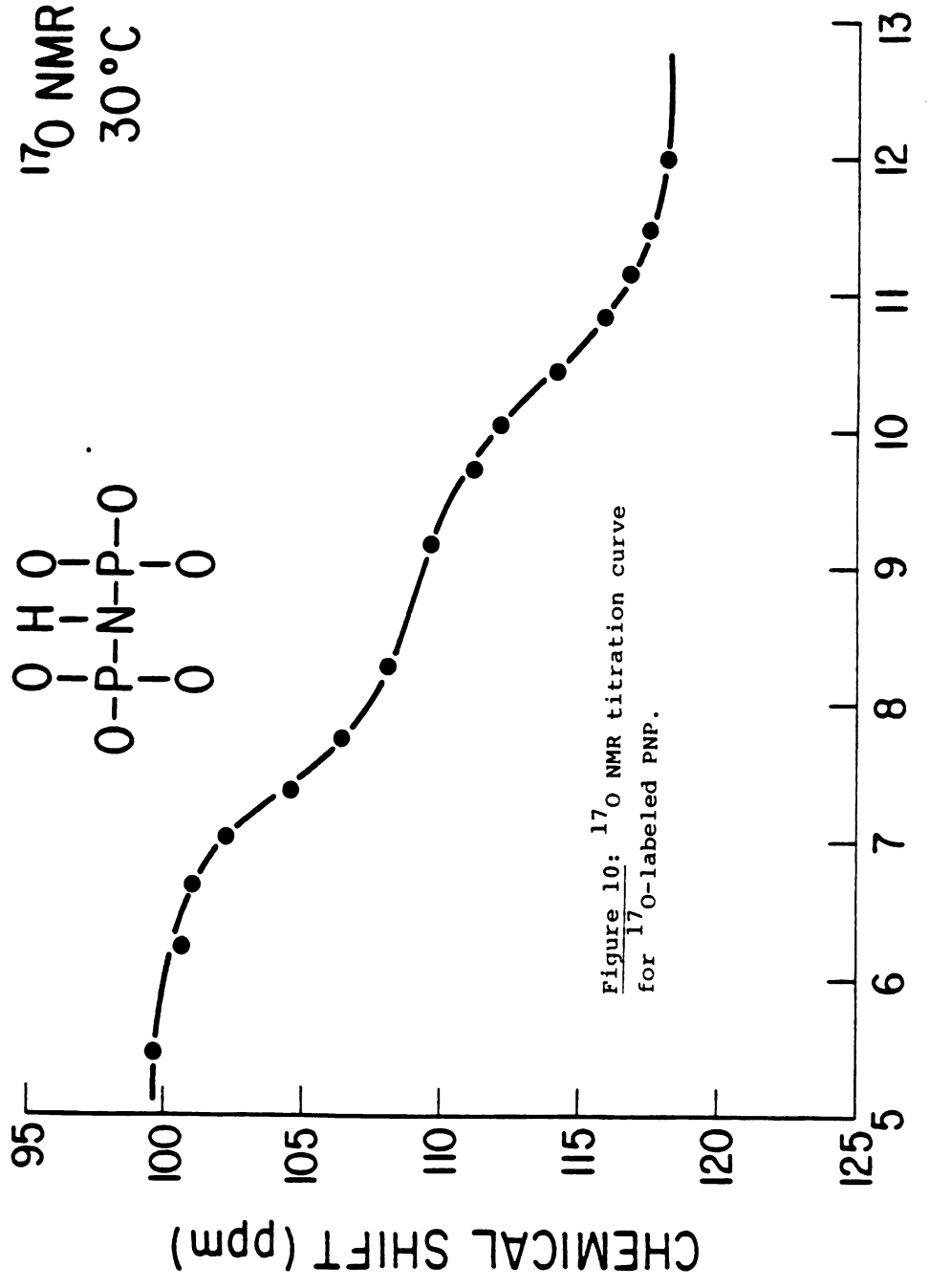


Figure 10: ^{17}O NMR titration curve for ^{17}O-labeled PNP.

with gated broadband ^{1}H decoupling off during data acquisition, and the results are shown in Figure 11. A large ^{15}N-^{1}H coupling constant of 72.6 Hz was measured, which is ample evidence for a proton directly bound to nitrogen at this pH. Therefore, the pKa of the N-H proton in PNP must be significantly higher than 11.0, which implies that the predominant structure of PNP in solution is the N-H tautomer as illustrated in structures 19 and 21.

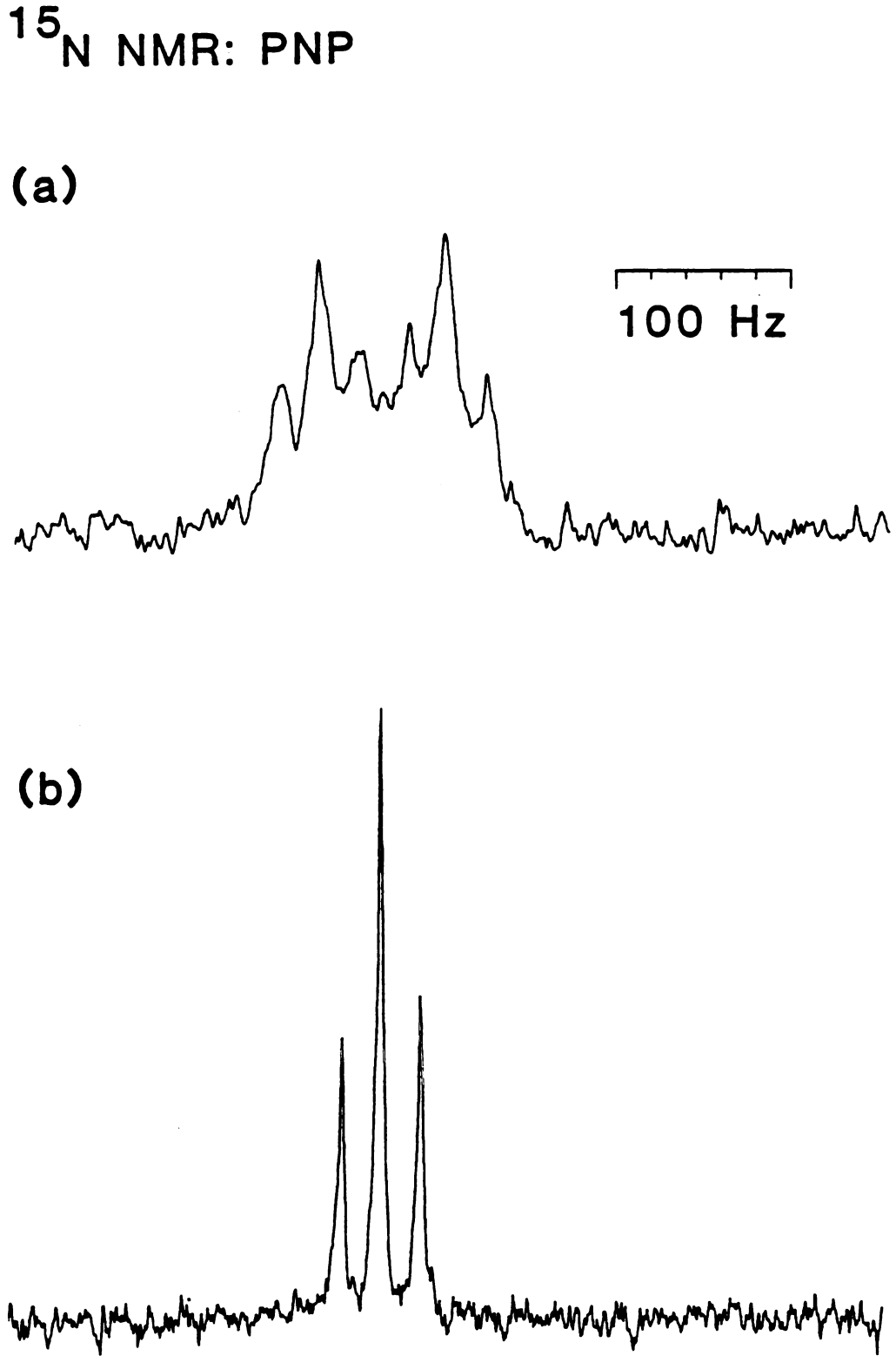


Figure 11: ^{15}N NMR spectra of ^{15}N-labeled PNP in 10% D_2O, pH 11.0: (a) without and (b) with broadband ^{1}H decoupling.

6. ^{17}O NMR Chemical Shifts versus pH for α-$^{17}O_1$- and β,γ-^{17}O AMP-PNP.

(α-$^{17}O_1$ AMP-PNP was prepared from unlabeled PNP and ^{17}O- AMP, which was a gift from John Gerlt. The ^{17}O NMR spectra at 36.6 MHz were taken by Professor Gerlt at Yale University.)

The ^{31}P NMR titration data for AMP-PNP reported by Tran-Dinh and Roux (1977) was confirmed in our laboratory. We found that upon protonation at 29°C the resonance associated with the β-phosphorus shifts upfield 3.28 ppm whereas the resonance associated with the γ-phosphorus shifts upfield 1.09 ppm. The pKas associated with the chemical shift changes are 8.23 + 0.02 and 8.26 + 0.01, respectively; these are in good agreement with the value of 8.41 measured by potentiometric titration (Pettit and Siddiqui, 1976). Our conclusion that ^{31}P NMR does not provide a quantitative measure of charge neutralization in PNP suggests that a similar situation could also apply to AMP-PNP.

AMP-PNP is less stable than ATP, particularly at elevated temperatures. Nonetheless, we have been able both to assign the resonances in the tetraanionic form of the β,γ -labeled sample of AMP-PNP at 50°C and also to determine complete ^{17}O NMR pH titration data at this temperature. The assignments of the resonances in the doubly labeled sample were accomplished by selective decoupling of the directly bonded ^{31}P nuclei. The essential spectra were obtained at pH 10.8 with a sample of the tetraethylammonium salt of β,γ-

^{17}O AMP-PNP, and these are shown in Figure 12. The spectra reveal the presence of small amounts of the hydrolysis products, β-^{17}O ADP-NH_2 (δ= 107 ppm) and inorganic phosphate (δ = 100 ppm). The chemical shift of the resonance associated with ADP-NH_2 is very similar to that of the resonance assignable to the β-nonbridging oxygens in AMP-PNP; however, the amount of ADP-NH_2 present in the sample does not interfere with the assignments. The decoupling conditions used to acquire these spectra are: spectrum a, broadband ^{31}P decoupling of both the β- and γ-nuclei; spectrum b, no ^{31}P decoupling; spectrum c, selective ^{31}P decoupling of the β-nuclei; and spectrum d, selective ^{31}P decoupling of the γ-nuclei. A ^{31}P decoupled spectrum of the hydrolysis products is shown in spectrum e. Decoupling of only the β-nuclei (spectrum c) produces a sharpening of the resonance at about 110 ppm whereas decoupling of only the γ-nuclei (spectrum b) produces a sharpening of the resonance at about 118 ppm. This behavior allows the downfield resonance to be assigned to the γ-oxygens and the upfield to the β-oxygens.

In preliminary experiments, we observed that the tetraethylammonium salt of AMP-PNP is too unstable at 50°C and neutral pH to allow ^{17}O NMR spectra to be obtained of the trianionic species (the elevated temperature is necessary to resolve the resonances in the β,γ-labeled sample). However, using the sodium salt of the doubly labeled sample, we obtained at 50°C the 67.8 MHz spectra shown in Figure 13. The spectrum at pH 11.60 is that of the tetraanion, and the spectrum at pH 6.95 is that of the

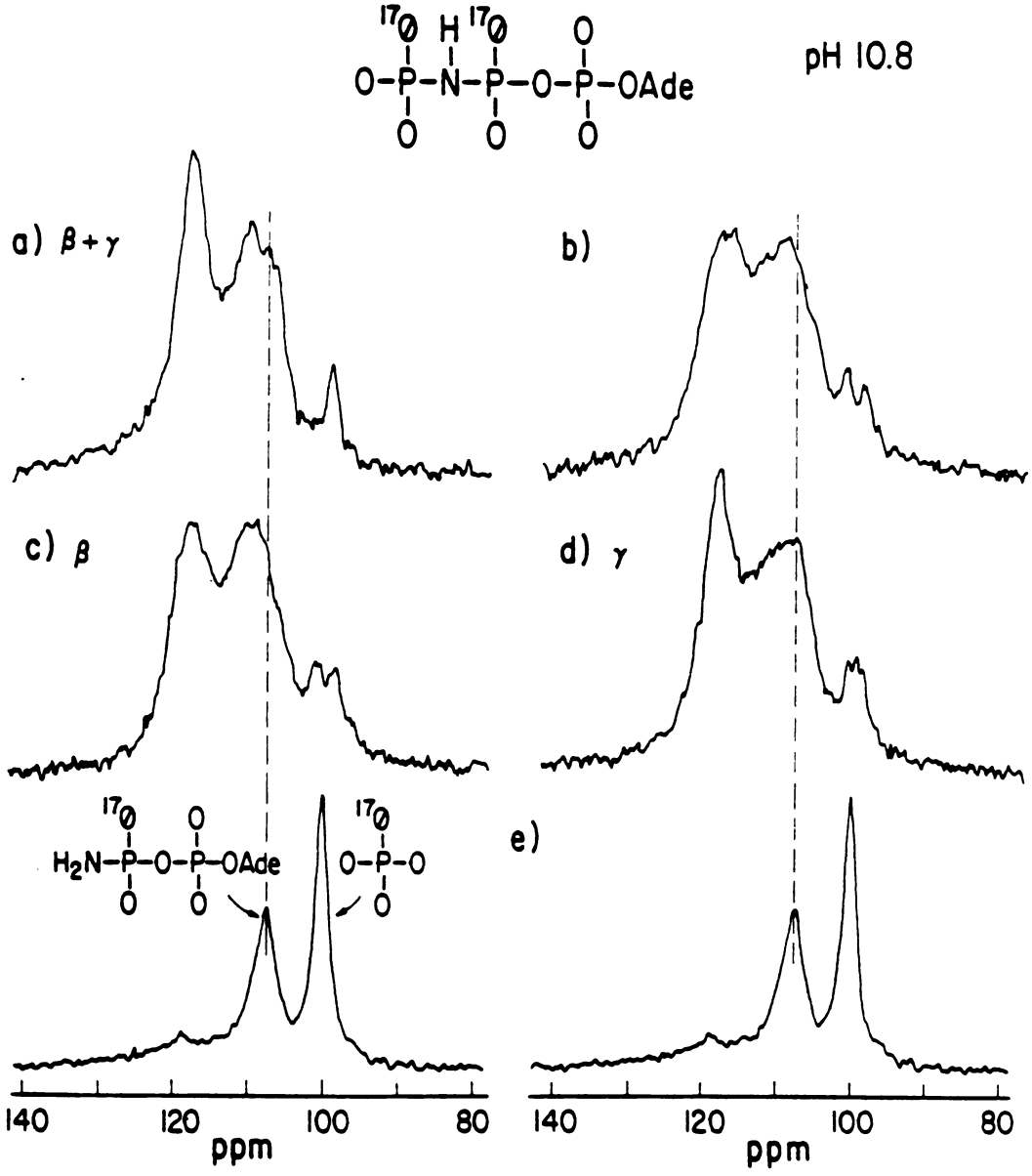


Figure 12: ^{17}O NMR spectra of ^{17}O-labeled AMP-PNP in 20% D_2O with and without selective ^{31}P decoupling as described in text.

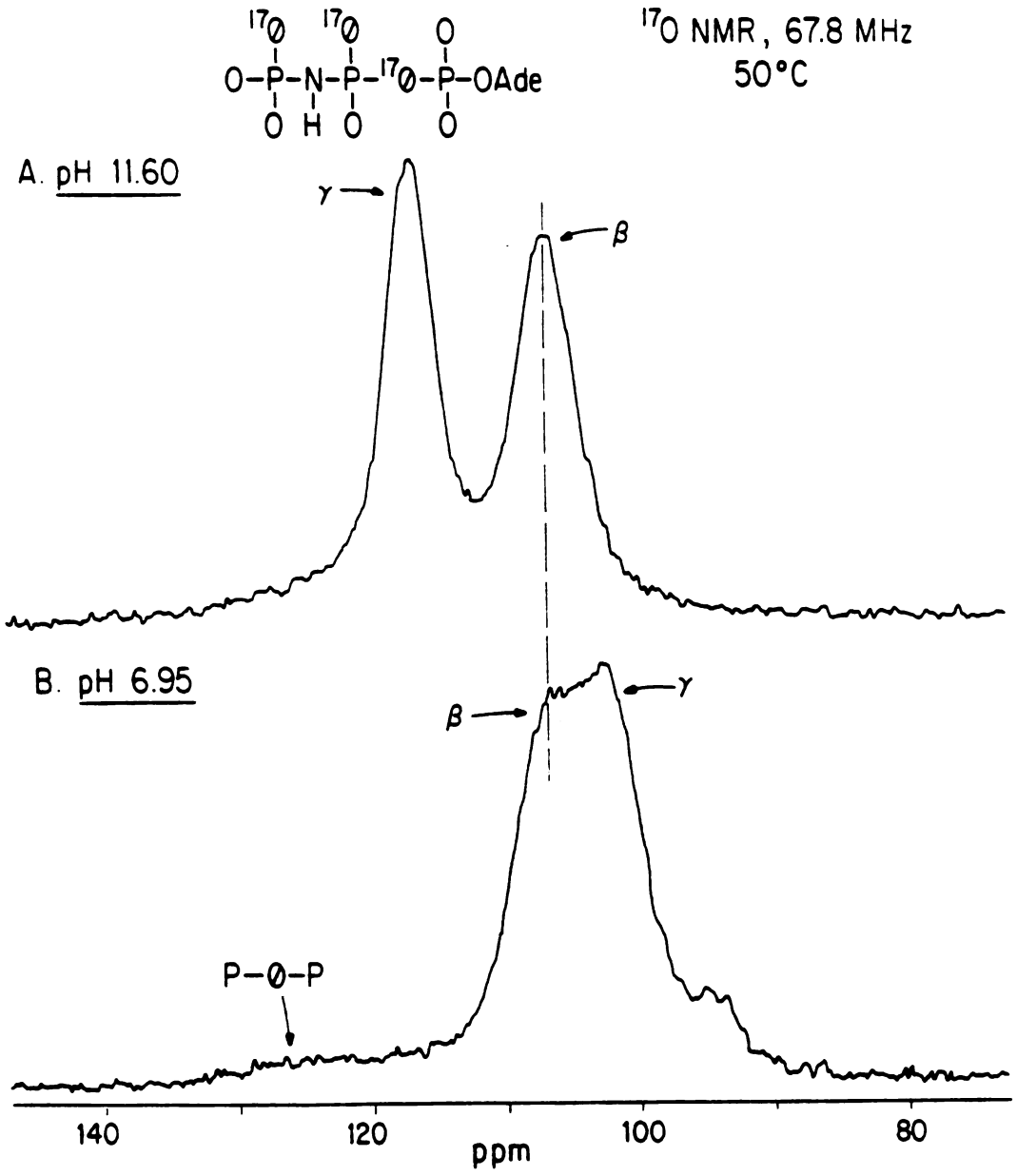


Figure 13: ^{17}O NMR spectra of ^{17}O-labeled AMP-PNP as described in text.

trianion; the resonance which was assigned to the γ-phosphoryl oxygen shifts upfield approximately 16 ppm upon protonation of the molecule whereas that assigned to the β-phosphoryl oxygens is essentially pH independent. The resonance associated with the α,β-bridging oxygen can be observed at about 125 ppm in the spectrum taken at pH 6.95, with this assignment being made by comparison with the chemical shift of the bridging oxygen in pyrophosphate (to be covered in Part 2 of this dissertation). (The weak doublet at about 94 ppm in the spectrum obtained at pH 6.95 can be attributed to inorganic phosphate which resulted from a small amount of hydrolysis of this doubly labeled sample.)

We subsequently determined the complete ^{17}O NMR pH titration behavior of the samples of AMP-PNP at 36.6 MHz, and the results are shown in Figure 14. The data for the β- and γ-phosphoryl oxygens were obtained with the sodium salt of the doubly labeled sample at 50°C, and those for the α-phosphoryl oxygens were obtained with the tetraethylammonium salt of α-^{17}O AMP-PNP at 30°C. As expected on the basis of the spectra shown in Figure 13, only the chemical shift associated with the γ-phosphoryl oxygens is sensitive to pH, with the upfield shift occurring on protonation being 15.6 ppm. The change in chemical shift for the γ-phosphoryl oxygens is 47 ppm per charge neutralized, a value slightly smaller than those found for PNP but in the range expected for full protonation occurring only at this position (Gerlt et al., 1982, submitted). The pKa derived from the pH titration data is 8.22 ± 0.03, which is in good agreement with those determined by both ^{31}P NMR

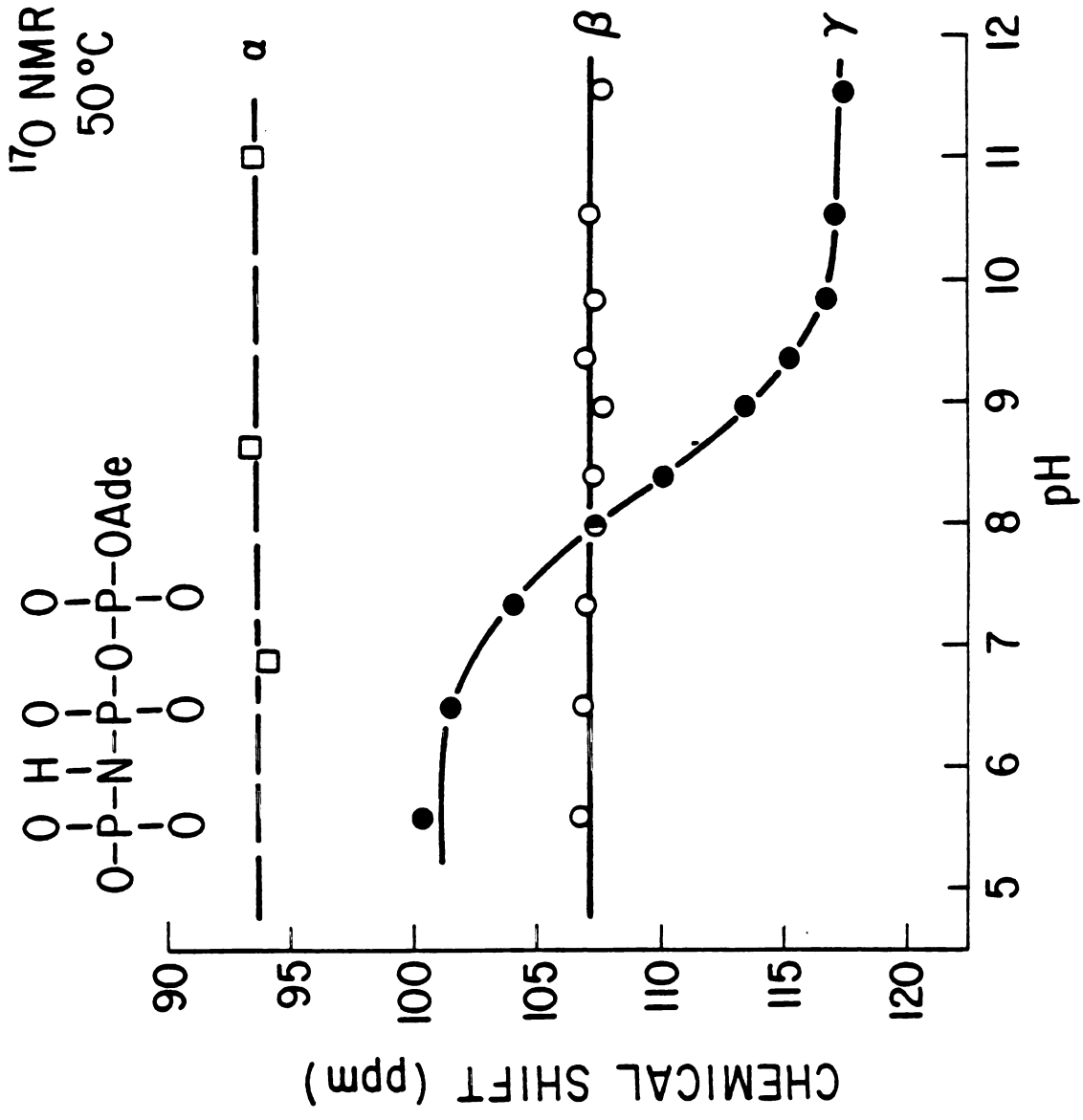


Figure 14: ^{17}O NMR titration curves for the α-, β- and γ-phosphoryl oxygens of ^{17}O-labeled AMP-PNP.

and potentiometric titrations. We conclude that the ^{17}O NMR pH titration behavior of AMP-PNP is in accord with proton tion occurring only on the γ-phosphoryl oxygens, thereby establishing that the imino tautomers shown in Figure 2 are of minimal importance.

7. ^{15}N NMR Chemical Shifts versus pH and Mg^{2+} for β,γ-^{15}N AMP-PNP.

The ^{15}N NMR spectra for β,γ-^{15}N AMP-PNP at pH 11.75 and at pH 7.63 are given in Figure 15. When the pH is decreased from 11.75 to 7.17, an upfield change in chemical shift of only about 1.1 ppm is observed. Unfortunately, it was not possible to obtain chemical shift data below pH 7.17 due to the increasing appearance of hydrolysis products, which make the spectra too complex to interpret at the field strength used. At high pH, the ^{15}N NMR spectrum appears as a doublet of doublets due to the different ^{31}P-^{15}N coupling constants to the β- and γ-phosphorus nuclei, which can be assigned from the ^{31}P NMR spectrum as 22 Hz and 32 Hz, respectively. At lower pH, these coupling constants approach an equivalent value of approximately 27 Hz, giving rise to an apparent triplet in the ^{15}N NMR spectrum.

Figure 16 shows the ^{15}N NMR spectra at pH 8.92 with and without ^{1}H decoupling. From these two spectra, a ^{15}N-^{1}H coupling constant of about 71 Hz is obtained, indicating that the proton resides primarily on nitrogen at this pH. The ^{1}H NMR chemical shift of the ^{15}N-^{1}H proton was estimated by ^{15}N NMR using narrow band ^{1}H decoupling; it was found to be about 4.4 ppm downfield from TSP [3-(trimethylsilyl)-1-propanesulfonic acid]. We conclude from this evidence that the N-H tautomer of AMP-PNP predominates even at high pH (see Figure 2, Ib). From the relationship (exchange rate) $< 2\pi J$ (Abragam, 1962), where J is the coupling constant between the two nuclei involved in

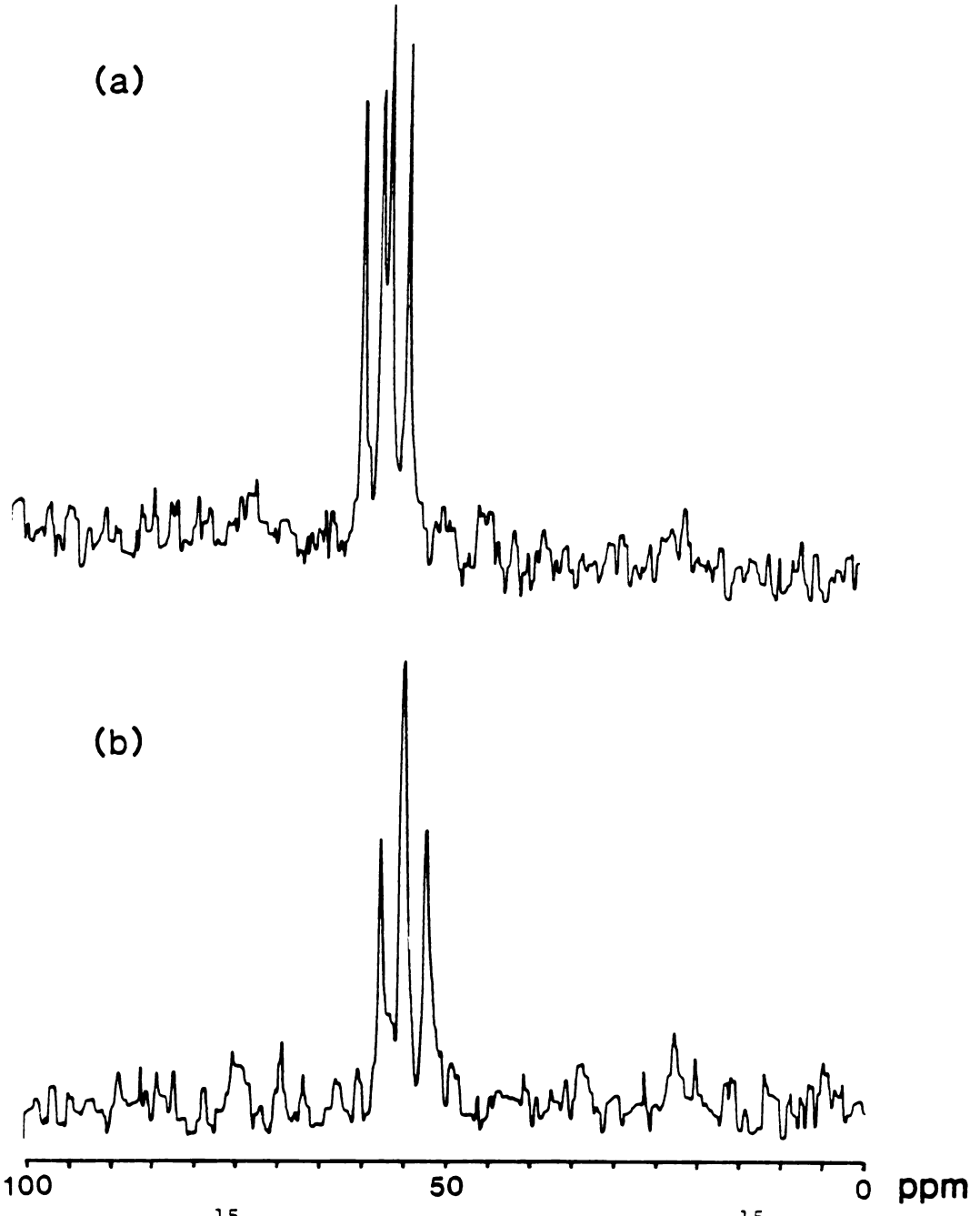


Figure 15: ^{15}N NMR spectra (proton decoupled) of ^{15}N-labeled AMP-PNP: (a) pH 11.75 and (b) pH 7.63.

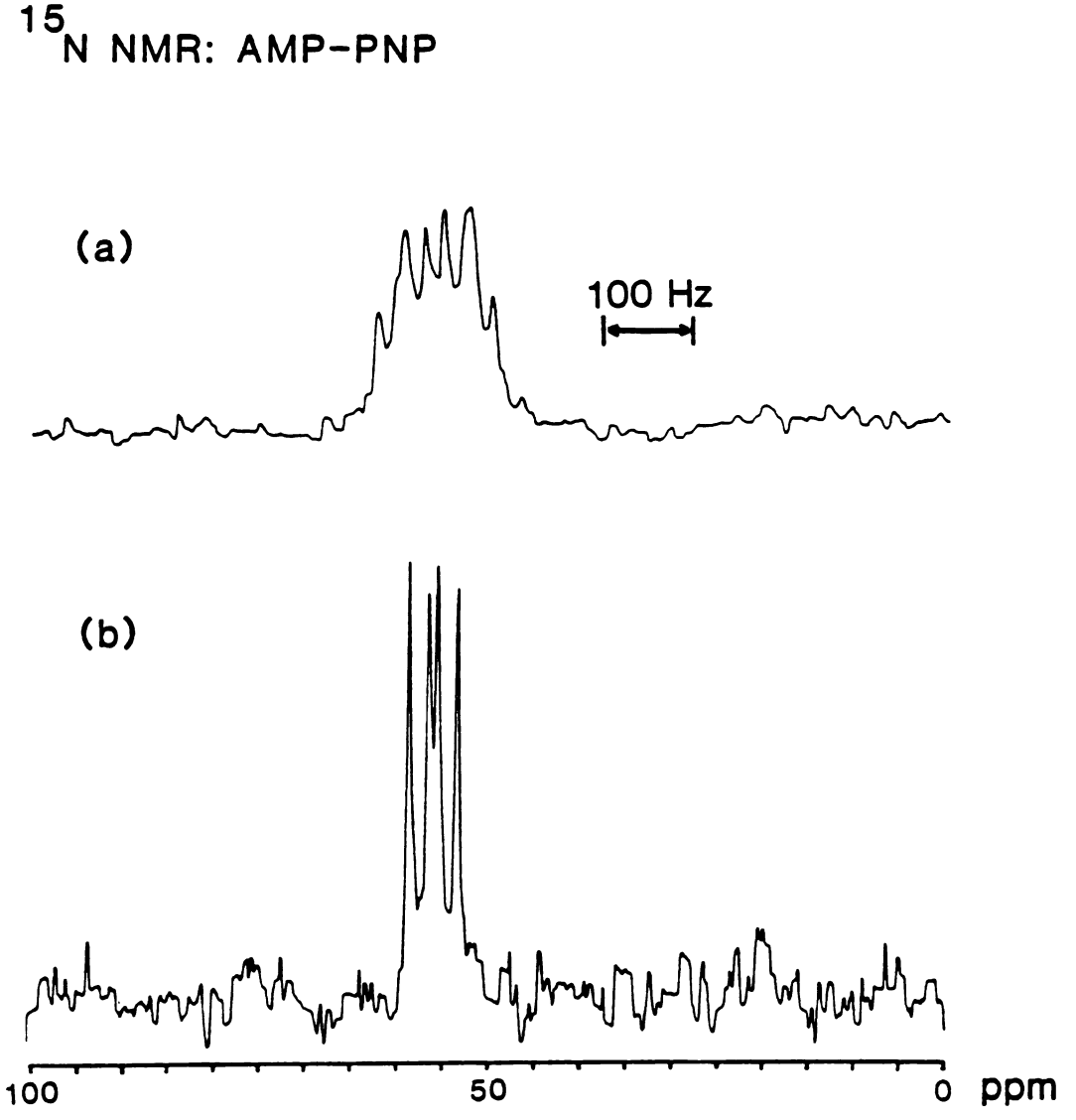


Figure 16: ^{15}N NMR spectra in 20% D_2O of ^{15}N-labeled AMP-PNP at pH 8.92: (a) without and (b) with ^{1}H decoupling.

the exchange process, we calculate that the N-H tautomer (Figure 2, Ib) is in slow exchange with the other possible structures (Figure 2, Ia, Ic, Id). A ^{15}N-^{1}H coupling constant of 71 Hz implies that this exchange rate can be no greater that 446 s^{-1}. These results show that the pKa for the N-H proton must be significantly higher than the highest pH measured (11.75).

Figure 17 shows the variation of the ^{15}N NMR chemical shift for β,γ-^{15}N AMP-PNP as a function of the ratio R= Mg^{2+}/ AMP-PNP at pH 9.4. No change in chemical shift is observed above R = 1.0, indicating that Mg^{2+} complexes with AMP-PNP in a 1:1 molar ratio. This conclusion regarding the stoichiometry of the AMP-PNP-Mg^{2+} complex was also made by Tran-Dinh and coworkers (1977) on the basis of ^{31}P NMR studies. However, Tran-Dinh's argument predicting complexation between Mg^{2+} and the β,γ-imido nitrogen of AMP-PNP is doubtful based on our experiments. The ^{15}N NMR spectra for β,γ-^{15}N AMP-PNP show a net change in chemical shift of only about 1.0 ppm when one equivalent of Mg^{2+} is added, and one could argue that a much larger shift would be expected if Mg^{2+} binds directly to the nitrogen of the P-N-P bridge. Unfortunately, our experiments with ^{15}N-labeled tetraethyl PNP failed to show whether or not ^{15}N NMR chemical shifts are sensitive to changes in net charge with P-N-P bridge systems, particularly in the case of fast exchange. It might be interesting to conduct future studies with one of the more tightly binding divalent metal ions (e.g., cobalt).

A ^{15}N NMR spectrum of β,γ-^{15}N AMP-PNP with R = 1.0 at

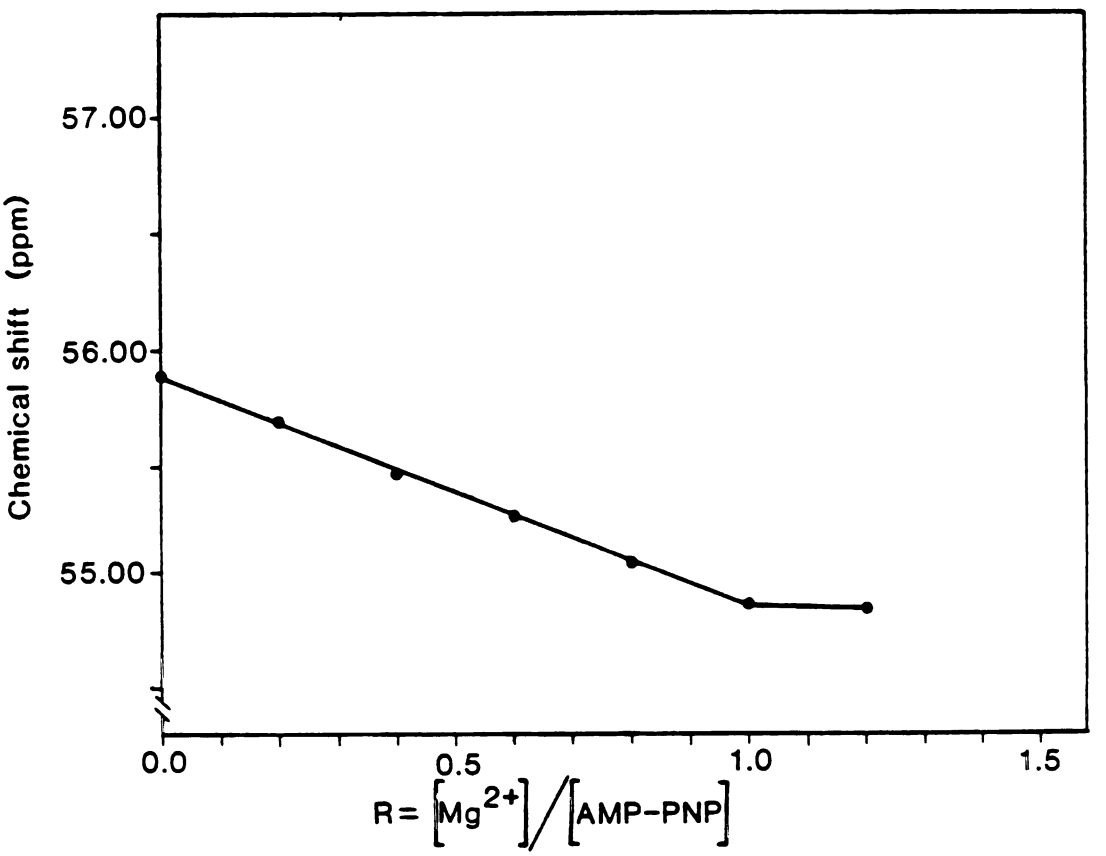


Figure 17: Plot of ^{15}N NMR chemical shift as a function of Mg^{2+} concentration for ^{15}N-labeled AMP-PNP at pH 9.4 in 20% D_2O.

pH 9.4 was taken in the absence of proton decoupling, again giving an ^{15}N-^{1}H coupling constant of about 70 Hz. When the pH of this sample was raised to 11.4, there was no change in chemical shift. Therefore, it is apparent that the β,γ -bridge nitrogen remains protonated in the presence of Mg^{2+} in the pH range studied.

8. Summary.

We have proposed that a P(O)-N(H)-P(O) bridge system is capable of tautomerizing to produce "imino" structures where the proton resides instead on oxygen provided that the N-H proton is sufficiently acidic. We have shown that the pKa of tetraethyl PNP is 3.8, and that the N-H proton is in fast exchange with one or more of these proposed tautomers. In the cases of PNP and AMP-PNP, however, we have shown that the pKa of the N-H proton is substantially above 14, and that the proton is predominantly on nitrogen in slow exchange.

Estimates for the pKas of PNP and AMP-PNP can be calculated by considering the addition of negative charges to tetraethyl PNP and by making the assumption that the addition of each successive negative charge increases the pKa by the same amount. This assumption appears to hold for the mono-, di- and tribasic forms of phosphoric acid, which possess pKas of 2.15, 7.20, and 12.38, respectively. Assuming, then, that the addition of each successive negative charge to tetraethyl PNP elevates the pKa by about 5 units, the pKas for PNP and AMP-PNP are calculated to be about 24 and 19, respectively.

IV. N-Alkyl Derivatives of AMP-PNP.

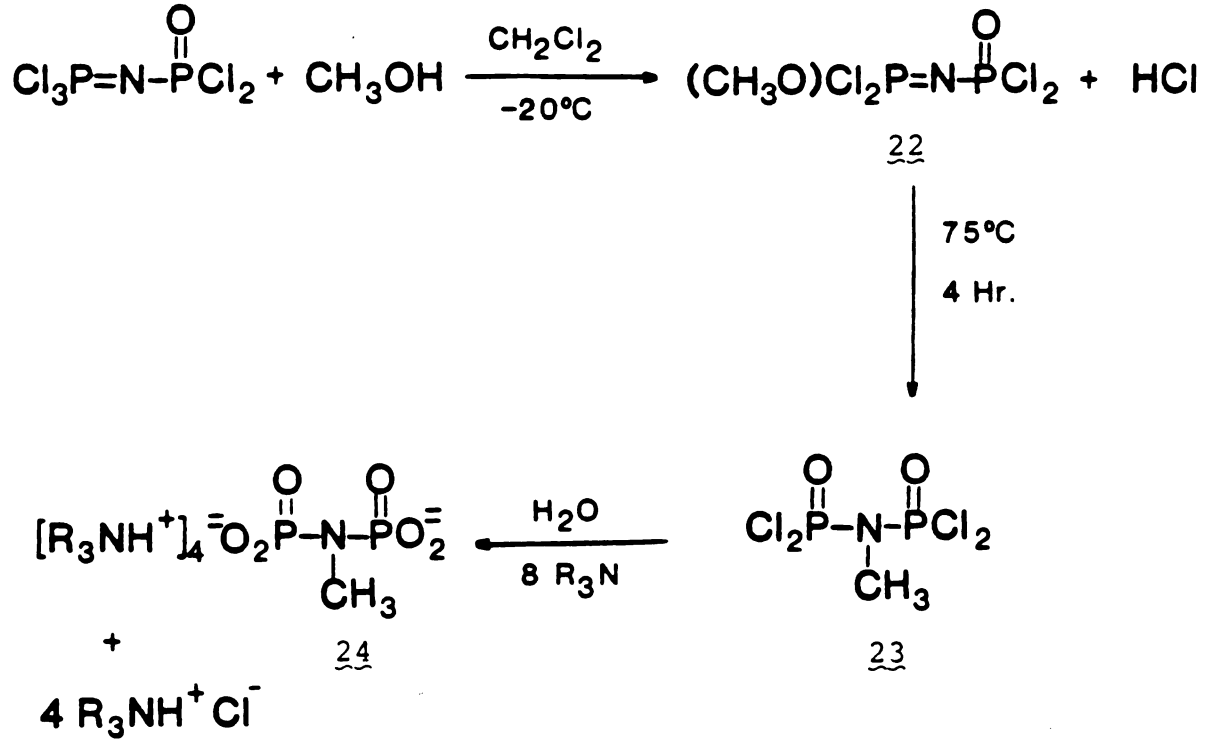
A. N-Methyl AMP-PNP.

1. Rationale for Synthesis.

N-Methyl AMP-PNP was prepared from N-methyl imidodiphosphate (N-methyl PNP) and AMP by the procedure of Michelson (see Section E).

The synthesis of N-methyl PNP begins with diethylphosphoryl triethoxyiminophosphorane according to Scheme IX. The first two steps were performed according to the procedure of Riesel et al. (1977b). In the first step, methanol undergoes nucleophilic displacement of chloride at the iminophosphorane position. Two reasons for methanol attacking at this position rather than at the dichlorophosphoryl position are: (1) less energy is required to break a P=N bond than a P=O bond, assuming the nucleophilic displacement proceeds by either an AE or Sn2(P) mechanism; and (2) addition at the imino phosphorus may be assisted by "Michael addition"-type conjugation with the phosphoryl group. In the second step, the intermediate (22) is rearranged to the more thermodynamically stable N-methyl imidodiphosphoryl tetrachloride (23). Hydrolysis of 23 in the presence of a tertiary amine such as trietylamine produces N-methyl PNP (24).

SCHEME IX



The ^{31}P NMR spectra of N-methyl PNP at pH 12 in the presence and absence of ^{1}H decoupling are shown in Figure 18. The chemical shift is 5.8 ppm from 85% H_3PO_4 (external standard), and the ^{31}P-^{1}H coupling constant is 9.4 Hz.

Unfortunately, we have not yet succeeded in purifying N-methyl AMP-PNP. In the method of purification used (anion exchange chromatography on QAE Sephadex A-25--see Section V), a side product resembling a linear triphosphate co-elutes with N-methyl AMP-PNP, along with small amounts of other contaminants. This is indicated in a ^{31}P NMR spectrum of this material taken at 97.3 MHz (Figure 19). Peaks corresponding to this linear triphosphate material are indicated with an "a"; the peaks indicated "b" are presumably ADP-$NHCH_3$, which is a hydrolysis product analogous to ADP-NH_2 from AMP-PNP. Based on ^{31}P NMR spectra

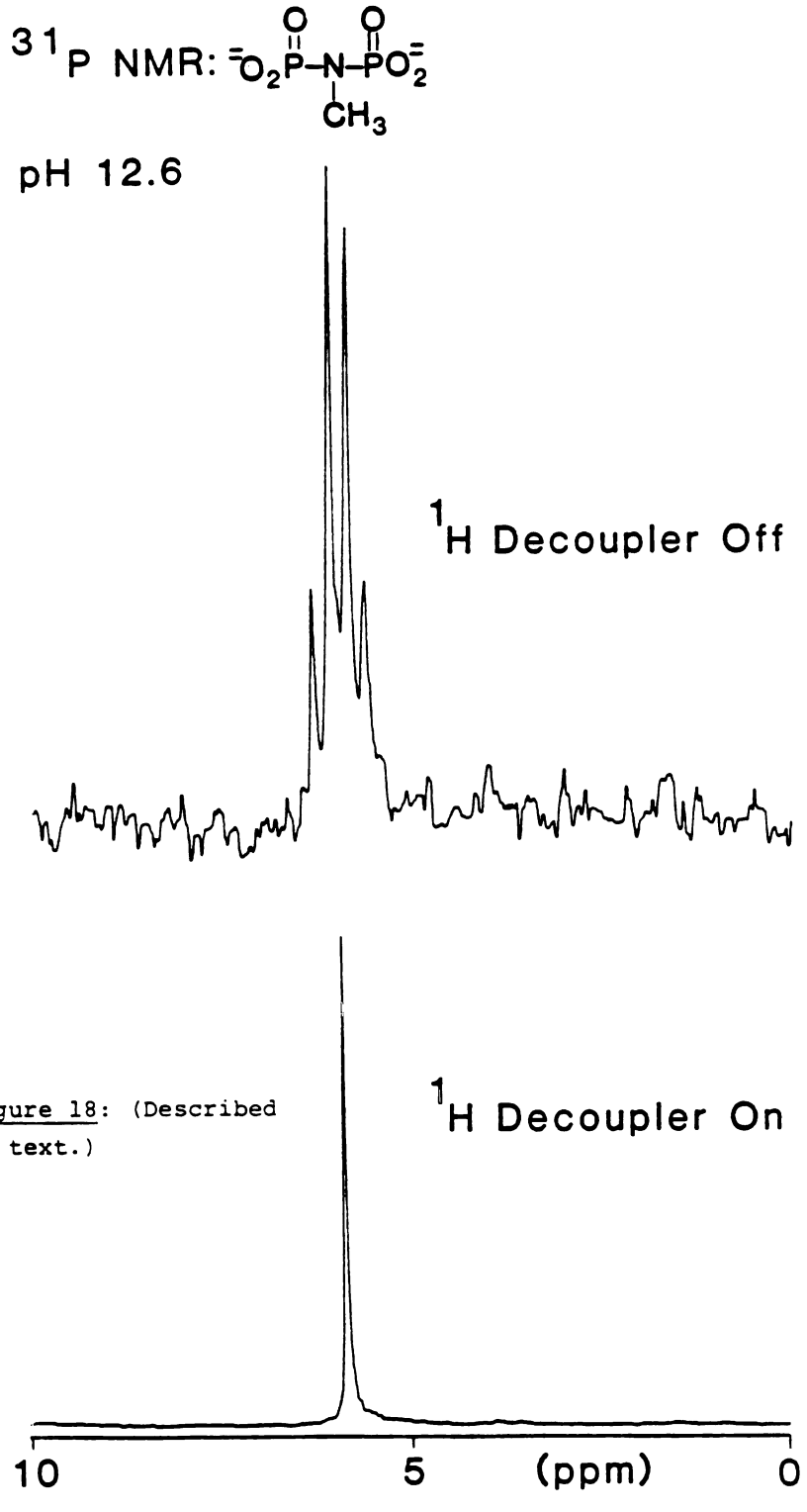


Figure 18: (Described in text.)

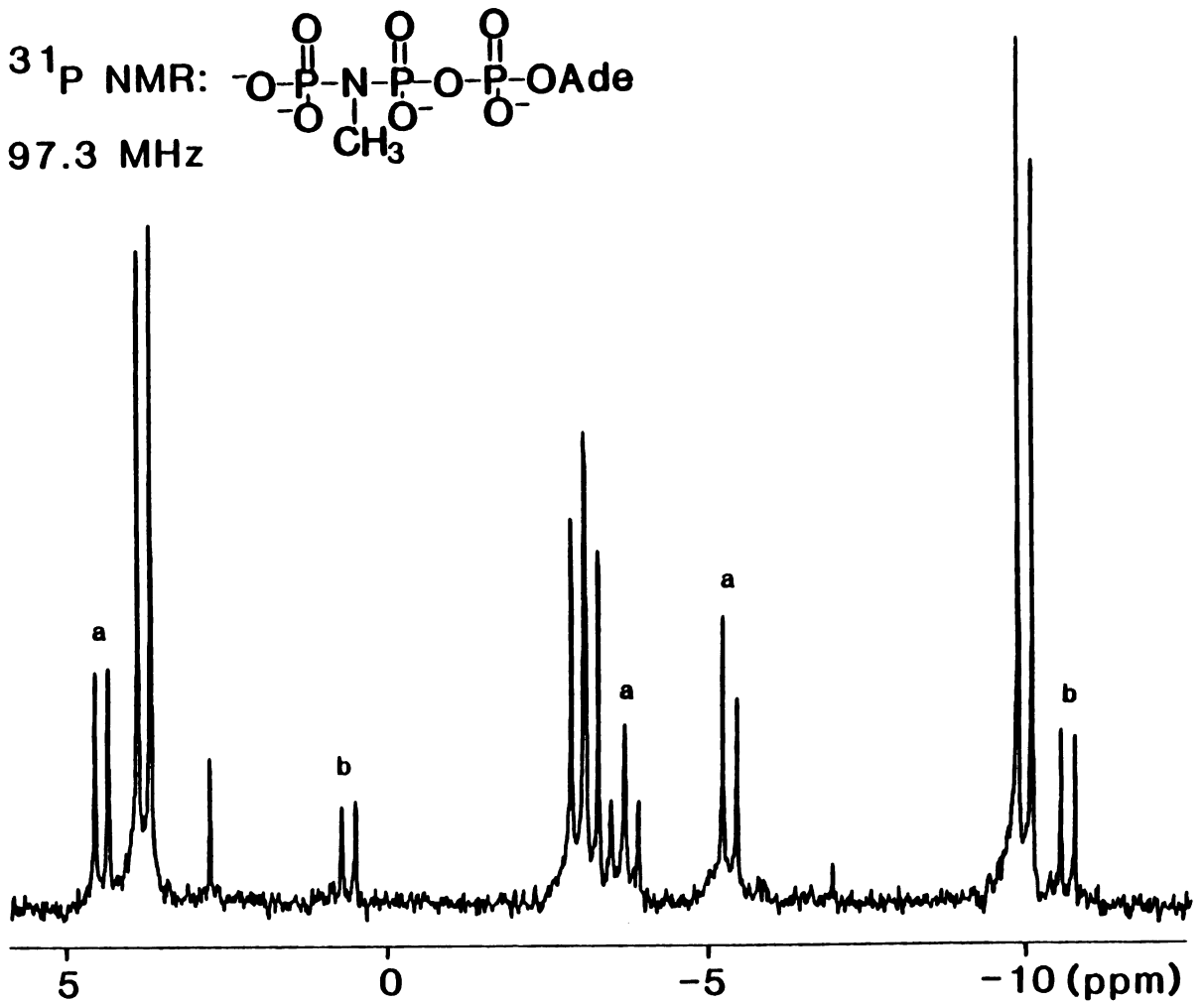


Figure 19: ^{31}P NMR spectrum of a sample of crude N-methyl AMP-PNP (proton decoupled) at pH 11.0.

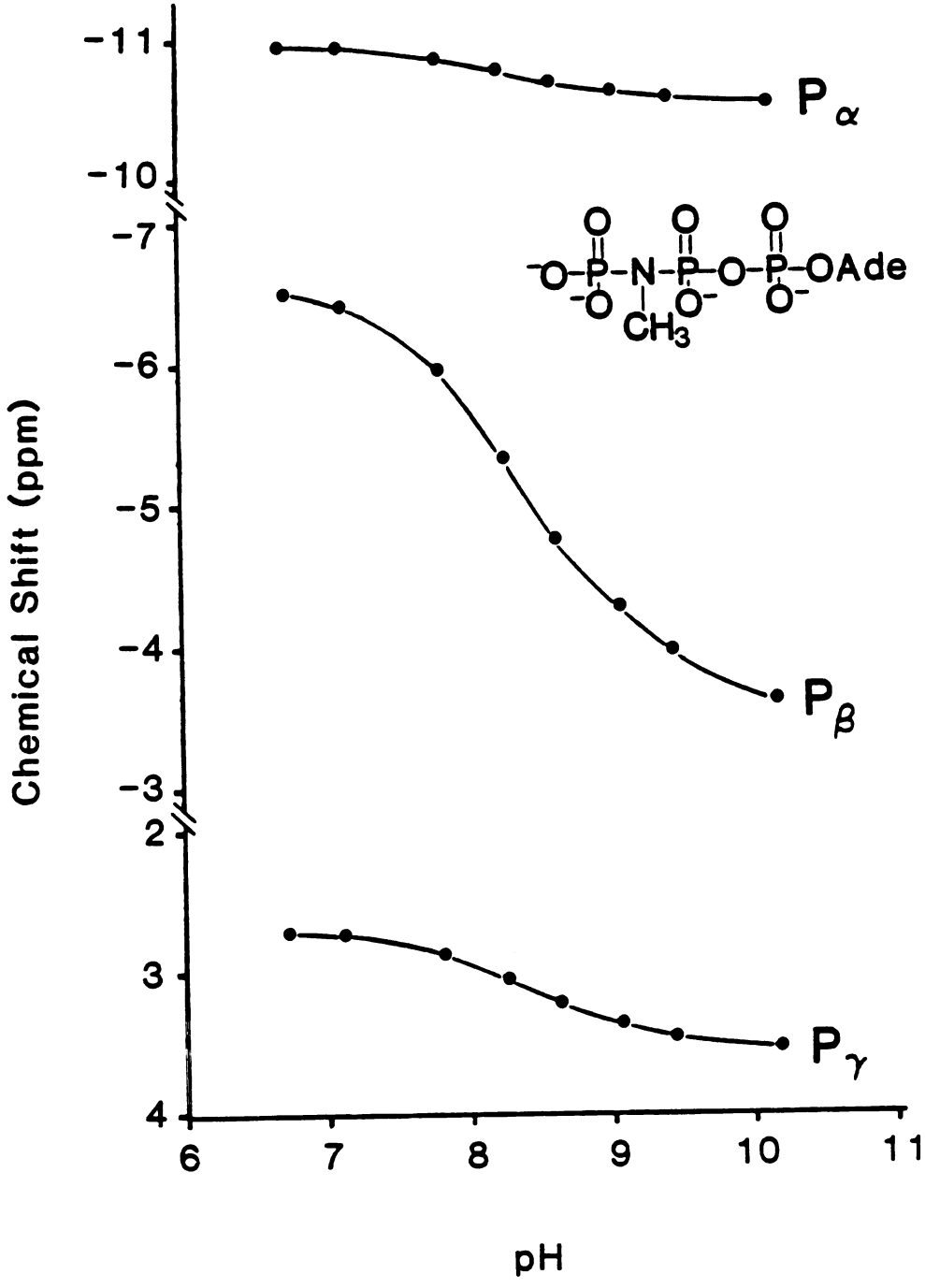
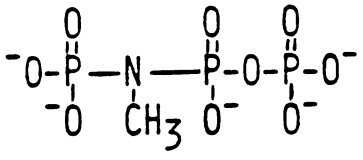


Figure 20: ^{31}P NMR titration curves for the α-, β- and γ-phosphoryl groups of N-methyl AMP-PNP.

taken without ^{1}H decoupling, we believe the linear triphosphate contaminant has the following structure (25).



25

In spite of the contaminants present in our preparation of N-methyl AMP-PNP, we were able to obtain a ^{31}P NMR titration plot versus pH for each phosphorus group, and the results are summarized in Figure 20. It is interesting to note that N-methyl AMP-PNP behaves very much like AMP-PNP in that the largest shift is seen for the β-phosphorus resonances upon protonation of the tetraanion.

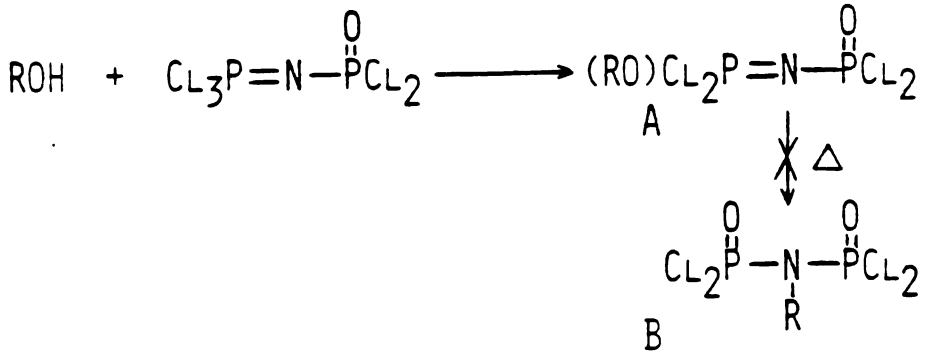
B. Attempts at Preparing other N-alkyl Analogs of PNP.

1. Synthetic Attempts Which Have Failed.

Our lack of success at preparing other N-alkyl derivatives of AMP-PNP was due to our inability to prepare the corresponding N-alkyl PNP derivatives. The method by which these preparations were attempted was analogous to the synthesis of N-methyl PNP (Scheme X). This procedure was tried with a variety of primary alcohols, including allyl alcohol, n-propyl alcohol, and benzyl alcohol. In each case there was evidence that addition of the alcohol to dichlorophosphoryl trichloroiminophosphorane at low temperature does produce the proposed intermediate (A). However, attempts to thermally rearrange this intermediate

to the N-alkylated compound (B) failed. In each case, polymerization was apparent, and a thick tar resulted. In the attempts with allyl alcohol this apparent polymerization occurred at room temperature. When the benzyl intermediate (A, R = $CH_2C_6H_5$) was heated a violent exothermic reaction ensued with the evolution of fumes having an odor resembling toluene.

SCHEME X.



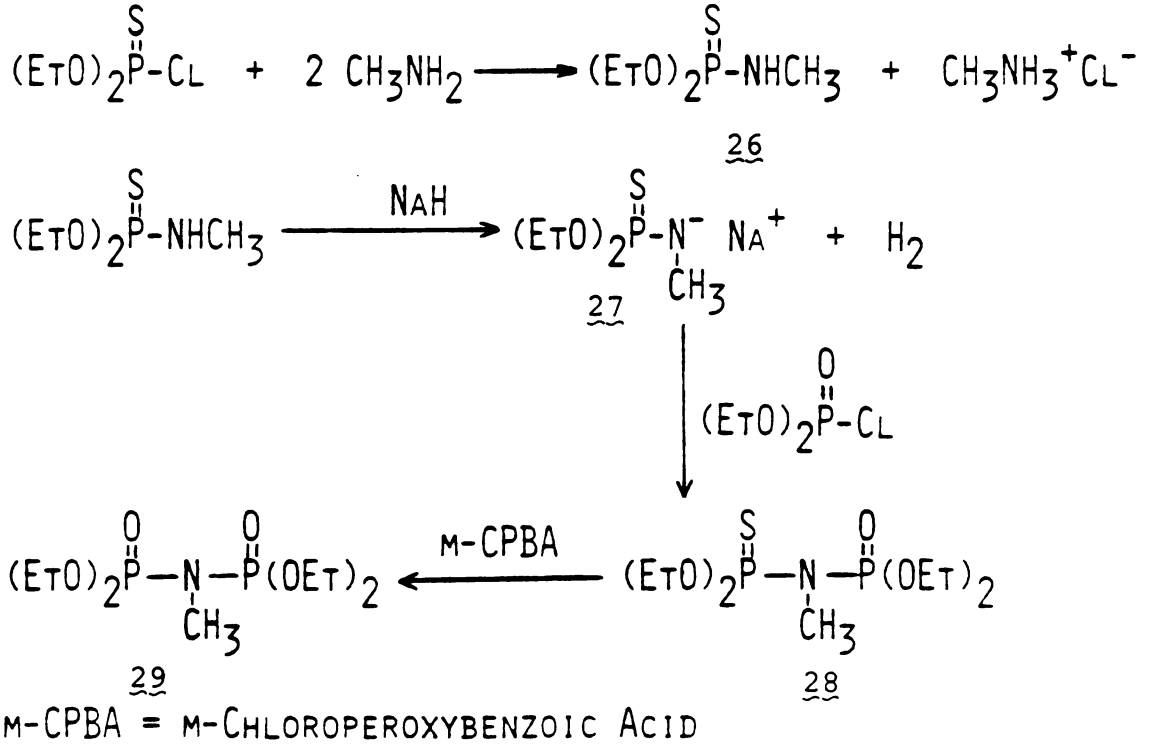
At this point, an alternate procedure will be proposed for the preparation of N-alkyl derivatives of PNP based on a method we have developed for the preparation of the tetraethyl ester of N-methyl PNP.

2. Synthesis of Tetraethyl N-Methyl PNP.

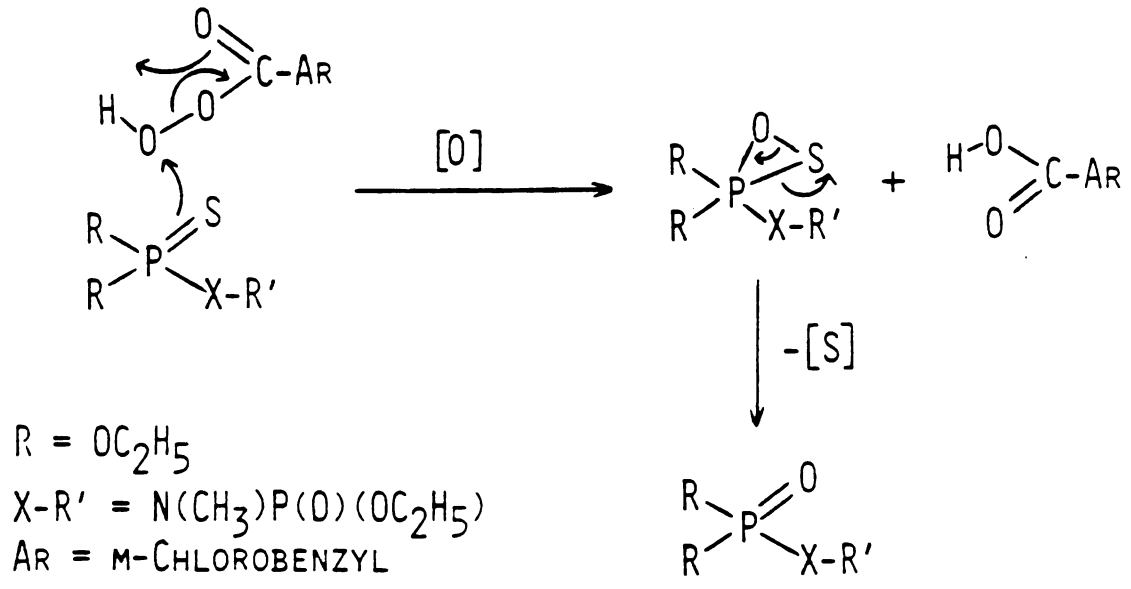
The synthesis of this compound was achieved according to Scheme XI. Preliminary attempts to couple the sodium salt of N-methyl diethylphosphoramidate with diethyl chlorophosphate were unsuccessful. (The attacking nucleophile appears to be the phosphoryl oxygen of N-methyl diethylphosphoramidate instead of the nitrogen.) However, when the thio-analog (27) was used the major product was

N-methyl diethylphosphorothioyl diethylphosphoramidate (28). Replacement of sulfur in 28 with oxygen was achieved with m-chloro peroxybenzoic acid to give tetraethyl N-methyl PNP (29). The reaction of m-chloro peroxybenzoic acid with other phosphorothioyl compounds has been reported by Bellet and Casida (1974). A proposed mechanism for this reaction appears in Scheme XII. In this mechanism, oxygen is inserted accross the P=S bond to give intermediate A. Rearrangement of this intermediate results in the formation of a P=O bond along with the release of elemental sulfur. We will see this reaction again in Part 2 of this dissertation.

SCHEME XI.



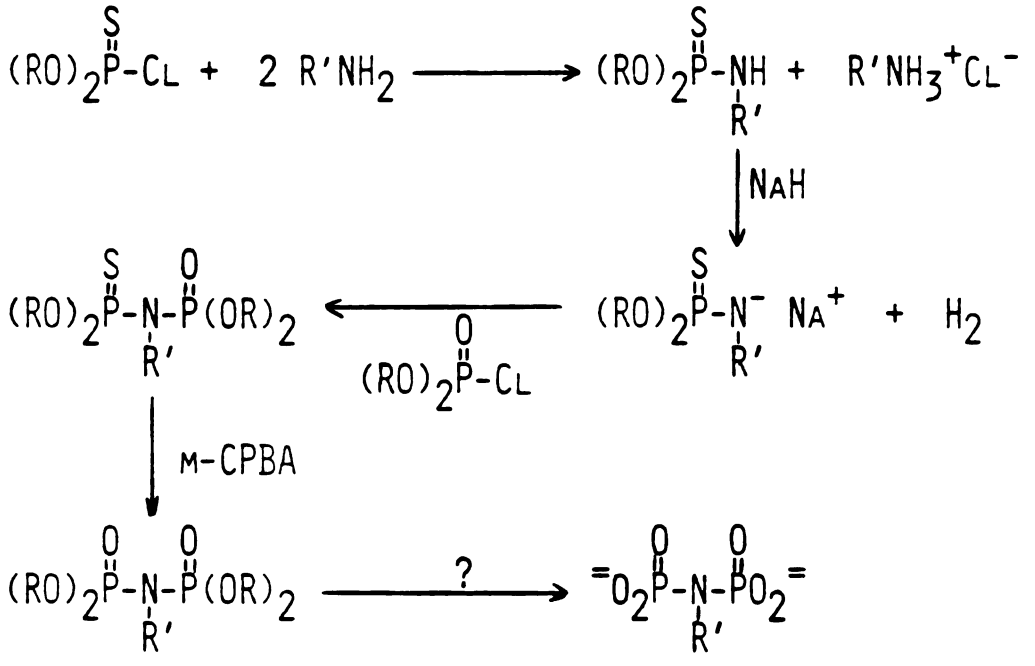
SCHEME XII.



3. Proposal for N-Alkyl Derivatives of PNP.

A general proposal for the preparation of N-alkyl PNP derivatives is given in Scheme XIII. The uncertain step is dealkylation of the proposed tetraalkyl N-alkyl' PNP derivative to N-alkyl PNP. Preliminary attempts to remove the ethyl groups in tetraethyl N-methyl PNP using trimethylsilyl iodide appeared to also give N-demethylation. Perhaps another dealkylation procedure, along with careful choice of alkyl ester groups, may one day lead to a successful synthesis of these interesting compounds.

SCHEME XIII.


(RO)2P(=S)-Cl + 2 R'NH2
(RO)2P(=S)-NH + R'NH3+Cl-
R'
NaH
(RO)2P(=S)-N- Na+ + H2
(RO)2P(=O)-Cl
(RO)2P(=S)-N-P(=O)(OR)2
m-CPBA
(RO)2P(=O)-N-P(=O)(OR)2
?
=O2P-N-PO2=

V. Experimental Section.

A. General Methods and Procedures.

1. Melting points, Microanalyses, IR Spectra, and Routine ^1H NMR Spectra.

Melting points were determined on a Thomas capillary melting point apparatus and are uncorrected. The pressure in all vacuum distillations was determined using a Mc Leod Guage. All boiling points are uncorrected. Microanalyses were performed by the Microanalytical Laboratory, University of California, Berkeley. Infrared spectra were run with neat samples using NaCl cells on a Perkin Elmer Model 457 spectrometer. Routine ^1H NMR spectra were taken on a Varian Model FT-80 spectrometer using either $CDCl_3$ as the solvent and tetramethylsilane (TMS) as the internal standard or D_2O and 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as the internal standard.

2. Anion Exchange Chromatography of Phosphate Compounds.

Phosphate compounds were separated by virture of differences in negative charge using anion exchange chromatography on either DEAE Sephadex A-25 or QAE Sephadex A-25. Column dimensions were 2.5 cm i.d. X 40 cm long. Elution was performed with a linear gradient of triethylammonium bicarbonate buffer, prepared by bubbling CO_2 through a fine glass frit into a stirring solution of

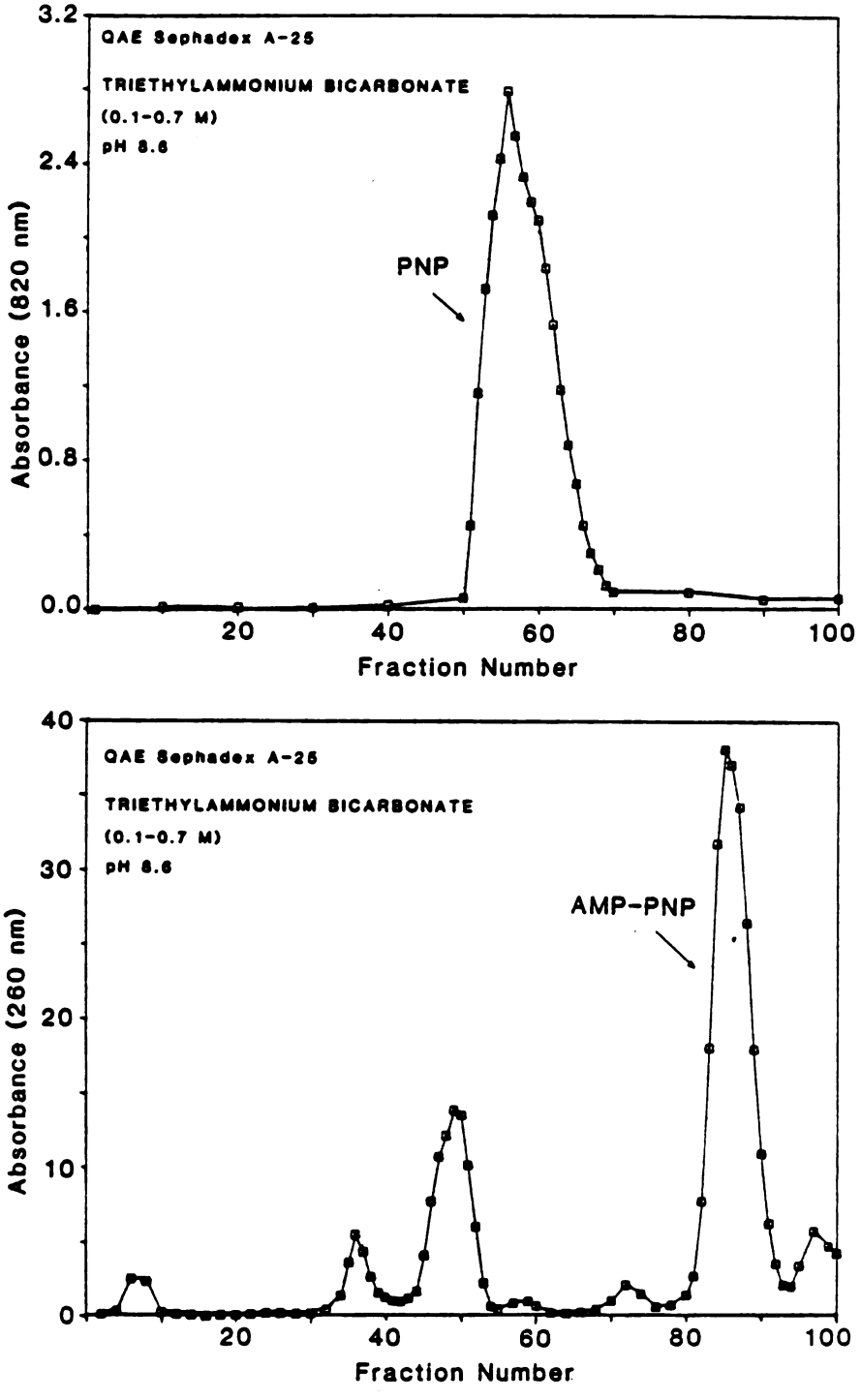


Figure 21: Representative anion-exchange elution profiles for PNP and AMP-PNP.

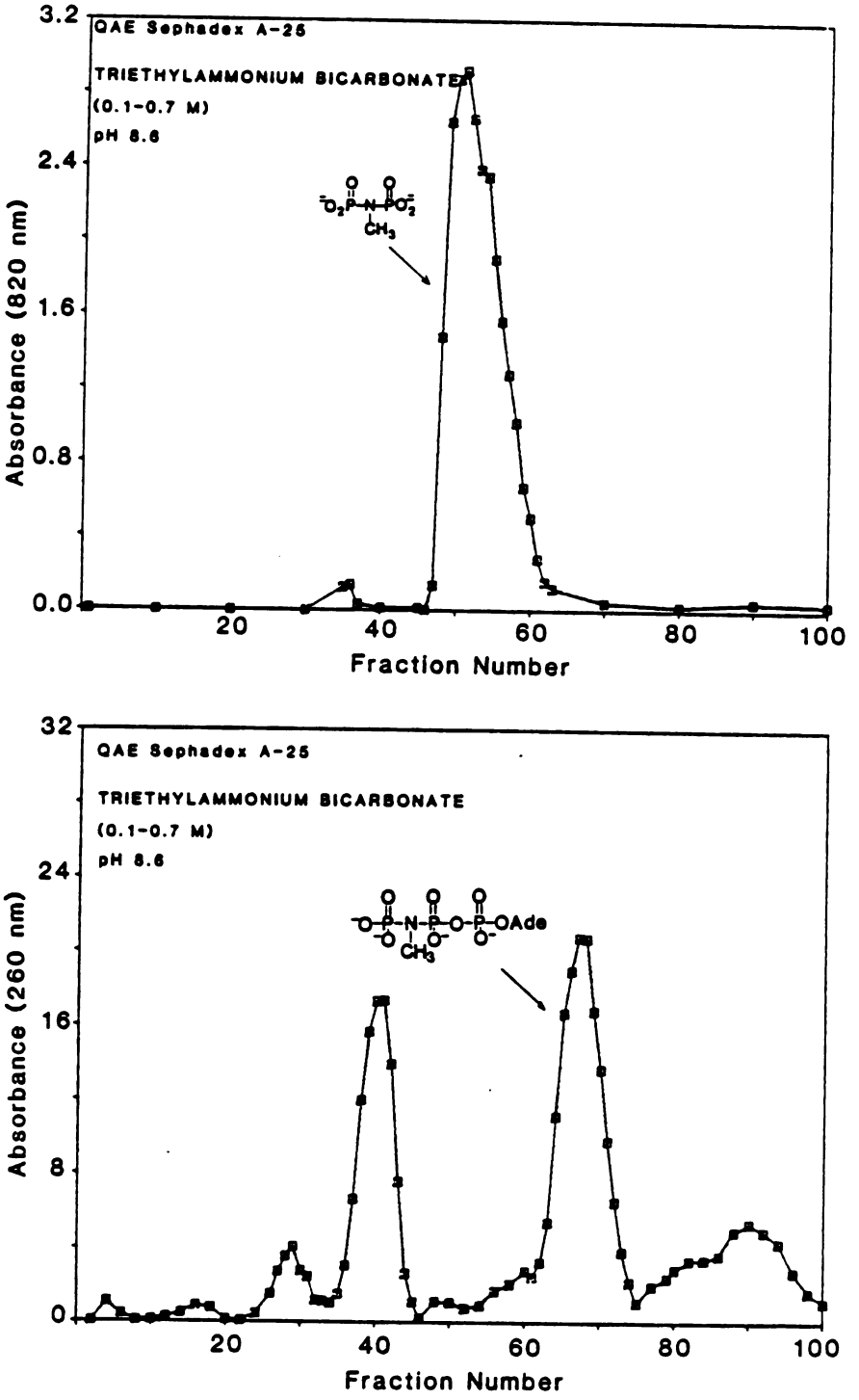


Figure 22: Representative anion-exchange elution profiles for N-methyl PNP and N-methyl AMP-PNP.

distilled deionized water and triethylamine until the desired pH was reached. Fractions (25 mL) were collected with the aid of a Gilson Fractionator. Representative elution profiles follow (Figures 21,22). Arrows indicate the appropriate fractions which were pooled.

3. Sample Preparation: NMR.

Routine samples prepared for ^{15}N or ^{31}P NMR analysis were dissolved in either 20% D_2O or $CDCl_3$ and transferred to 12 mm NMR tubes (for use on the Varian XL-100 spectrometer).

The sodium salt of β,γ-^{15}N AMP-PNP or ^{15}N PNP was dissolved in 1.7 mL of 20% D_2O to give a final solute concentration of about 70 mM. For pH titration experiments, EGTA was added to a final concentration of 0.1 mM. In experiments involving Mg^{2+}, the nucleotide solution was freed from adventitious divalent metal ions by shaking with Chelex-100 (Na^+) and filtering. Mg^{2+} was added from a stock solution prepared by dissolving $MgCl_2 \cdot 6\ H_2O$ in distilled, deionized water.

The ^{17}O -labeled samples were either percolated through columns of Chelex-100 (tetraethylammonium) and lyophilized or used directly as their sodium salts to prepare 2 mL samples of 40 mM solutes dissolved in 20% D_2O containing 1 mM EGTA. The 10 mm NMR tubes were made metal free by soaking them overnight in a 1:1 solution of concentrated nitric acid and sulfuric acid and then rinsing thoroughly with distilled deionized water.

4. ^{15}N NMR Measurements.

^{15}N NMR spectra were taken either at 10.14 MHz using a Varian XL-100 NMR spectrometer or at 24.36 MHz using the UCSF Wide Bore 240 MHz spectrometer. The spectra at 10.14 MHz were taken with a spectral width of 2000 Hz and 4096 data points were used to acquire the free induction decay; a time delay between pulses of 3 s was employed. The spectra at 24.36 MHz were taken with a spectral width of 3000 Hz and 8192 data points were used with a time delay between pulses of 1 s. Five hundred transients were usually collected. An exponential linebroadening factor of either 2 Hz or 3 Hz was applied to the total free induction decay prior to Fourier transformation. In most cases, spectra were taken with broad band ^{1}H decoupling. When it was desired to measure ^{1}H-^{15}N coupling, a gated method was used which turned off the decoupler during data acquisition. Chemical shifts are measured relative to ^{15}N ammonium sulfate used as an external standard.

5. ^{17}O NMR Measurements.

^{17}O NMR spectra at 36.6 MHz were recorded as previously described with a Bruker WH-270 NMR spectrometer using a 14085 Hz sweep width (Gerlt et al., 1982) except that in some experiments 1024 data points were used to acquire the free induction decay, resulting in a 0.0358 sec recycle time; following multiplication by an exponential line-broadening factor, the free induction decay was zero filled to 4096 data points and Fourier transformed. This

method of data treatment was not observed to alter the lineshapes, but, as expected, the signal-to-noise ratio improved noticeably.

^{17}O NMR spectra at 67.8 MHz were obtained using the Bruker WM-500 NMR spectrometer located in the Southern California Regional NMR Facility at the California Institute of Technology. With this spectrometer, 2048 data points were used to acquire the free induction decay using a spectral width of 25 KHz, resulting in a 0.0410 sec recycle time; following multiplication by an exponential linebroadening factor, the free induction decay was zero filled to 8192 data points and Fourier transformed. This spectrometer does not have the capability to decouple directly bonded ^{31}P nuclei.

^{17}O NMR chemical shifts are measured relative to natural abundance $H_2{}^{17}O$ (0.57 ppm downfield of natural abundance ^{17}O in the 20% D_2O used as solvent).

6. ^{31}P NMR Measurements.

^{31}P NMR spectra were recorded at 40.5 MHz using a Varian XL-100 NMR spectrometer. Broadband proton decoupling was routinely employed; unless otherwise stated, spectra were taken at 25°C. Chemical shifts are measured relative to 85% H_3PO_4 with positive shifts being downfield of the reference.

7. NMR Titration Data Analysis.

Values for the pKas and chemical shifts associated with species differing in state of protonation were fit to the experimental data using computer program written by Professor W.W. Cleland, University of Wisconsin, and by Thomas M. Marschner, University of California, San Francisco.

8. Materials.

Ammonia-^{15}N (99% ^{15}N) was purchased from KOR Isotopes, Inc. ^{15}N Ammonium sulfate was purchased from MSD Isotopes in 99% isotopic purity. $H_2{}^{17}O$ (13%^{16}O, 52%^{17}O, 35%^{18}O) was obtained from Monsanto. $H_2{}^{17}O$ (33.6%^{16}O, 39.1%^{17}O, 27.3%^{18}O) was purchased from Cambridge Isotopes. Diethyl chlorophosphate, triethyl phosphite, triphenyl phosphite, diphenyl chlorophosphate, diethyl chlorothiophosphate, diethyl chlorophosphite, thionyl chloride, and trimethylsilyl iodide were all from Aldrich Chemical Co. Adenosine-5'-monophosphoric acid was the product of Sigma Chemical Co. Unlabeled AMP-PNP and tetrasodium imidodiphosphate (PNP) were purchased from Boehringer-Mannheim. DEAE Sephadex A-25 and QAE Sephadex A-25 were purchased from Pharmacii- Chemicals. All other chemicals were of the finest quality commercially available (i.e, whatever happened to be on the shelf).

B. Chemical Synthesis. Compounds Referred to in Section III.

1. Preparation of ^{15}N Tetraethyl PNP. (Refer to Scheme I.)

a) ^{15}N Diethyl Phosphoramidate (14, see Scheme IIIa).

Ammonia-^{15}N (2.0 L, 89.3 mmol) was transferred on a vacuum line to a vessel containing 8.92 mL of distilled, deionized water to make a 10 M solution. This solution was transferred to a 25 mL flask containing a magnetic stir bar and equipped with an addition funnel. Diethyl chlorophosphate (7.69 g, 44.6 mmol) was added dropwise from the addition funnel with vigorous stirring at 0°C. After complete addition the reaction mixture was allowed to warm to room temperature and stirring was continued for 1 Hr. More water (10 mL) was added, and the product was extracted with four successive additions of 50 mL portions of CH_2Cl_2. The CH_2Cl_2 layer was dried over $MgSO_4$. After removal of solvent at reduced pressure, 15 mL of benzene was added and similarly removed to rid the product of traces of water. A flaky, white solid was obtained which melted between 49 - 51°C [literature m.p. 52°C, Goehring and Niedenzu, 1956]. The product weighed 4.37 g (64% of the theoretical amount). ^1H NMR ($CDCl_3$): δ 1.32 (6H, t, J_{HH}= 7.1 Hz), 4.09 (4H, apparent quintet). ^{31}P NMR ($CDCl_3$, proton decoupled): δ = 16.07 (d, J_{PN} = 38.2 Hz).

b) ^{15}N Diethyl Phosphoro(thionylamidate).

Thionyl chloride was heated at reflux with triphenhyl phosphite (20 mL per 200 mL $SOCl_2$) and then freshly

distilled (b.p. 76°C). Benzene was dried by distilling off the first 10% by volume in a nitrogen atmosphere and retaining the rest. ^{15}N Diethyl phosphoramidate (3.16 g, 20.6 mmol) was added to 10 mL of dry benzene in a flask equipped with a reflux condenser and $CaCl_2$ drying tube. $SOCl_2$ (2.46 g, 20.6 mmol) was then added, and the apparatus was purged with dry N_2. Using an oil bath, the temperature was raised slowly to 70°C. HCl gas began to evolve above 45°C. Heating was continued until HCl gas evolution ceased (ca. 2 hrs.). The cooled mixture was filtered through activated carbon, and the benzene was removed under reduced pressure to give a pale yellow oil. This crude product was purified by vacuum distillation at 72 - 74°C (0.3 torr) [literature b.p. 77°C at 1 torr, Wieczorkowski, 1963]. This fraction weighed 2.84 g (69% of the theoretical amount).

c) ^{15}N Diethylphosphoryl Triethoxyiminophosphorane.

Triethyl phosphite (7.11 g, 42.8 mmol) was dissolved in 6 mL of dry benzene, and ^{15}N diethyl phosphoro(thionylamidate) (2.84 g, 14.3 mmol) in 5 mL dry benzene was added dropwise with stirring under a purge of dry N_2. Stirring was continued for several hours at room temperature following complete addition, and the benzene was removed at reduced pressure. The side products, triethyl phosphate and triethyl thiophosphate, distilled together at 50 - 53°C (0.2 torr) [literature b.p. 42°C at 0.2 torr, Wieczorkowski, 1965] and weighed 4.65 g. The desired product, ^{15}N diethyphosphoryl triethoxyiminophosphorane, distilled at 95°C (0.005 torr,

oil diffusion pump) and weighed 2.78 g (61% of the theoretical amount). ^{1}H NMR ($CDCl_3$): δ 1.29 (6H, t, J_{HH}= 7.1 Hz), 1.33 (9H, t, J_{HH}= 7.1 Hz), 4.09 (10 H, m). ^{31}P NMR ($CDCl_3$, proton decoupled): δ -2.33 (apparent doublet of doublets, see Figure 23b).

Anal. Calcd. for $C_{10}H_{25}{}^{15}NO_6P_2$: C, 37.74; H, 7.92; ^{15}N, 4.71; P, 19.46. Found: C, 37.52; H, 7.85; ^{15}N, 4.70; P, 19.40.

d) ^{15}N Tetraethyl Imidodiphosphate (7).

^{15}N Diethylphosphoryl triethoxyiminophosphorane (1.00 g, 3.1 mmol) was dissolved in 2 mL of dry benzene and the solution was cooled to 0°C. Dry HCl (passed through conc. H_2SO_4) was introduced over a 15 min. period at 0°C, followed by 15 min. at room temperature. The reaction mixture was then let stand at room temperature for 1 Hr., and the benzene was removed under reduced pressure. Additional dry benzene (5 mL) was added and then likewise removed under reduced pressure after 1 Hr. The final traces of HCl were removed under high vacuum (about 0.1 torr) at room temperature. ^{31}P NMR revealed that the reaction had proceeded with quantitative yield of the desired product (7). In one instance, however, the product (7) was purified by vacuum distillation at 112 - 117°C (0.003 torr, oil diffusion pump). ^{1}H NMR ($CDCl_3$): δ 1.32 (12H, t, J_{HH}= 7.1 Hz), 4.10 (8H, apparent quintet). ^{31}P NMR ($CDCl_3$, proton decoupled): δ -0.743 (d, J_{PN}= 33.6 Hz). ^{15}N NMR ($CDCl_3$, proton decoupled): δ -52.72 (t, J_{PN}= 33.6 Hz) (see Figure 8).

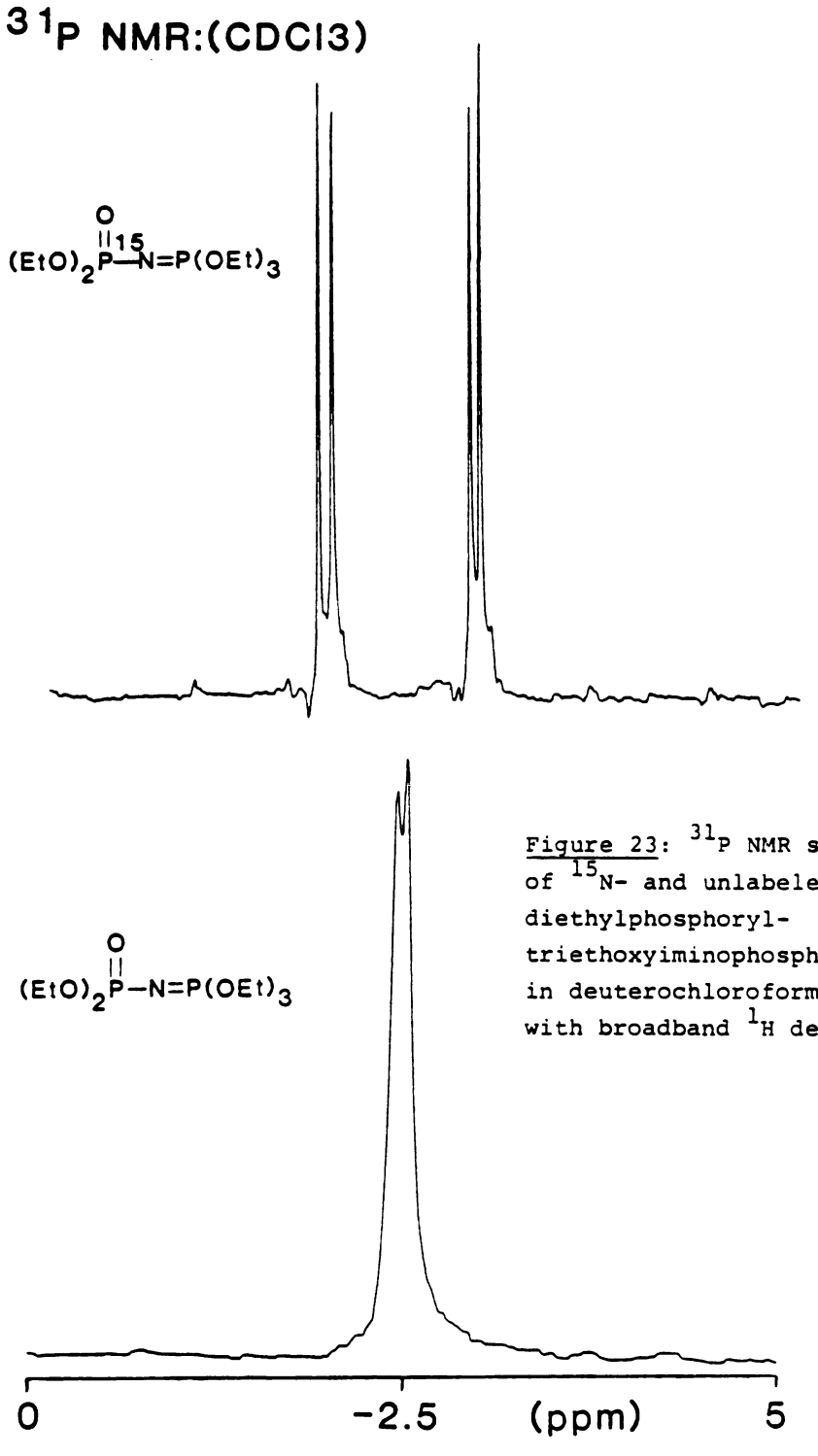


Figure 23: ^{31}P NMR spectra of ^{15}N- and unlabeled diethylphosphoryl-triethoxyiminophosphorane in deuterochloroform with broadband ^{1}H decoupling.

Anal. Calcd. for $C_8H_{21}{}^{15}NO_6P_2$: C, 33.11; H, 7.29; ^{15}N, 5.17; P, 21.35. Found: C, 33.83; H, 7.27; ^{15}N, 4.94; P, 20.90.

2. Preparation of ^{17}O Tetraethyl PNP (10). (See Schemes I, IIIb.)

a) ^{17}O Diethyl Phosphite (17).

Triethylamine and diethyl chlorophosphite were freshly distilled under dry N_2 atmosphere directly into a 3-necked 200 mL flask equipped with a dry magnetic stir bar, rubber septum, and a line leading to a Firestone valve. The reaction vessel was then stoppered and purged with N_2 using the Firestone valve. Triethylamine (7.0 mL, 50 mmol) was added using a glass syringe, and the vessel was cooled to 0° C in an ice bath. Next, diethyl chlorophosphite (7.0 mL, 49 mmol) was added slowly dropwise using a glass syringe with vigorous stirring. When the addition was complete the vessel was allowed to warm to room temperature and the Firestone valve was replaced with a glass stopper. Stirring was continued at room temperature overnight. Triethylammonium chloride was then removed by filtration through a fine glass scintered funnel, and the solvent was removed under reduced pressure. The product (17) was purified by vacuum distillation at 73°C (14 torr) using a water aspirator. The yield was 5.83 g (85%). The infrared spectrum was identical to that of authentic diethyl phosphite with a characteristic P-H stretch at 2425 cm^{-1}.

b) ^{17}O Diethyl Chlorophosphate.

^{17}O Diethyl phosphite (5.73 g, 41.2 mmol) and CCl_4 (12.67 g, 82.4 mmol) were weighed into a 50 mL round bottom flask containing a magnetic stir bar. The vessel was purged

with N_2, and a rubber septum was attached. Next, a needle was inserted which was connected to a small $CaCl_2$ drying tube, and the vessel was cooled to 0°C in an ice bath. Triethylamine (0.66 mL, 4.74 mmol) was then added slowly with stirring using a glass syringe. After 15 min. at 0°C, the reaction vessel was allowed to warm slowly to room temperature and stirring was continued for 3 hours. The reaction mixture was then filtered through a fine glass scintered funnel to remove a small amount of triethylammonium chloride which precipitated during the reaction. The low boiling components in the reaction mixture were removed under reduced pressure. The product was purified by vacuum distillation at 70°C (4 torr) [literature b.p. 64°C, 6-7 torr, Steinberg, 1950]. The yield was 5.58 g (78% of the theoretical yield).

c) ^{17}O Diethyl Phosphoramidate.

^{17}O Diethyl chlorophosphate (5.58 g, 32.2 mmol) was added slowly dropwise to a cooled, stirred solution of conc. NH_3/H_2O (29.6% NH_3 by wt., 5.54 g, 96.5 mmol) in an ice bath. After completed addition the reaction mixture was allowed to warm to room temperature and stirring was continued for 1 Hr. More water (10 mL) was then added, and the product was extracted with four successive additions of 50 mL portions of CH_2Cl_2. The CH_2Cl_2 layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure to give a white flaky solid. The yield was 2.84 g (69% of the theoretical amount).

d) ^{17}O Diethyl Phosphoro(thionylamidate).

This synthesis was identical to the synthesis of ^{15}N diethyl phosphoryl(thionylamidate) (preparation 1b, this section).

e) ^{17}O Diethylphosphoryl Triethoxyiminophosphorane.

This synthesis was identical to that described for ^{15}N diethylphosphoryl triethoxyiminophosphorane (preparation 1c, this section).

f) ^{17}O Tetraethyl PNP.

Again, this synthesis was identical to the preparation of ^{15}N tetraethyl PNP (preparation 1d, this section).

3. Preparation of ^{15}N PNP (19).

The synthesis of ^{15}N PNP was achieved by two different methods.

Method A: Via ^{15}N Tetraethyl PNP (Scheme VI).

^{15}N Tetraethyl PNP (1.00 g, 3.45 mmol) was dissolved in 1.0 mL of dry CH_2Cl_2, and the resulting solution was cooled to ca -40°C using a dry ice-acetone bath. Trimethylsilyl iodide (2.76 g, 13.8 mmol) was added dropwise with stirring over the course of ca. 15 min., and the cooled mixture was stirred for an additional 45 min. Stirring was continued at room temperature overnight. Water (10 mL) was then added, and the mixture was stirred at room temperature for 30 min. Next, a 50% aqueous NaOH solution (2.21 g, 27.6 mmol) was added. The aqueous phase was then washed with 5 ml CH_2Cl_2 and diluted with an additional 10 mL H_2O. The product (white needles) was precipitated by dropwise addition of ethanol at 0°C. After one recrystallization from aq. EtOH, the needles weighed 1.2 g (78.1% yeild).

Anal. Calcd. for $Na_4H^{15}NO_6P_2 \cdot 10\ H_2O$: H, 4.74; ^{15}N, 3.36; P, 13.89; Na, 20.62. Found: H, 4.60; ^{15}N, 2.81; P, 13.90; Na, 21.00.

Method B: Via ^{15}N Dichlorophosphoryl Trichloroiminophosphorane (20, Scheme VIIa).

a) Preparation of ^{15}N Dichlorophosphoryl Trichloroiminophosphorane.

^{15}N Ammonium sulfate (2.00 g, 14.91 mmol) and PCl_5 (13.97 g, 67.10 mmol) were weighed into a dry 100 mL round bottom flask equipped with a magnetic stir bar. s-Tetachloroethane (30 mL) was added, and a $CaCl_2$ reflux condenser and $CaCl_2$ drying tube were attached. The reaction mixture was heated in an oil bath with stirring for exactly 60 min.; the reaction vessel was then cooled in an ice bath. The cooled mixture was filtered to remove a small amount of unreacted $(^{15}NH_4)_2SO_4$, and the solvent was removed under reduced pressure with a bath temperature no greater than 40° C. The product was purified by vacuum distillation, b.p. 83-85°C (0.02 torr) [literature b.p. 110-115°C, 0.1 torr, Emsley et al., 1971]. The pure material solidified after several minutes to give a white glassy solid, m.p. 32°C [literature m.p. 32°C, Emsley et al., 1971], and weighed 5.31 g (66% of the theoretical yield).

b) Preparation of ^{15}N PNP, Tetrasodium Salt, Decahydrate.

^{15}N Dichlorophosphoryl trichloroiminophosphorane (0.5405 g, 2 mmol) was treated with 1 N NaOH (19.0 mL, 19.0 mmol) in an ice bath for two hours. This sample was purified by anion-exchange chromatography at 2-4°C on QAE Sephadex A-25 (HCO_3^-) using a 3 L linear gradient of 0.1 - 0.7 M triethylammonium bicarbonate, pH 8.6, as eluent. Fractions were assayed for acid-labile phosphate according to the procedure of Ames (1966). Imidodiphosphate is eluted from the column by 0.4 M triethylammonium bicarbonate under these conditions. The pooled fractions were concentrated to a syrup on a rotary evaporator using a vacuum pump and a dry

ice/ ethanol trap with a bath temperature below 25°C. One mL of tributylamine was added to the syrup, and residual water and triethylammonium bicarbonate were removed by repeated evaporation of 25 mL aliquots of absolute methanol under reduced pressure. The resulting syrup was transferred to a 30 mL glass centrifuge tube with three 2 mL rinses of methanol. The sodium salt of ^{15}N imidodiphosphate was precipitated by addition of 10-12 equivalents of 1 M NaI in acetone and recovered by centrifugation. After washing the precipitate twice with cold acetone, it was dissolved in about 15 mL cold water. The resulting solution was adjusted to pH 11.5 with concentrated NaOH, and the product was precipitated by addition of ethanol to give white needles (0.7953 g, 89%).

^{31}P NMR (20% D_2O, pH 9.15, proton decoupled): δ 2.91 (d, J_{PN}= 22.6 Hz). ^{15}N NMR (10% D_2O, pH 11.00, proton decoupled): δ 56.90 (t, J_{PN}= 22.6 Hz). (See Figure 11).

4. Preparation of ^{17}O PNP (21). (See Sheme VII).

Tetrasodium ^{17}O imidodiphosphate, decahydrate was prepared by dropwise addition of a solution of dichlorophosphoryl trichloroiminophosphorane (0.5385 g, 2 mmol) in 2 mL of dry methylene chloride to a stirred mixture of 1 mL $H_2{}^{17}O$, triethylamine (2.509 mL, 18 mmol), and 2 mL dry methylene chloride at 0°C. The mixture was stirred for 0.5 Hr. at 0°C and then for 2 hours at room temperature. The solvent was removed by rotary evaporation using a water aspirator and a bath temperature of 20°C. The resulting viscous solution was purified by chromatography on a column of QAE Sephadex A-25 (HCO_3^-). Purification was accomplished as described for ^{15}N imidodiphosphate to give needles (0.6048 g, 67%). This material was approximately 25% enriched with ^{17}O in each of the oxygens as judged by integration of the ^{31}P NMR spectrum of the β,γ-^{17}O AMP-PNP prepared from this sample.

5.[β,γ-^{15}N]-,[α-$^{17}O_1$]-, and[β,γ-^{17}O]AMP-PNP were prepared from either the corresponding labeled PNP or from[$^{17}O_1$]AMP in a procedure that closely paralleled that of Yount and coworkers (1971a, see Scheme V) in yields of 38%, 38%, and 40%, respectively. The purification procedure for these samples of AMP-PNP differed from that reported by Yount in two respects: QAE Sephadex A-25 (HCO_3^-) was used in place of DEAE Sephadex A-25 (HCO_3^-), and a linear gradient of 0.1 - 0.7 M triethylammonium bicarbonate, pH 8.6, was used for elution instead of a gradient of 0.0 - 0.4 M

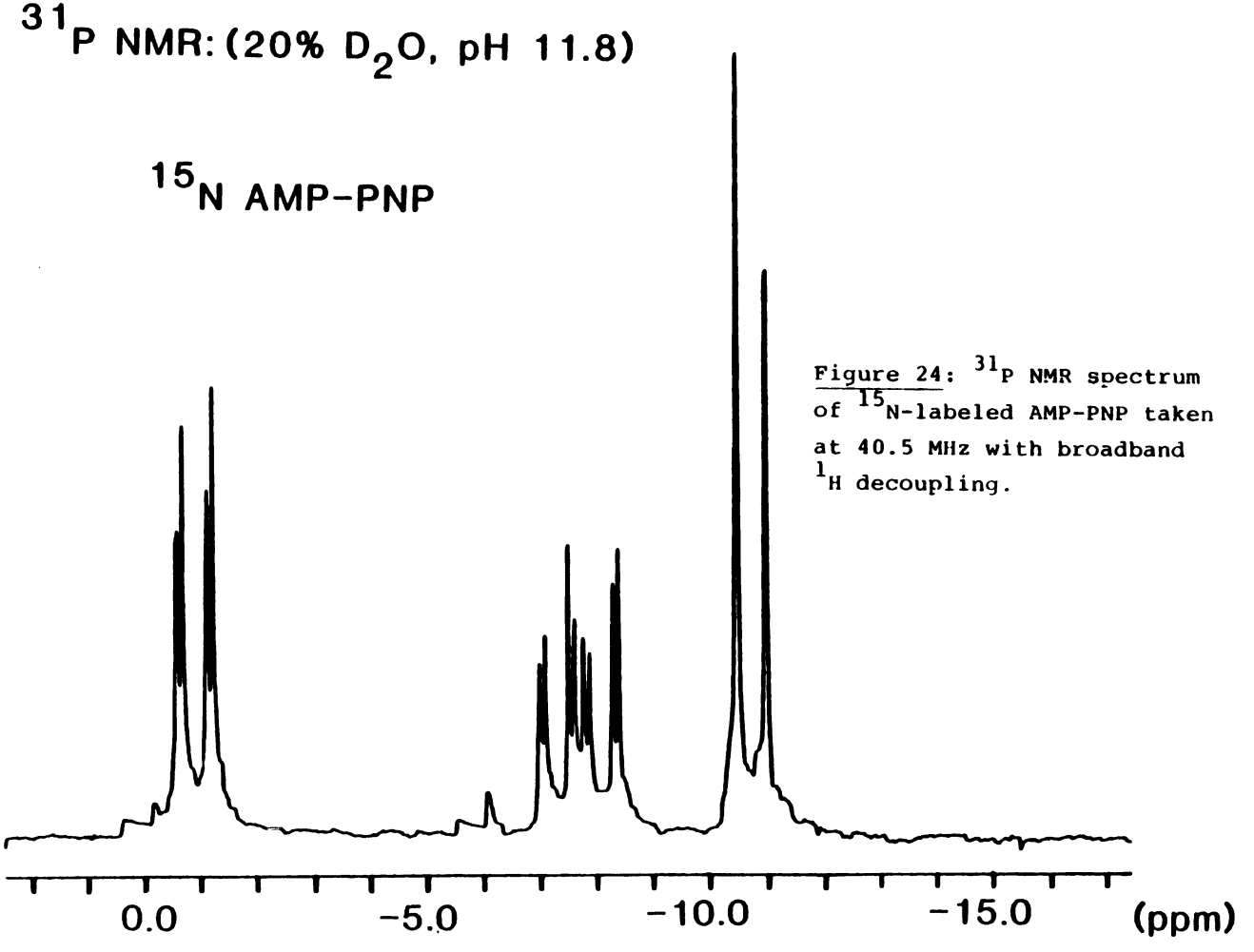


Figure 24: ^{31}P NMR spectrum of ^{15}N-labeled AMP-PNP taken at 40.5 MHz with broadband ^{1}H decoupling.

triethylammonium bicarbonate, pH 7-8. (AMP-PNP is more stable at the higher pH.) All three labeled samples of AMP-PNP had identical Rf values (0.34) when compared to authentic unlabeled AMP-PNP using polyethylenimine cellulose TLC with 1.2 N LiCl as eluent (Rowley and Kenyon, 1974).

The α-^{17}O AMP-PNP is predicted to have an ^{17}O -enrichment of 32% in one of the α-nonbridging oxygens. Integration of the ^{31}P NMR resonances of β,γ-^{17}O AMP-PNP relative to an internal standard of AMP was consistent with an ^{17}O -enrichment of approximately 25% in each of the oxygens derived from ^{17}O-PNP.

C. Chemical Synthesis: Compounds Referred to in Section IV.

1. Preparation of N-Methyl AMP-PNP.

a) Unlabeled Dichlorophosphoryl Trichloroiminophosphorane.

This synthesis was identical to the one previously given for ^{15}N dichlorophosphoryl trichloroiminophosphorane.

b) N-Methyl Imidodiphosphoryl Tetrachloride (23) (Scheme IX).

CH_2Cl_2 was dried by heating at reflux over CaH_2. Dichlorophosphoryl trichloroiminophosphorane (15.00 g, 55.7 mmol) was dissolved in 50 mL of dry CH_2Cl_2 in a 200 mL round bottom flask equipped with a magnetic stir bar, addition funnel, and $CaCl_2$ drying tube. The reaction vessel was cooled to ca. -20°C using a dry ice/ acetone bath. Next, absolute methanol (1.785 g, 55.7 mmol), dissolved initially in 25 mL of dry CH_2Cl_2, was added dropwise with stirring using the addition funnel. Stirring was continued at -20°C for 30 min. and then at room temperature for 2 hours. The solvent was removed under reduced pressure, and the last traces of HCl were removed by application of high vacuum (0.1 torr) at room temperature. The resulting oil was then heated at 60-75°C using an oil bath for 4 hours, protected from moisture with a $CaCl_2$ drying tube. The product was purified by vacuum distillation at 72-73°C (0.01 torr) [literature b.p. 95-98°C, 0.3 torr, Riesel et al., 1977b]. This material rapidly solidified to give a white glassy solid, m.p. 49-50°C [literature m.p. 50-51°C, Riesel et al.,

1977b] which weighed 8.78 g (59.5% of the theoretical amount). ^{1}H NMR ($CDCl_3$): δ 3.38 (t, J_{PN}= 13.6 Hz).

Anal. Calcd. for $C_1H_3NO_2P_2Cl_4$: C, 4.54; H, 1.14; N, 5.29; P, 23.39. Found: C, 4.40; H, 1.16; N, 5.14; P, 23.18.

c) N-Methyl Imidodiphosphate, Tributylammonium Salt (24).

N-Methyl imidodiphosphoryl tetrachloride (0.5296 g, 2 mmol) was treated with 1 N NaOH (19.0 mL, 19.0 mmol) in an ice bath for 1 hour. The reaction was then warmed to room temperature with stirring until all of the white solid had dissolved. This sample was purified by anion-exchange chromatography at 2-4°C on QAE Sephadex A-25 (HCO_3^-) using a 3 L linear gradient of 0.1 - 0.7 M triethylammonium bicarbonate, pH 8.5, as eluent. Fractions were assayed for acid-labile phosphate according to the procedure of Ames (1966). N-Methyl PNP is eluted from the column by 0.36 M triethylammonium bicarbonate under these conditions. The appropriate fractions were pooled, 1 mL of tributylamine was added, and the resulting solution was concentrated to a syrup on a rotary evaporator using a vacuum pump and a dry ice/ ethanol trap with a bath temperature below 25°C. An additional 1 mL of tributylamine was added, and the syrup was stripped of residual water and triethylammonium bicarbonate by repeated evaporation of 25 mL aliquots of absolute methanol under reduced pressure. ^{31}P NMR (20% D_2O, pH 12.6, proton decoupled): δ 5.83 (s); (proton coupled): (quartet, J_{PH}= 9.4 Hz).

d) N-Methyl AMP-PNP.

This compound was prepared from N-methyl PNP and AMP as described for the ^{15}N- and ^{17}O-labeled AMP-PNP analogs. Purification was performed by anion exchange chromatography on QAE Sephadex A-25 (HCO_3^-) using a 3 L linear gradient of 0.1 - 0.7 M triethylammonium bicarbonate buffer (pH 8.6). N-methyl AMP-PNP is eluted from the column by 0.48 M triethylammonium bicarbonate under these conditions. The appropriate fractions were pooled, and 4 equiv. of tetrabutylammonium hydroxide (from a 40% aqueous solution) was added. The buffer was removed, and precipitation of the Na^+ salt of N-methyl AMP-PNP was done as described for the other AMP-PNP analogs. The yield of N-methyl AMP-PNP was 20.4%, as determined by UV absorption at 260 nm using a molar absorbancy index (Adenine) of 1.54×10^4. As described in the text, this sample was found by ^{31}P NMR to be contaminated with other impurities.

2. Preparation of Tetraethyl N-Methyl PNP (29) (Scheme XI).

a) N-Methyl Diethylphosphorothioylamidate (26).

Monomethylamine (19.19 g, 0.6179 mole) was bubbled through a glass frit into 250 mL of CH_2Cl_2 at 0°C. This solution was transfered to a 500 mL round bottom flask equipped with a magnetic stir bar and an addition funnel. Diethyl chlorothiophosphate (58.27 g, 0.3089 mole), dissolved initially in 50 mL of CH_2Cl_2, was added dropwise with stirring at 0°C. Stirring was continued for 1 hour at 0°C and then overnight at room temperature. Next, precipitated monomethylammonium chloride was removed by filtration, and the solvent was removed under reduced pressure to give an oil (58.3 g). The product was purified by vacuum distillation at 68-70°C (0.1 torr) and weighed 55.23 g (97.6% of the theoretical amount).

Anal. Calcd. for $C_5H_{14}NO_2PS$: C, 32.77; H, 7.70; N, 7.65; P, 16.91; S, 17.50. Found: C, 32.95; H, 7.68; N, 7.58; P, 16.94; S, 17.38.

b) N-Methyl Diethylphosphorothioyl Diethylphosphoramidate.

Dimethoxyethane (ethylene glycol, dimethyl ether) was heated at reflux and distilled over $LiAlH_4$ prior to use. NaH (4.32 g, 90.1 mmol, as a 50% oil dispersion) was activated by washing with hexane under dry N_2 atmosphere, quickly transferred to a 300 mL round bottom flask, and covered with 100 mL of dry dimethoxyethane. A magnetic stir bar was added and an addition funnel an $CaCl_2$ drying tube were attached after purging the apparatus with dry N_2.

N-Methyl diethylphosphorothioylamidate (15.00 g, 81.8 mmol), dissolved initially in 25 mL of dry dimethoxyethane, was added dropwise using the addition funnel with stirring at 0° C. Stirring was continued for 30 min. at 0°C and then for 1 and 1/2 hours at room temperature. Next, diethylchlorophosphate (14.11 g, 81.8 mmol), dissolved initially in 25 mL of dry dimethoxyethane, was added dropwise using the addition funnel with stirring at 0°C. After 15 min. at 0°C, stirring was continued at room temperature for 48 hours. The reaction mixture was then filtered to remove NaCl, and the solvent was removed under reduced pressure. The product was purified by fractional vacuum distillation, b.p. 106-109°C (0.15 torr) [literature b.p. 120°C, 1.0 torr, Arbuzov et al., 1954] and weighed 17.53 g (67 % of the theoretical yield).

Anal. Calcd. for $C_9H_{23}NO_5P_2S$: C, 33.85; H, 7.26; N, 4.39; P, 19.40; S, 10.04. Found: C, 34.01; H, 7.24; N, 4.35; P, 19.30; S, 10.13.

c) N-Methyl Diethylphosphoryl Diethylphosphoramidate (29).

A solution of m-chloroperoxybenzoic acid (11.00 g, 63.7 mmol) in 120 mL of CH_2Cl_2 was added dropwise with stirring to N-methyl diethylphosphorothioyl diethylphosphoramidate (5.00 g, 15.7 mmol) in a 250 mL round bottom flask. A $CaCl_2$ drying tube was then attached and stirring was continued for 30 min. The reaction mixture was filtered, and then washed with 80 mL of 10% sodium sulfite followed by 2 X 30 mL of saturated $NaHCO_3$. The organic layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure. The

resulting residue was filtered to give a colorless oil which weighed 5.6 g. The product was purified by vacuum distillation at 109-110°C (0.25 torr) and weighed 2.54 g (53.4% of the theoretical amount). $\underline{^1H\ NMR\ (CDCl_3)}$: δ 1.35 (12H, t, J_{HH}= 7.0 Hz), 2.95 (3H, J_{PH}= 9.7 Hz), 4.16 (10H, m).

Anal. Calcd. for $C_9H_{23}NO_6P_2$: C, 35.65; H, 7.64; N, 4.62; P, 20.43. Found: C, 36.02; H, 7.49; N, 4.39; P, 20.16.

D. Chemical Synthesis: Miscellaneous Compounds.

1. N-Methyl Diethylphosphoramidate.

Monomethylamine (6.04 g, 0.1945 mole) was bubbled through a glass frit into 250 mL of CCl_4 at 0°C. This solution was transferred to a 500 mL round bottom flask equipped with a magnetic stir bar, addition funnel, and $CaCl_2$ drying tube. A solution of diethyl phosphite (13.43 g, 0.0972 mole), dissolved initially in 25 mL of CCl_4, was added dropwise with stirring at 0°C using the addition funnel. The reaction mixture was then stirred at room temperature for 1 1/2 hours. Precipitated monomethylammonium chloride was removed by filtration, and the solvent was removed under reduced pressure. The product was purified by vacuum distillation at 77-78°C (0.4 torr) and weighed 15.33 g (94.3% of the theoretical amount). ^1H NMR ($CDCl_3$): δ 1.33 (6H, t, J_{HH}= 7.0 Hz), 2.58 (3H, quartet, J_{HH}= 12.0 Hz, J = 5.6 Hz), 3.03 (1H, broad singlet, exchangeable with D_2O), 4.06 (4H, apparent quintet).

2. N-Benzyl Diethylphosphoramidate.

This compound was prepared from benzylamine and diethyl phosphite in a procedure identical to that described for N-methyl diethylphosphoramidate. The product was purified by vacuum distillation at 149-150°C (0.25 torr), 98% yield.

Anal. Calcd. for $C_{11}H_{18}NO_3P$: C, 54.31; H, 7.46; N, 5.76; P, 12.74. Found: C, 54.53; H, 7.37; N, 5.78; P, 12.77.

3. N-Allyl Diethylphosphoramidate.

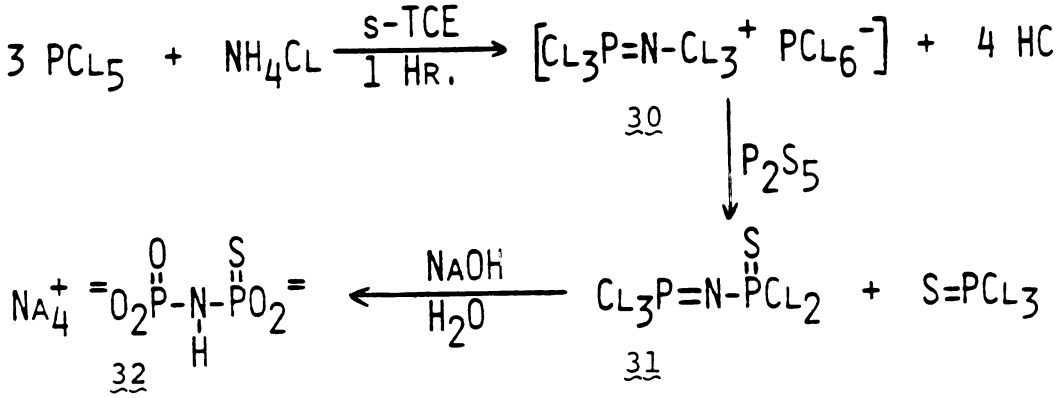
This material was prepared from allylamine and diethyl phosphite according to the procedure given above for N-methyl diethylphosphoramidate. The product was purified by vacuum distillation at 100-103°C (0.5 torr), 97% yield.

Anal. Calcd. for $C_7H_{16}NO_3P$: C, 43.52; H, 8.35; N, 7.25; P, 16.03. Found: C, 43.72; H, 8.28; N, 7.21; P, 16.12.

4. Preparation of Phosphoroimidophosphorothioate (32).

The method used to prepare 32 is shown in Scheme XIV.

SCHEME XIV.



a) $[Cl_3PNPCl_3^+][PCl_6^-]$ (30) was prepared according to the procedure of Emsley and Udy (1970).

b) Dichlorophosphorothioyl Trichloroiminophosphorane (31) was prepared according to the procedure of Khodak and Gilyarov (1979). ^{31}P NMR ($CDCl_3$, proton decoupled): δ 28.5 (broad singlet), -5.5 (broad singlet); P-P coupling was not resolved.

c) Phosphoroimidophosphorothioate (32).

Dichlorophosphoryl trichloroiminophosphorane (31, 0.5706 g, 2 mmol) was treated with 1 N NaOH (18 mL, 18 mmol) at 0°C. The solution was then stirred at room temperature until all solid material had dissolved (ca. 12 hours). The resulting solution was analyzed by ^{31}P NMR (pH 12): δ 1.71 (d, J_{PP}= 5.6 Hz), 34.97 (d, J_{PP}= 5.7 Hz). This material was shown by ^{31}P NMR to hydrolyze rapidly below pH 7.5 to phosphoramidate (δ = 5.5) and thiophosphate (δ = 35.4). Attempts to purify 32 by anion exchange chromatography were unsuccessful.

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Part 2:[β,γ-^{18}O]- and[β,γ-^{17}O]-Bridge Labeled ATP: Synthesis and Positional Isotope Exchange Studies Using ^{31}P NMR

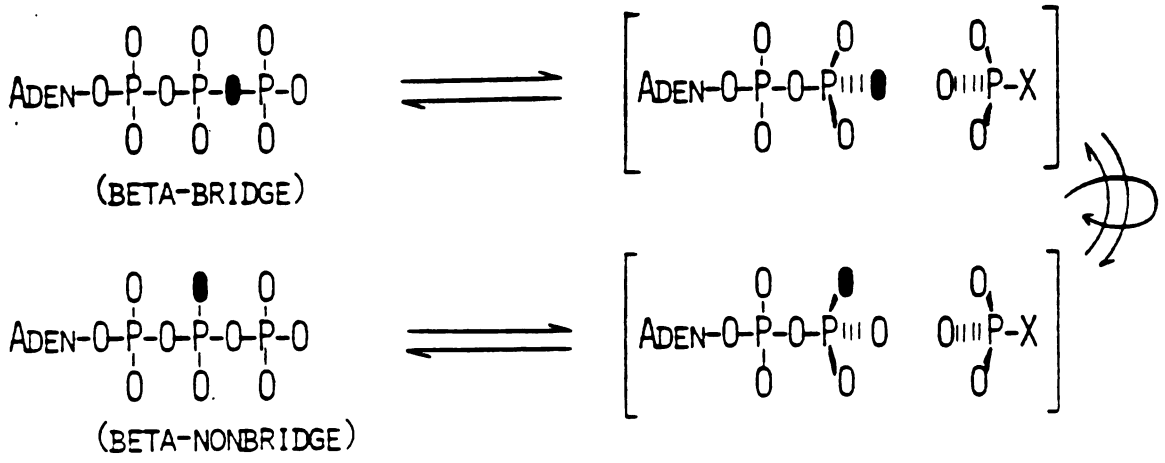
I. Introduction.

In Part 1 of this dissertation we mentioned the use of ATP analogs as competitive inhibitors of adenosine triphosphatases and kinases which normally catalyze either the hydrolysis or transfer of the γ-phosphoryl group of ATP. In Part 2 we will discuss the method of positional isotope exchange which has been developed to study the chemical processes which occur on the enzyme during the phosphotransfer reaction.

The positional isotope exchange (PIX) technique of Midelfort and Rose (1976) was developed to detect either a phosphoenzyme intermediate for phosphotransfer reactions in cases where ATP⇌ADP exchange reactions cannot otherwise be observed. That is, the ADP is generated at the active site of the enzyme and is not released during the phosphotransfer step. Briefly, this method follows the posotional exchange of ^{18}O in ATP from a bridge (β,γ) to a nonbridge (β) position during the re-formation of ATP from enzyme-bound ADP and the proposed phosphoenzyme or phosphorylated substrate (Scheme I). Assuming free rotation of the β-phosphoryl group of enzyme-bound ADP, there is a 67% probability that the labeled oxygen will be found in one of the β-nonbridge positions in the back reaction. Detection of this exchange was originally done using mass spectrometry, which required a series of enzymatic and

derivatization steps to prepare the sample for analysis.

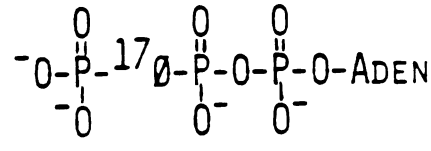
SCHEME I: GENERAL SCHEME FOR POSITIONAL ISOTOPE EXCHANGE



Recently, it has been shown that ^{31}P NMR resonances in ATP are shifted upfield by about 0.02 ppm when an ^{16}O atom is replaced by ^{18}O (Cohn and Hu, 1978; Lowe and Sproat, 1978a; Lutz et al., 1979). These principles have enabled the determination of PIX to be performed using ^{31}P NMR spectroscopy. An important advantage of ^{31}P NMR as applied to PIX experiments is that it provides a non-destructive means of detection while the reaction is in progress. In the practical application of this method, however, a high frequency NMR instrument is desirable to resolve the individual peaks.

Independently, Tsai and coworkers (1979, 1980) have demonstrated that the line-broadening effect of an ^{17}O nucleus directly bonded to phosphorus causes a quantitative decrease in the ^{31}P NMR signal in direct proportion to the amount of ^{17}O enrichment. We were particularly interested in Tsai's proposal (1980) that the effect of ^{17}O on the ^{31}P NMR signal could be utilized in performing PIX experiments.

It was apparent from this work that if an ATP analog could be made with an exclusive ^{17}O label in the β,γ-bridge position (i.e., 1), then the positional isotope exchange of this label to a β-nonbridge position could be observed by an increase in the intensity of the $^{31}P_{\gamma}$-resonance on a lower frequency NMR instrument, which Tsai calls the $^{31}P(^{17}O)$ NMR method. It was also felt that the corresponding analog with an ^{18}O label (i.e., 2) would be useful in the more traditional, higher field $^{31}P(^{18}O)$ method.



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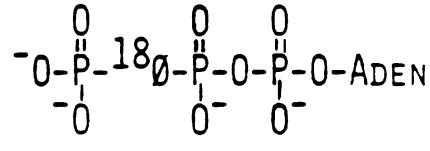

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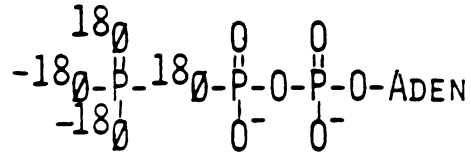
To date, no synthesis of ATP with an exclusive ^{17}O or ^{18}O label in the β,γ-bridge has been available. Thus, in the PIX experiments reported by Midelfort and Rose (1976), Wimmer et al. (1979) and Raushel and Villafranca (1980), a multiply labeled analog, γ-$^{18}O_4$-ATP (3), was used. Alternatively, PIX experiments have been performed with an ATP analog possessing multiple ^{18}O labels in the α,β-bridge and in the two β-nonbridge positions (4), as was used, for example, by Lowe and Sproat (1978b) in a study of pyruvate kinase.



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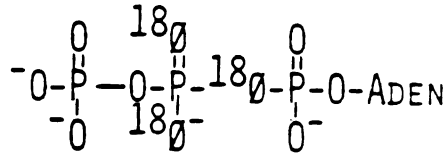

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In Part 2 we will report a synthetic method for preparing specific ^{17}O and ^{18}O labeled ATP analogs in sufficient enrichment at the β,γ-bridge for PIX to be readily observed using either the $^{31}P(^{18}O)$ or $^{31}P(^{17}O)$ NMR methods. We will also report the use of these compounds in prototype PIX experiments with carbamoyl phosphate synthetase from E. coli and bicarbonate as the phosphorylated substrate.

II. Theoretical Background.

A. ^{18}O Isotope Sifts in ^{31}P NMR.

Oxygen 18, with its two spin-paired neutrons outside a magnetically inert ^{16}O nuclear core, has zero spin. Thus, ^{18}O-atoms are unable to exert a magnetic influence on ^{31}P nuclear magnetic resonances. Nevertheless, their influence on ^{31}P NMR chemical shifts was hinted at over 30 years ago by Ramsey and Purcell (1952) who first predicted the effect of isotopic substitution on the magnetic shielding of nuclei. This effect has been observed in a variety of systems (see Risley and Van Etten, 1980). With few exceptions (Kanazawa et al., 1965), substitution by a heavier isotope results in a small upfield shift in the NMR signal of a neighboring nucleus. It has been shown (Batiz-Hernandez and Bernheim, 1967) that the magnitude of this shift is related to the fractional change in mass, the chemical shift range of the nucleus being observed, and the structure of the compounds.

The ^{18}O isotope effect in ^{31}P NMR was first reported by Cohn and Hu (1978) and others (see Introduction). For example, five peaks were resolved in a ^{31}P NMR spectrum of randomized ^{18}O-labeled inorganic phosphate, corresponding to the five isotopic isomers: $^{16}O_4$, $^{16}O_3{}^{18}O$, $^{16}O_2{}^{18}O_2$, $^{16}O{}^{18}O_3$, and $^{18}O_4$. It was observed that the magnitude of the upfield chemical shift is directly related to the number of ^{16}O atoms replaced by ^{18}O.

As mentioned in the introduction, the magnitude of

upfield chemical shift for a ^{31}P resonance exerted by a directly bound ^{18}O-atom can be correlated with the bond order between these two nuclei. Table 1 lists the contributions to upfield shifts in ^{31}P resonances for ATP labeled with ^{18}O at various positions, as determined from multiply labeled ^{18}O-ATP analogs by Cohn and Hu (1980). Based on this data, we predicted that the P_β and P_γ -resonances for β-^{18}O-nonbridge labeled ATP would be easily distinguishable from those for β,γ-^{18}O-bridge labeled ATP, provided that ^{31}P NMR spectra of sufficient resolution were obtained. For example, the ^{18}O-nonbridge and ^{18}O-bridge P_β -resonances at 97.3 MHz are predicted to be separated by about 1.1 Hz, for which linewidths on the order of 0.5 Hz are desirable for quantitative results.

Table 1. ^{31}P Chemical Shift (ppm) (Upfield) per ^{18}O Bonded to P Atoms of ATP (from Cohn and Hu, 1980).

Site of ^{18}O-Label	Predicted Upfield Shift (ppm)
α,β-bridge	0.0172 (P_α)
	0.0165 (P_β)
β-nonbridge	0.0281 (P_β)
β,γ-bridge	0.0165 (P_β)
	0.0220 (P_γ)
γ-nonbridge	0.0220 (P_γ)

B. ^{17}O Line Broadening Effects in ^{31}P NMR.

An ^{17}O nucleus possesses a spin of I = 5/2, and thus is termed quadrupolar. The nature of the magnetic interaction between ^{31}P and ^{17}O has been discussed in detail by Tsai (1980, 1982). Briefly, the line shape of a ^{31}P resonance (I = 1/2, no quadrupole moment) directly coupled to ^{17}O is dependent on the quadrupolar relaxation time T_q of ^{17}O and the spin-spin coupling constant J between the two nuclei (termed "scalar relaxation of the second kind" by Abragam, 1961). Depending on the magnitude of the product T_qJ, four cases may be distinguished: (1) T_qJ is large, the ^{31}P signal is split by spin-spin coupling, as in the case of $P^{17}O\ Cl_3$; (2) T_qJ decreases, lines of the multiplet broaden; (3) T_qJ is small, the ^{31}P resonance becomes a broad singlet; and (4) T_qJ is very small, the quadrupolar effect is not detectable. Most phosphate compounds of biological interest fall into categories (2) and (3). In case (3), the ^{31}P linewidth is inversely proportional to the spin-spin relaxation time, T_2:

$$\frac{1}{T_2} = \frac{4\pi^2 J^2 I(I+1)}{3} T_q \qquad \text{Where } I = 5/2$$

The value of J depends primarily on the functional group, and does not vary much among phosphate compounds. However, T_q varies widely because it is a function primarily of the rotational correlation time and secondarily of molecular symmetry.

The ^{31}P linewidths observed by Tsai *et al.* (1980) for ^{17}O-labeled ATP analogs ranged from 50 Hz for multiple labeling at P_α or P_γ to ≥350 Hz for multiple labeling at P_β. In practice, narrow resonances are also observed due to incomplete labeling at these positions (only 60 atom%

^{17}O-water is currently available for use in preparing these compounds). Thus, the intensity of ^{31}P resonances in ^{17}O-labeled ATP samples can be measured to have decreased with respect to those of unlabeled ATP, provided that the broad resonances due to bound ^{17}O species are not included in the integration.

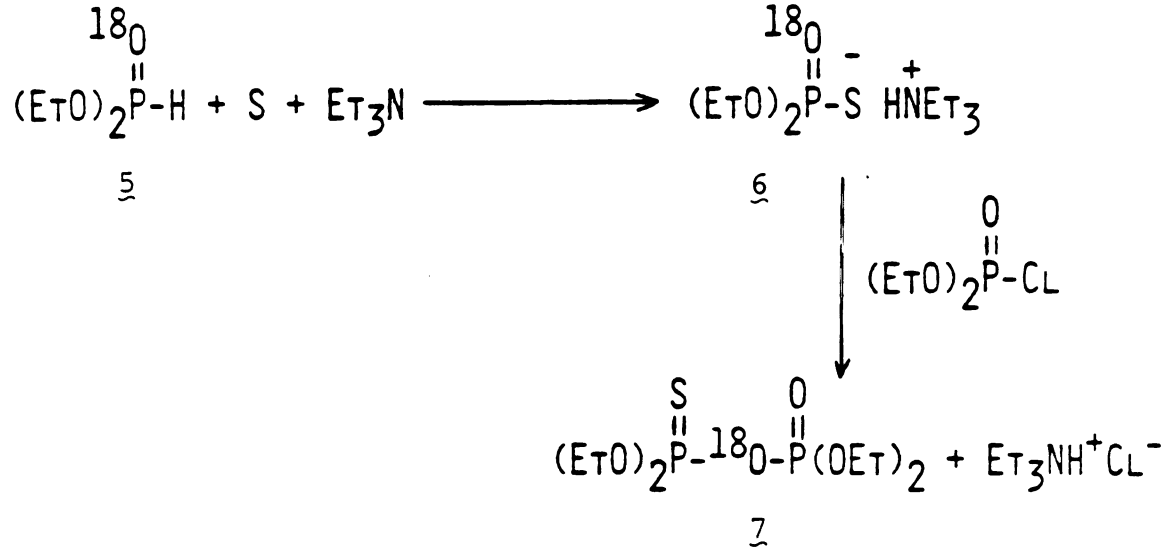
The principles of ^{17}O line broadening in ^{31}P NMR are readily adapted to the observation of positional isotope exchange using β,γ-^{17}O-bridge labeled ATP (1). As β,γ-bridge to β-nonbridge positional isotope exchange proceeds, the number of ATP molecules having an ^{17}O atom directly bonded to P_γ will decrease, thereby giving rise to an increase in the intensity of the narrow P_γ peaks in the ^{31}P NMR spectrum. The intensity of the P_β peaks will remain unchanged, since the ^{17}O-atom remains bound at P_β after PIX has occurred.

III. [β,γ-^{17}O]- and [β,γ-^{18}O]-Labeled ATP: Synthesis and Analysis by ^{17}O and ^{31}P NMR.

A. General Procedure for Preparing Bridge Labeled Pyrophosphate.

The synthesis of ^{18}O-bridge labeled tetraethyl thiopyrophosphate (7) was performed as described in Scheme II. The preparation of ^{17}O-labeled diethyl phosphite was described in Part 1 of this dissertation. This same procedure was used to make the corresponding ^{18}O-labeled analog (5).

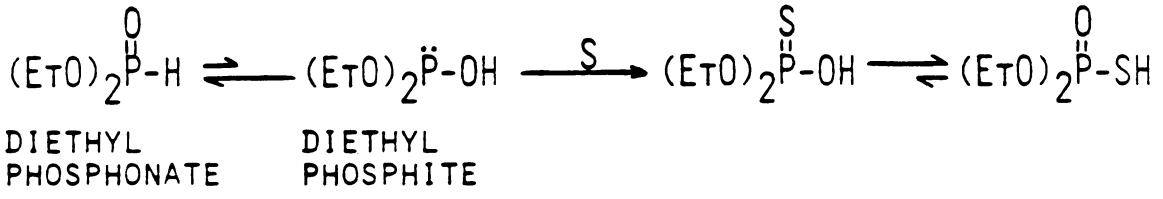
SCHEME II.



The reaction of sulfur with diethyl phosphite in the presence of an equimolar amount of triethylamine was first described by Arbuzov *et al.* (1954). It should be pointed out that the term diethyl phosphite is actually a misnomer. Infrared and ^{31}P NMR evidence have shown that the predominant structure of this molecule is better termed

diethyl phosphonate. Nevertheless, the reaction with sulfur is best understood as occurring with the "diethyl phosphite" form (Scheme III). Presumably, triethylamine assists in pulling this reaction forward by abstracting a proton from diethyl phosphite either before or during the reaction with sulfur.

SCHEME III.

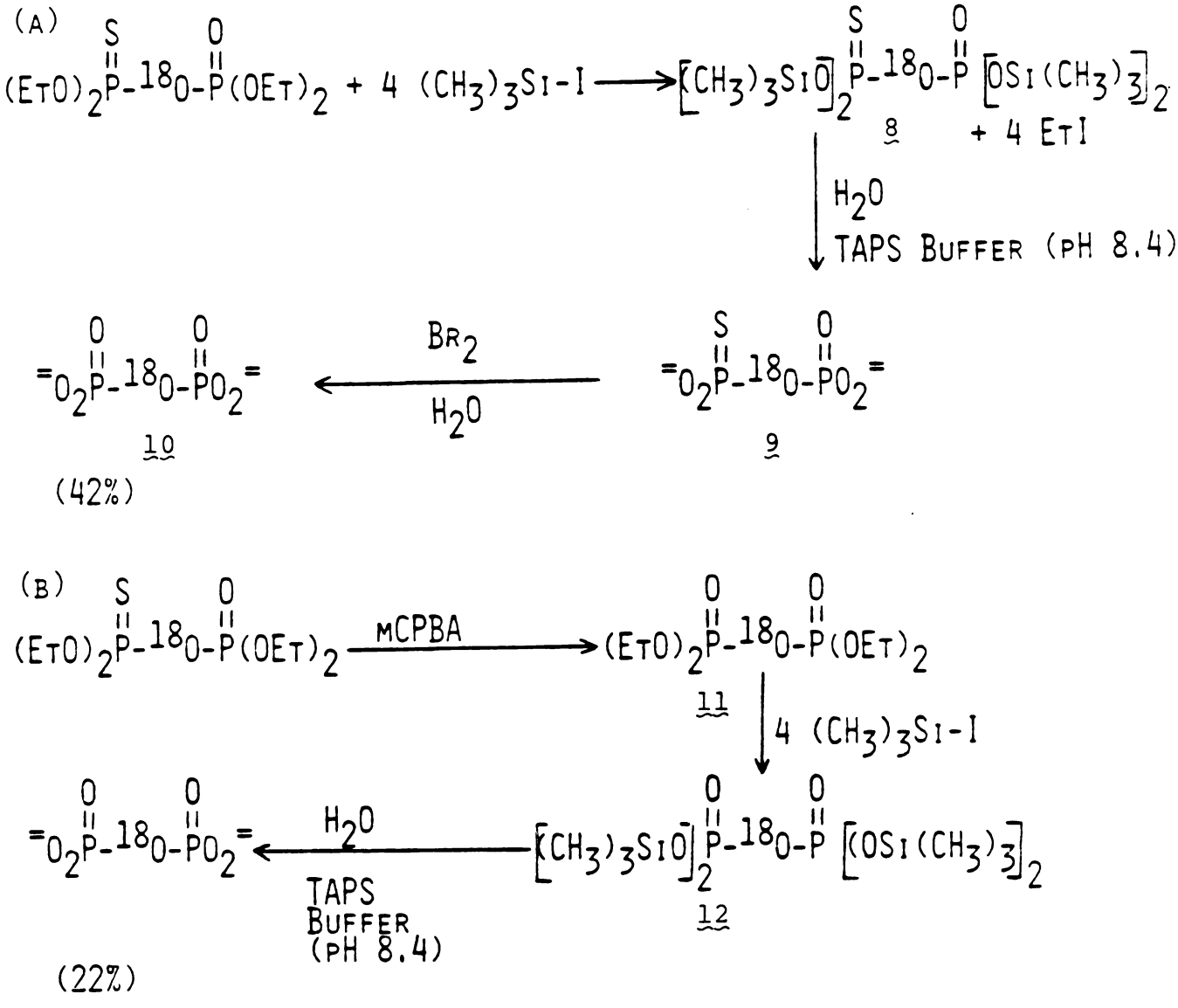


The key to this synthetic scheme is the reaction of the triethylammonium salt of labeled diethyl thiophosphate (6) with diethyl chlorophosphate. Since oxygen is a much better nucleophile toward phosphorus than sulfur, the reaction proceeds to give oxygen-bridge labeled tetraethyl thiopyrophosphate (7) as the sole product.

Dealkylation of ^{18}O-bridge labeled tetraethyl thiopyrophosphate (7) was achieved by two separate routes (Schemes IVa,b). In both methods, dealkylation was adapted from the procedure of Chojnowski et al. (1978).

The trimethylsilyl derivative of thiopyrophosphate (8) is readily hydrolyzed in TAPS buffer [tris(hydroxymethyl) methylaminopropane sulfonic acid] at pH 8.4 to give the labeled derivative of thiopyrophosphate (9). It is important to note that 9 is not very stable, even at this pH, and should not be kept longer than 5 min. or so at room temperature before the final reaction is undertaken.

SCHEME IV.

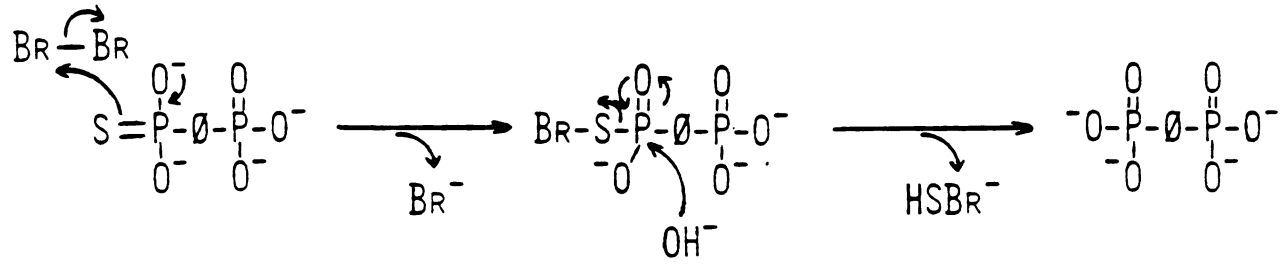


mCPBA = m-Chloroperoxybenzoic Acid

The use of bromine to replace sulfur with oxygen in thiopyrophosphate was adapted from the procedure that Lowe et al. (1982) used in the replacement of sulfur with ^{17}O in the synthesis of adenosine-5'-[(R)$_\alpha$-^{17}O]-triphosphate. A proposed mechanism for this reaction appears in Scheme V. TAPS buffer is used because the net reaction of thiopyrophosphate with Br_2 and a molecule of water results

in the formation of a molecule each of HBr and HSBr. (Thiopyrophosphate is even less stable at low pH.)

SCHEME V.



Other methods to replace sulfur with oxygen in phosphate compounds have been described. For example, Connally et al. (1982) have succeeded in replacing sulfur with ^{18}O by reacting various nucleoside phosphorothioates with N-bromosuccinimide in dioxane with $H_2{}^{18}O$. A similar reaction was reported by Sammons et al. (1982) using cyanogen bromide in $H_2{}^{18}O$. Both reactions have been shown to proceed with inversion of configuration at phosphorus.

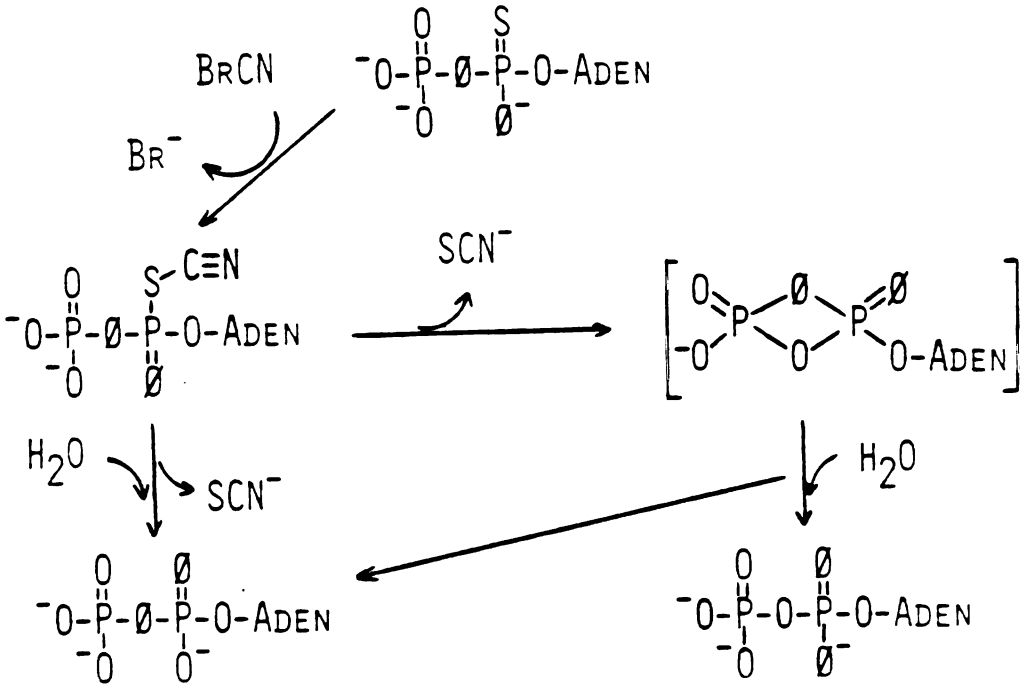
In addition, a cyclic intermediate has been implicated in the reaction of α_1-α,β_1-^{18}O-adenosine-5'-(1-thiophosphate) with H_2O in the presence of cyanogen bromide (Scheme VI).

In Scheme IVb, ^{18}O-bridge labeled tetraethyl thiopyrophosphate is converted to ^{18}O-bridge labeled tetraethyl pyrophosphate (11) using m-chloroperoxybenzoic acid in CH_2Cl_2. This reaction has already been described for the conversion of N-methyl diethylphosphorothioyl diethylphosphoramidate to N-methyl tetraethyl imidodiphosphate (see Part 1, Section IV). The yield of ^{18}O-labeled pyrophosphate obtained by this route (22%) was

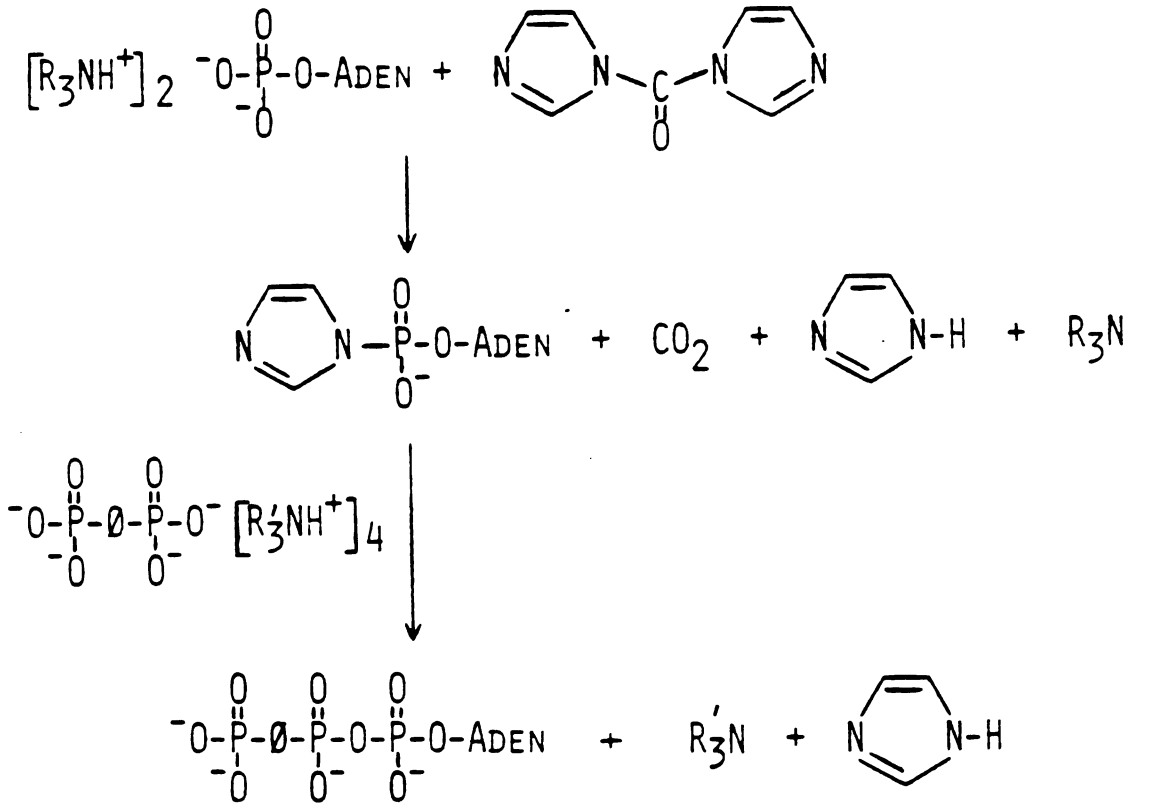
significantly less than that obtained according to Scheme IVa (42%).

The procedures used to prepare ^{17}O-bridge labeled tetraethyl thiopyrophosphate were identical to those just described for the ^{18}O-analog. Conversion of this intermediate to ^{17}O-bridge labeled pyrophosphate was performed as described in Scheme IVa.

SCHEME VI.



SCHEME VII.



R = n-Octyl

R'= n-Butyl

B. General Procedure for Preparing β,γ-Bridge Labeled ATP from Bridge Labeled Pyrophosphate.

β,γ-^{17}O- or ^{18}O-labeled ATP analogs were prepared from AMP and the corresponding labeled pyrophosphate samples according to the procedure of Hoard and Ott (1965) using 1,1'-carbonyldiimidazole (Scheme VII). The trioctylammonium salt of AMP was used due to difficulties in drying the tributylammonium salt: occasionally a white precipitate was obtained which could not be redissolved in dry dimethylformamide. This procedure was chosen over the Michelson procedure (see Part 1) because a linear triphosphate side product resulted from the latter procedure (as determined by ^{31}P NMR) which could not be separated from the desired ATP analog by anion exchange chromatography.

C. Results and Discussion.

A ^{31}P NMR spectrum taken at 97.3 MHz of a sample of ^{18}O-labeled pyrophosphate prepared using the reactions shown in Scheme IVa is given in Figure 1a. The presence of small amounts of unlabeled and ^{18}O-nonbridge pyrophosphate is indicated (13% and 11%, respectively) in addition to the desired ^{18}O-bridge material. The resonance corresponding to ^{18}O-bridge pyrophosphate appears 0.019 ppm upfield from that corresponding to unlabeled pyrophosphate, in reasonable agreement with the magnitude of an upfield chemical shift associated with an ^{18}O-bridging oxygen in ATP (Cohn and Hu, 1980, see Table 1).

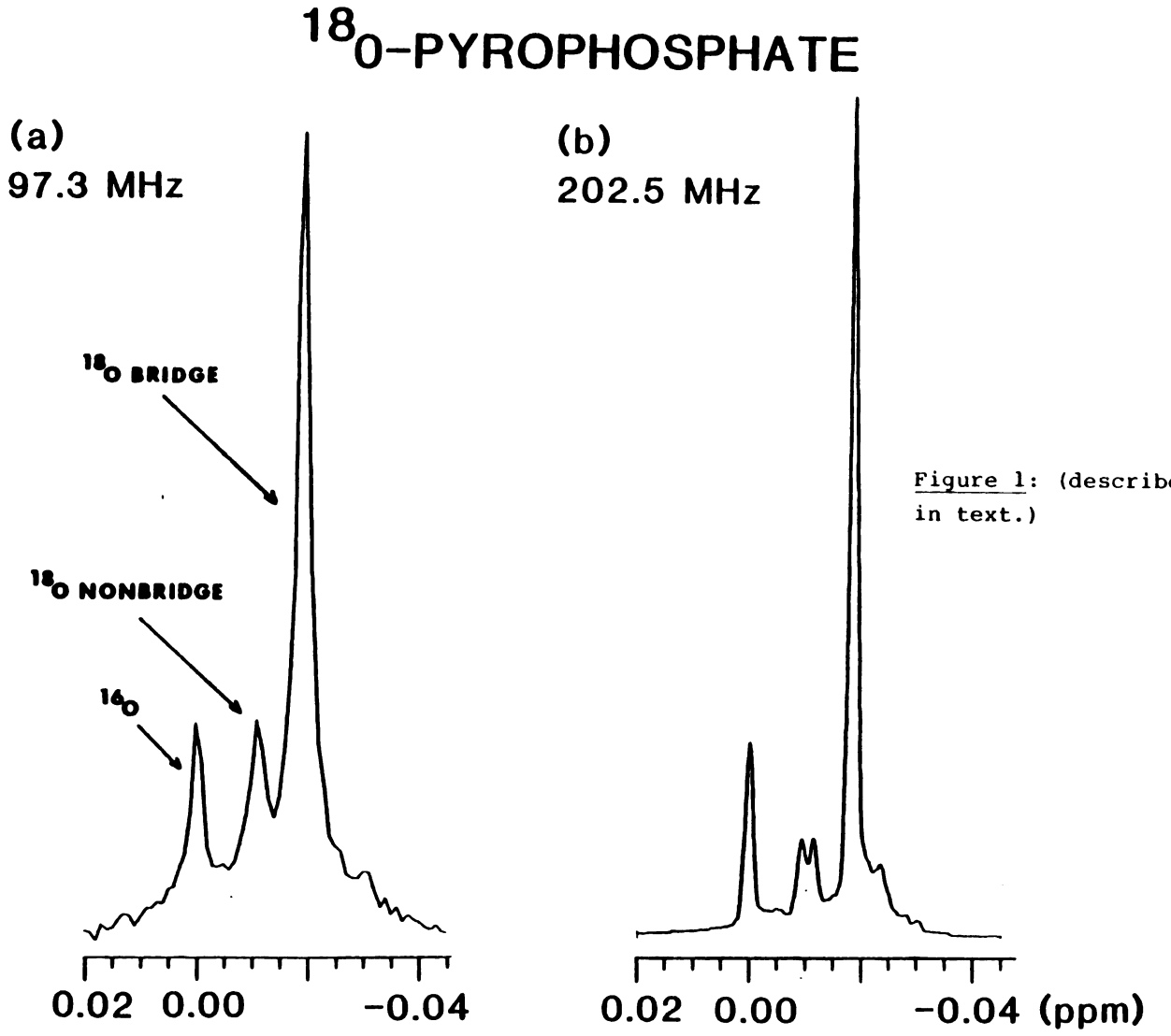


Figure 1: (described in text.)

At first glance it was unexpected that a peak corresponding to ^{18}O-nonbridge pyrophosphate does not appear at higher field from the peak for the ^{18}O-bridge species. What must be taken into consideration is that for ^{18}O-nonbridge pyrophosphate the phosphorus atoms are nonequivalent and are expected to be strongly coupled (approximately 20 Hz based on the coupling constants observed in ATP). Thus they will give rise to an AB splitting pattern. At 97.3 MHz, the peaks associated with this splitting pattern are collapsed into an apparent singlet at 0.011 ppm upfield from the resonance for unlabeled pyrophosphate, which is the calculated average of two isolated ^{16}O- and ^{18}O-nonbridge phosphoryl resonances separated by 0.022 ppm. As expected for such an AB splitting pattern, at 202.5 MHz the two innermost peaks of this splitting pattern were resolved, giving rise to an apparent doublet with a separation of about 0.5 Hz (Figure 1b).

Samples of ^{18}O-labeled pyrophosphate prepared by either Scheme IVa of Scheme IVb were shown to have the same relative amounts of unlabeled, ^{18}O-nonbridge, and ^{18}O-bridge species, provided that the same sample of ^{18}O-labeled tetraethyl thiopyrophosphate (TETPP) was used. Different preparations of ^{18}O-labeled TETPP yielded ^{18}O-pyrophosphate with percentages of unlabeled and ^{18}O-nonbridge species ranging from 13% and 11% to 22% and 20%, respectively. In each case, the relative amounts of unlabeled and ^{18}O-nonbridge species were similar in magnitude, suggesting that both of these minor amounts of undesired side products

may have originated by way of a common scrambling mechanism in the synthesis of ^{18}O-labeled TETPP. The same results were obtained when ^{18}O-labeled TETPP was prepared from purified triethylammonium diethyl-^{18}O-thiopyrophosphate and diethyl chlorophosphate, suggesting that unreacted sulfur is not responsible for the observed scrambling.

A ^{31}P NMR spectrum taken at 97.3 MHz of the resonances associated with the diethylphosphoryl group of a sample of ^{18}O-labeled TETPP is shown in Figure 2 along with a spectrum taken at 40.5 MHz of the entire molecule. In the spectrum taken at 97.3 MHz, a small doublet (peaks labeled "a") is observed 0.017 ppm upfield from the major doublet; we assume this smaller doublet to be associated with unlabeled TETPP. The relative amount of unlabeled TETPP in this sample, based on peak intensities, is about 18%. It is also likely that there is some ^{18}O-scrambled TETPP in this sample, although the peaks corresponding to this species were not resolved. Therefore, it seems reasonable to assume that the scrambling mechanism leading to unlabeled and ^{18}O-nonbridge pyrophosphate has occurred during or prior to the synthesis of ^{18}O-labeled TETPP. The exact nature of this scrambling mechanism must await further study.

When ^{18}O-nonbridge pyrophosphate is coupled with AMP to make ATP, the undesired ^{18}O-nonbridge atom is found at either the P_β- or P_γ-position with equal probability. Thus, the percentage of ^{18}O-nonbridge label found at the P_β- or P_γ-position of ATP is (fortunately) only half that found in the pyrophosphate starting material. Also, the ^{16}O-ATP which results serves as a convenient internal standard.

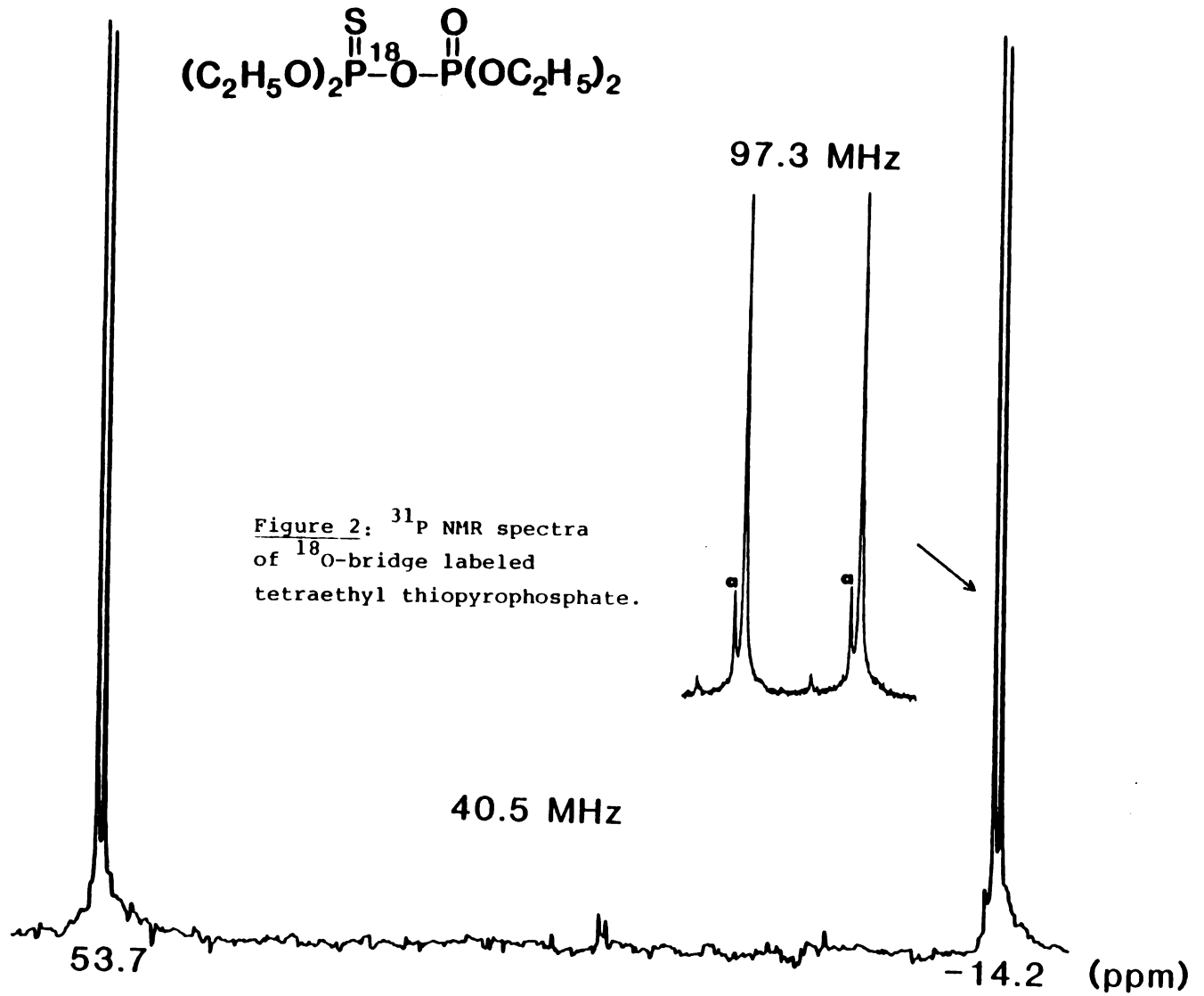


Figure 2: ^{31}P NMR spectra of ^{18}O-bridge labeled tetraethyl thiopyrophosphate.

The ^{18}O-labeled ATP synthesized was shown by high resolution ^{31}P NMR to consist of unlabeled, ^{18}O-nonbridge, and β,γ-^{18}O-bridge species at the β-phosphorus position of 18%, 6% and 76%, respectively (see Figure 3). The β-phosphorus resonances corresponding to ^{18}O-bridge and ^{18}O-nonbridge label appear 0.016 and 0.026 ppm upfield from those corresponding to unlabeled material, in agreement with published literature values (see Table 1). It is significant that the relative amount of ^{18}O-nonbridge label indicated is consistent with our prediction based on the peak assignment for ^{18}O-nonbridge pyrophosphate.

In the synthesis of ^{17}O-labeled pyrophosphate, the $H_2{}^{17}O$ used was not isotopically pure (it contained 33.6%^{16}O and 27.3%^{18}O), which led to concomitant formation of substantial amounts of ^{16}O- and ^{18}O-labeled material. Therefore, it was possible to detect the presence of nonbridge labeled pyrophosphate by high resolution ^{31}P NMR. The ratio of peak areas corresponding to ^{18}O-nonbridge pyrophosphate in the ^{31}P NMR spectrum taken at 97.3 MHz gave a value of 21% for the percentage of nonbridge label out of total label for either given oxygen isotope (^{17}O or ^{18}O). In the ^{17}O NMR spectrum of this sample at pH 10.5 (Figure 4) the broad ^{17}O-bridge resonance appears at 126.6 ppm and the narrower ^{17}O-nonbridge resonance appears at 108.5 ppm. These peak assignments were previously reported by Gerlt et al. (submitted for publication).

In summary, the ^{17}O- and ^{18}O-labeled ATP analogs prepared according to the synthetic schemes described in this section were shown by ^{31}P NMR to be highly enriched at

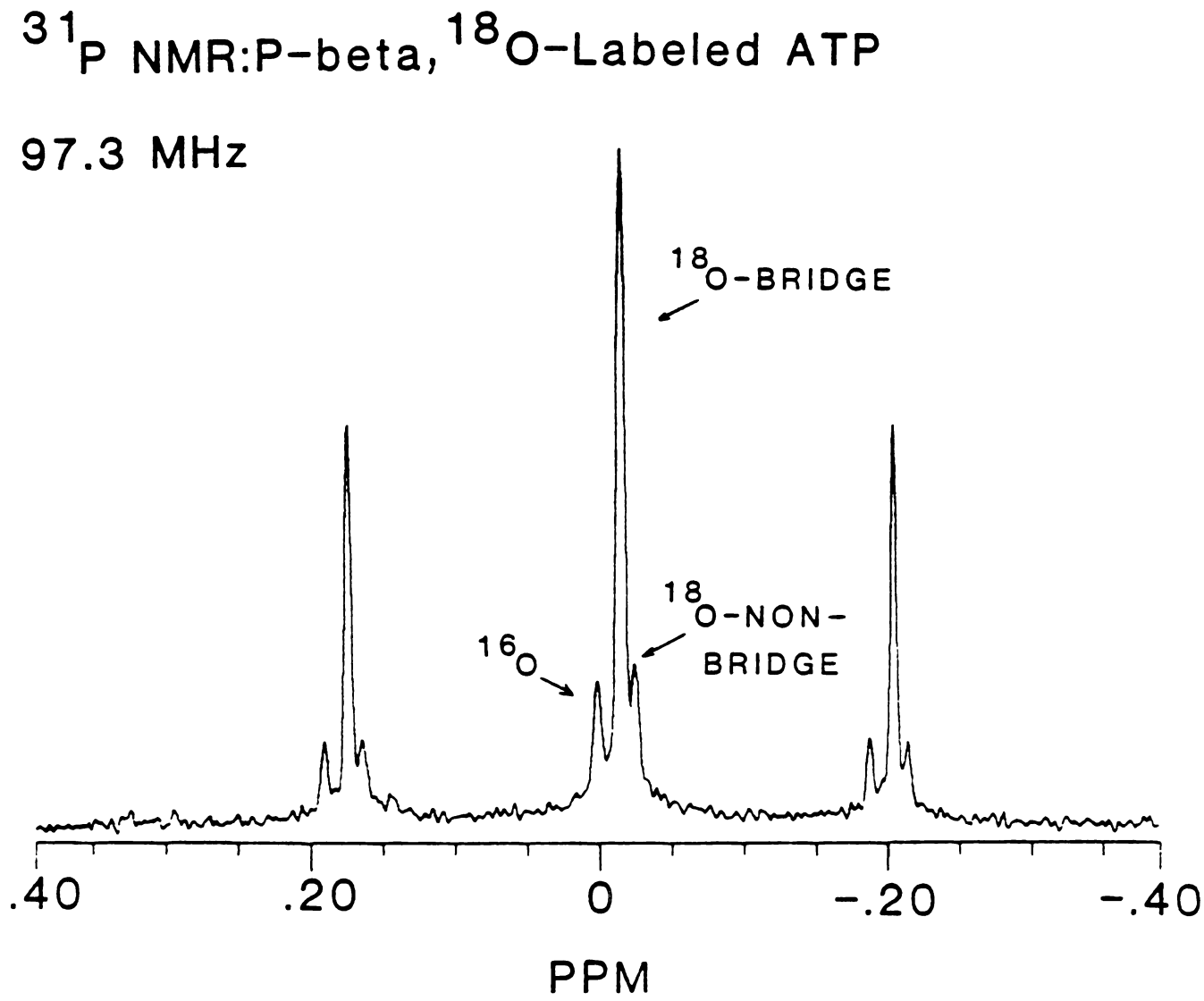


Figure 3: ^{31}P NMR spectrum of the β-phosphoryl resonances of ^{18}O-labeled ATP.

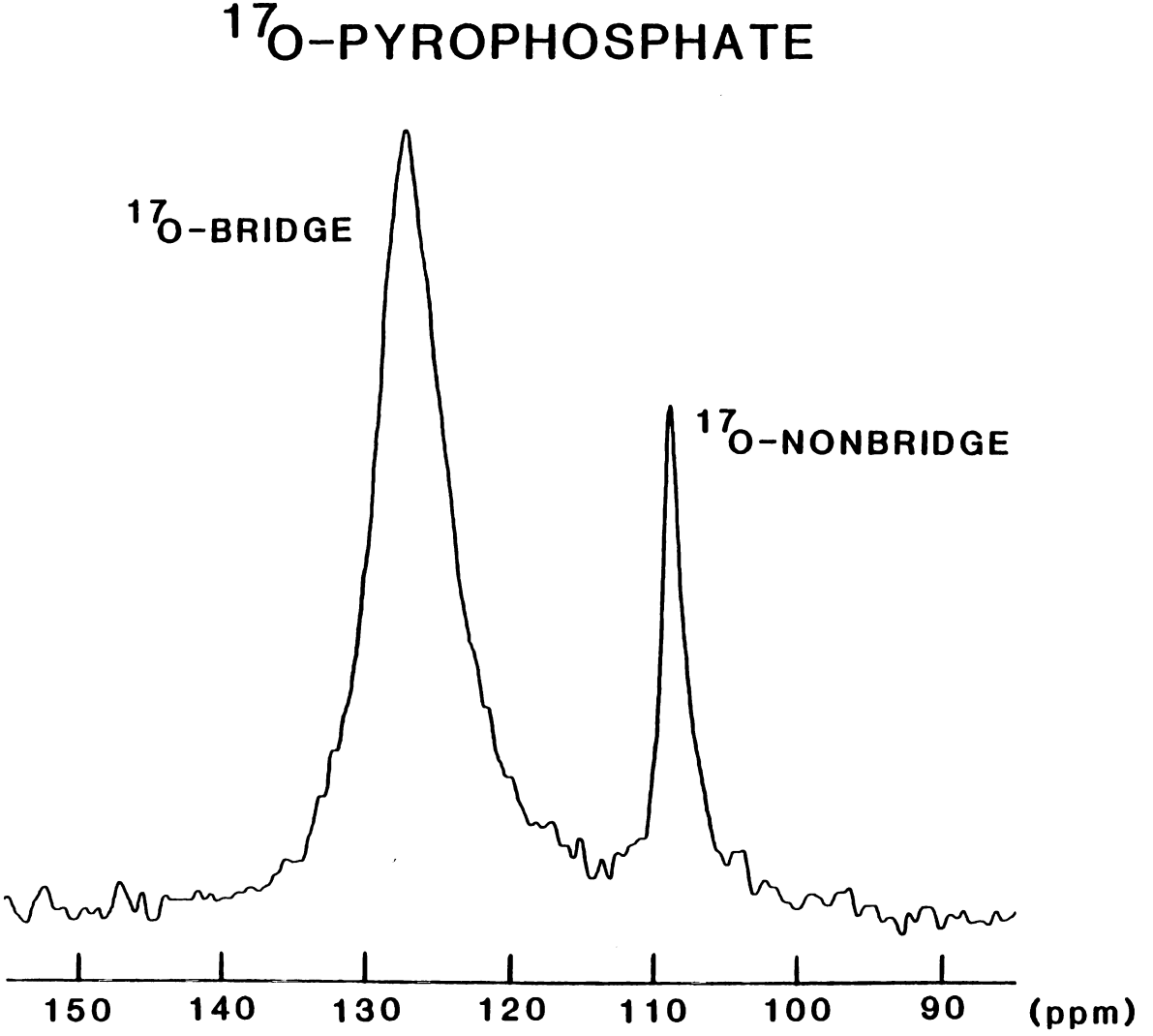


Figure 4: ^{17}O NMR spectrum of ^{17}O-labeled pyrophosphate at pH 10.5 (^{31}P-decoupled).

the β,γ-bridge position. These samples were used to measure positional isotope exchange in the bicarbonate-dependent ATPase reaction catalyzed by carbamoyl phosphate synthetase, which will be described in the next section.

IV. ^{31}P NMR Measurements of Positional Isotope Exchange in ^{17}O- or ^{18}O-Bridge Labeled ATP Catalyzed by Carbamoyl Phosphate Synthetase.

A. Historical Background.

Positional isotope exchange in ATP with carbamoyl phosphate synthetase from E. coli was first reported by Wimmer et al. (1979). The overall reaction catalyzed by this enzyme is as follows:

$$2\ MgATP + HCO_3^- + glutamine \longrightarrow$$
$$2\ MgADP + Pi + glutamate + carbamoyl\text{-}P$$

In addition to the overall reaction the enzyme also catalyzes a bicarbonate-dependent ATPase reaction. Since PIX was observed in this reaction, a carboxyphosphate intermediate was proposed. This scrambling process is illustrated in Scheme VIII with our β,γ-bridge labeled ATP analogs.

Wimmer et al. used mass spectral analysis with γ-$^{18}O_4$ -labeled ATP to measure this PIX reaction. As stated in the introduction, detection by this method involves an extensive series of enzymatic degradations of the ATP, followed by mass spectral analysis of the products (Midelfort and Rose, 1976).

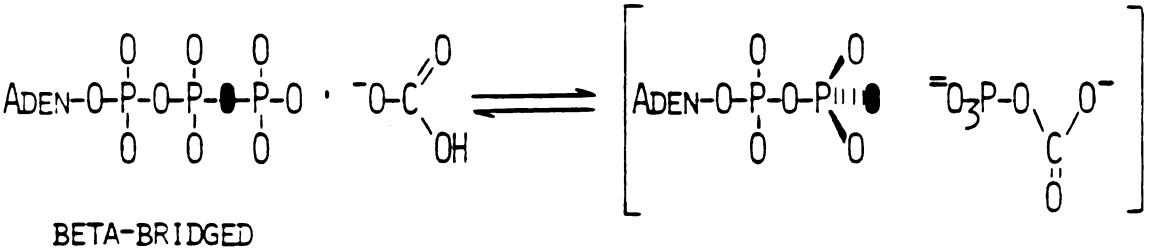
More recently, ^{31}P NMR analysis has been applied to measure PIX under the same conditions used by Wimmer et al. (Raushel and Villafranca, 1980). (This technique was referred to in the introduction as the $^{31}P(^{18}O)$ NMR method.)

SCHEME VIII. PIX IN CARBAMOYL PHOSPHATE SYNTHETASE WITH BETA, GAMMA-BRIDGE LABELED ATP.

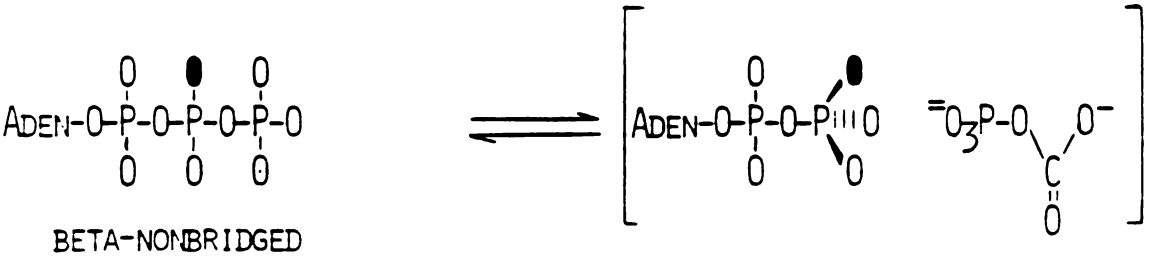
ATPASE REACTION:

$$MgATP \xrightarrow{HCO_3^-} MgADP + P_i$$

PROPOSED PIX WITH CARBOXY PHOSPHATE INTERMEDIATE:



BETA-BRIDGED



BETA-NONBRIDGED

The ATP analog used was γ-$^{18}O_4$-ATP, and the formation of γ-$^{18}O_3$-β-$^{18}O_1$(nonbridge)-ATP was followed by taking spectra of the P_γ-resonances at 81.01 MHz.

We have repeated the PIX experiments of Raushel and Villafranca using either ^{17}O- or ^{18}O-bridge labeled ATP, which were prepared as described in the preceding section. We now wish to report the measurement of PIX with these analogs using either the $^{31}P(^{18}O)$ or $^{31}P(^{17}O)$ NMR methods.

B. Results and Discussion.

Figure 5 shows the ^{31}P NMR spectra taken at 97.3 MHz of the P_β-resonances of ^{18}O-labeled ATP after incubation with bicarbonate in the presence and absence of enzyme. No PIX was observed in the sample in which enzyme was excluded. However, in the sample incubated with enzyme, a marked increase in the intensity of the triplet corresponding to ^{18}O-nonbridge ATP was observed, along with a decrease in the intensity of the triplet corresponding to ^{18}O-bridge ATP, indicating that positional isotope exchange (bridge to nonbridge) has occurred. The ratio of micromoles of ATP exchanged to micromoles of ADP produced was found to be 1.22 (Table 2i). Wimmer *et al.* (1979) have determined this ratio to be 1.4-1.7 at 37°C using mass spectral analysis, whereas Raushel and Villafranca have obtained a value of 0.42 under the same conditions using ^{31}P NMR analysis.

Positional Isotope exchange upon incubation of the ^{17}O-labeled ATP sample (containing also substantial ^{18}O label) in the presence of carbamoyl phosphate synthetase and

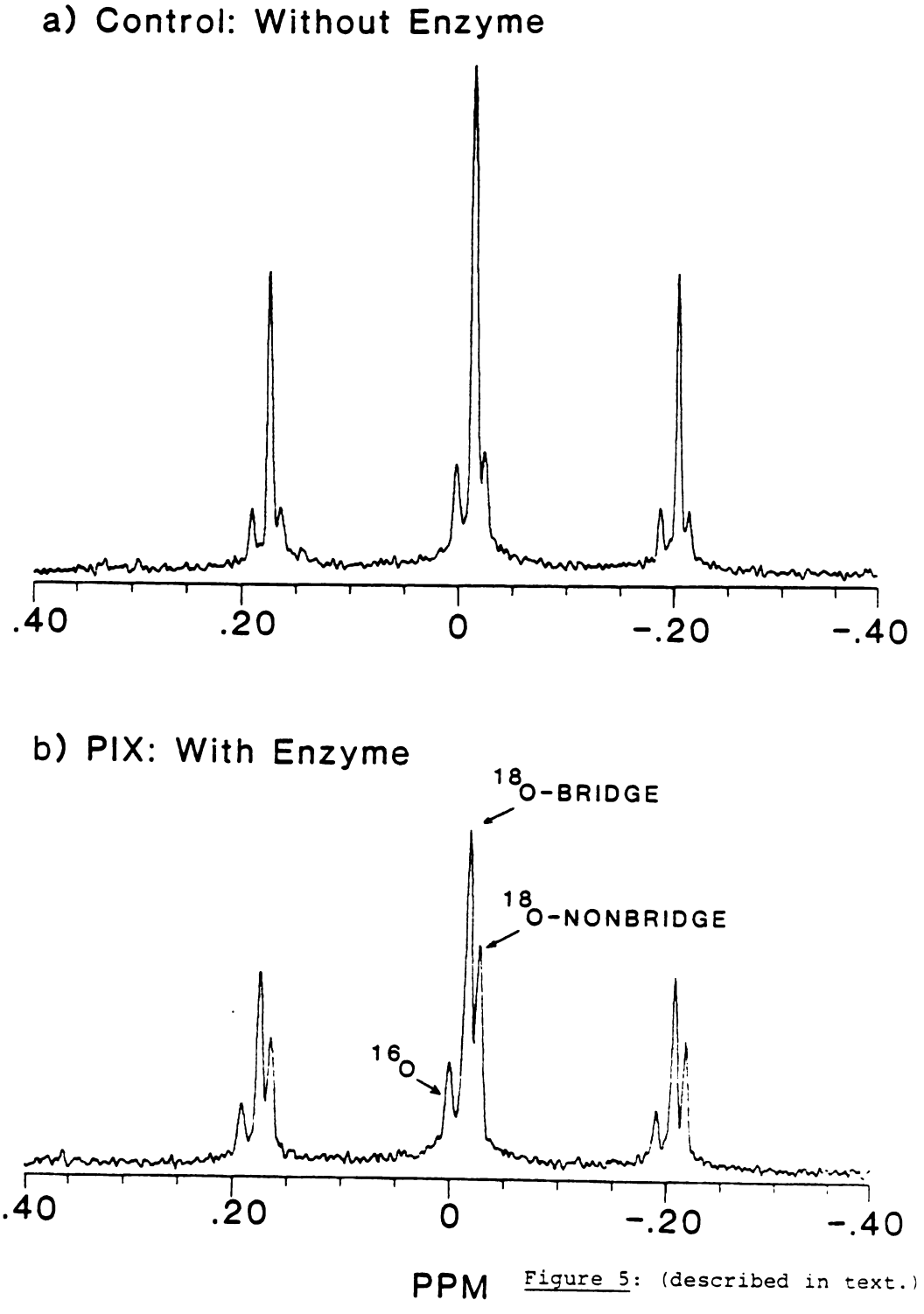


Figure 5: (described in text.)

bicarbonate was determined using both the $^{31}P(^{17}O)$ and $^{31}P(^{18}O)$ NMR methods. The ratio of micromoles of ATP exchanged t micromoles of ADP produced was determined by the $^{31}P(^{18}O)$ NMR method to be 1.28 (Table 2ii), in good agreement with the value of 1.22 obtained from the previous experiment using ^{18}O-labeled ATP. In the application of the $^{31}P(^{17}O)$ NMR method, the intensity of the P_γ-resonances of the ATP sample were monitored at 40.5 MHz during the course of the incubation. Although the individual unlabeled, ^{18}O-nonbridge and ^{18}O-bridge resonances were not resolved in the P_γ-doublet, the intensity of this doublet definitely increased with time as compared to the intensity of the P_α-doublet (Figure 6). The intensity of the P_β-triplet remained the same relative to that of the P_α-doublet, as expected, since the ^{17}O atom remains bound to the β-phosphorus after bridge to nonbridge positional isotope exchange has occurred. Assuming the increase in area under each peak of the P_γ-doublet was due entirely to ^{17}O positional isotope exchange from the β,γ-bridge to the β-nonbridge position, the ratio of micromoles of ATP exchanged to micromoles of ADP produced was determined to be 0.9 (Table 2iii). This value cannot be assumed to be so accurate as that obtained by the $^{31}P(^{18}O)$ NMR method, since accurate comparisons of peak areas are particularly dependent on the quality of the baseline resolution of each peak when two separate spectra are being considered. Nevertheless, both qualitatively and semi-quantitatively, the $^{31}P(^{17}O)$ NMR method shows that significant positional isotope exchange has occurred.

TABLE 2

Positional Isotope Exchange of β,γ- ^{18}O- and β,γ-^{17}O-Labeled ATP Catalyzed by Carbamoyl Phosphate Synthetase in the Presence of Bicarbonate.[a]

ATP Analog	Method	Fraction of Chemical Reaction[b]	Fraction of Exchange Reaction[c]	μmol of ATP exch./ μmol of ADP prod.[d]
(i) ^{18}O ATP	$^{31}P(^{18}O)$ NMR	0.48	0.55	1.22
(ii) ^{17}O ATP	$^{31}P(^{18}O)$ NMR	0.67	0.76	1.28
(iii) ^{17}O ATP	$^{31}P(^{17}O)$ NMR	0.67	0.63[e]	0.9[e]

a) Incubation conditions were described in the text.

b) The fraction of chemical reaction, X, is defined as $X = (ADP)_t/(ATP_{initial})$ where t = time at which the incubation was terminated.

c) In the $^{31}P(^{18}O)$ NMR method, the fraction of the exchange reaction, F, is defined as $F = (P_t - P_o)/(P_\infty - P_o)$ where P refers to the percentage of ^{18}O-nonbridge label at P_β at times t, zero, and at equilibrium. In the $^{31}P(^{17}O)$ NMR method, $F = (A_t - A_o)/(A_\infty - A_o)$, where A refers to the summed areas of the peaks in the P_γ doublet, normalized with respect to the P_α peaks at times t, zero, and at equilibrium (∞).

d) As derived by Litwin and Wimmer (1979), micromoles of ATP exchanged = $X/(\ln(1 - X))[ATP_{initial}] \ln(1 - F)$, where X = fraction of ATP lost F = fraction of equilibrium attained in the final ATP pool during positional isotope exchange.

e) Assuming a 2% error in our measurements of peak areas in the $^{31}P(^{17}O)$ NMR method, the calculated values for F would range between 0.79 and 0.47 and the calculated values for μmoles ATP exchanged/ μmoles ADP produced would range between 1.4 and 0.6.

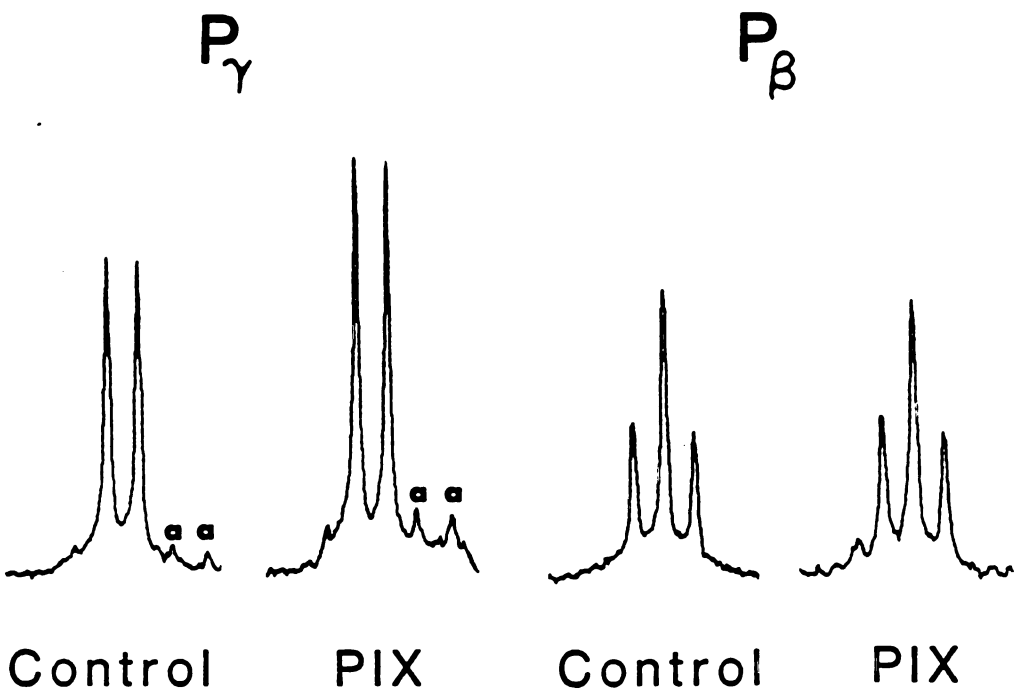


Figure 6: ^{31}P NMR spectra taken at 40.5 MHz of the β- and γ-resonances of a sample of ATP enriched with ^{17}O in the β,γ-bridge before and after positional isotope exchange catalyzed by carbamoyl phosphate synthetase in the presence of bicarbonate.

C. Conclusions.

The usefulness of the $^{31}P(^{17}O)$ NMR method in obtaining reliable quantitative data for PIX experiments will undoubtedly improve as higher isotopic enrichments of $H_2{}^{17}O$ become available. Currently, $H_2{}^{17}O$ is available with 60 atom% ^{17}O enrichment, although it is in short supply. The most attractive feature of the $^{31}P(^{17}O)$ NMR method at present, as applied to PIX experiments, is that it can be performed on virtually any NMR spectrometer which can be tuned to observe ^{31}P resonances. In practice, one can screen for the PIX phenomenon using the $^{31}P(^{17}O)$ NMR method and then obtain more quantitative data on exchange rates using the $^{31}P(^{18}O)$ NMR method with a higher field NMR instrument.

The ability to synthesize pyrophosphate isotopically enriched in the bridge position may play a significant role in the application of PIX experiments to enzymes which utilize pyrophosphate. We have demonstrated that bridge and nonbridge labeled pyrophosphate can easily be distinguished using either ^{31}P NMR or ^{17}O NMR.

V. Experimental Section.

A. General Methods and Procedures.

(Methods and procedures are described here which either have not previously been described or which differ from those described in Part 1.)

1. Positional Isotope Exchange in β,γ-^{17}O-ATP and β,γ-^{18}O-ATP.

Conditions were similar to those described by Raushel and Villafranca (1980). Carbamoyl phosphate synthetase (5 mg) was incubated with 67 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 133 mM KCl, 13% D_2O, 17 mM HCO_3^-, and either 12.5 mM β,γ-^{18}O-ATP (125 micromoles) or 17.5 mM β,γ-^{17}O-ATP (175 micromoles) at 30°C in a total volume of 10 mL. The bicarbonate-dependent ATPase reaction was followed by ^{31}P NMR at 40.5 MHz. After the reaction had proceeded to about 50% completion (3-4 hours), the solutions were cooled in ice, applied immediately to a DEAE Sephadex A-25 column, and the ATP samples were reisolated as their sodium salts according to the procedure described below. Controls without enzyme were incubated at 30°C for the same lengths of time, and the resulting ATP samples were recovered in the same manner.

2. Sample Preparation. The ^{18}O-bridged and ^{17}O-bridged pyrophosphate samples (55 micromoles) and the PIX and Control ^{18}O- and ^{17}O-ATP samples were percolated through

columns of Chelex-100 (Na^+ form) and lyophilized. These samples were then dissolved in 0.4 mL of 50% D_2O containing 0.5 mM EGTA. The 5 mm NMR tubes were made metal free by soaking overnight in 1:1 conc. HNO_3/conc. H_2SO_4 and then rinsing thoroughly with distilled deionized water.

3. NMR Measurements. ^{31}P NMR spectra of the tetraethyl esters of thiopyrophosphate and pyrophosphate were taken at 40.5 MHz in 12 mm NMR tubes using a Varian XL-100 spectrometer with $CDCl_3$ as the solvent. A spectral width of 4000 Hz and 8192 data points were used to acquire the free induction decay; a 90° pulse with a time delay between pulses of 5 s. was employed. All spectra were taken with broadband ^1H decoupling. Chemical shifts were measured relative to 85% H_3PO_4 as an external standard.

The course of the ATP ADP reaction in the PIX assays was monitored using ^{31}P NMR at 40.5 MHz on the varian XL-100 spectrometer in 12 mm tubes using a spectral width of 1000 Hz, 4096 data points, and one thousand data acquisitions. High resolution ^{31}P NMR spectra of the labeled pyrophosphate, tetraethyl thiopyrophosphate, and ATP samples were taken in 5 mm NMR tubes (metal-free) at 97.3 MHz using the UCSF Wide Bore 240 MHz spectrometer or at 202.5 MHz using a Bruker 500 MHz spectrometer located in the Southern California Regional NMR Facility at the California Institute of Technology. For these experiments 8192 data points were used with a spectral width of 400 Hz (at 97.3 MHz) or 1000 Hz (at 202.5 MHz) to acquire the free induction decays, which after processing gave digital resolutions of 0.05

Hz/data point and 0.03 Hz/data point, respectively. Typically, 128 data acquisitions were collected for the ATP samples, and 32 acquisitions were taken for the pyrophosphate samples.

^{17}O NMR spectra at 36.6 MHz were recorded on a Bruker WH-270 spectrometer at Yale University as previously described (Gerlt et al., submitted for publication). Chemical shifts were measured relative to ^{17}O water.

4. Materials. $H_2{}^{18}O$ (98 atom%) and $H_2{}^{17}O$ (containing 33.6% ^{16}O, 39.1%^{17}O, and 27.3%^{18}O) were purchased from Cambridge Isotopes. DEAE Sephadex A-25 was purchased from Pharmacia. Iodotrimethylsilane was from Aldrich, and adenosine-5'-monophosphoric acid was from Sigma. Finely powdered elemental sulfur was stored in a vacuum desiccator over sodium hydroxide pellets prior to use. Diethyl chlorophosphite, diethyl chlorophosphate, and triethylamine were distilled under nitrogen prior to use. Pyridine, dimethylformamide, and dichloromethane were heated at reflux over calcium hydride and freshly distilled. Diethyl ether was heated at reflux over sodium filings in a nitrogen atmosphere, distilled and used immediately. Carbamoyl phosphate synthetase from E. coli was purified as described earlier (Raushel and Villafranca, 1980) and was a generous gift from Dr. Joseph J. Villafranca. Other reagents were of the finest quality available. All reactions were performed under nitrogen unless stated otherwise.

B. Chemical Synthesis.

1. ^{17}O- and ^{18}O-Diethyl Phosphite.

These two compounds were prepared according to the procedure previously described for ^{17}O-diethyl phosphite (see Experimental Section, Part 1).

2. ^{18}O-Bridged Tetraethyl Thiopyrophosphate was prepared using a modification of the procedure of Arbuzov (1954) (Scheme II). Powdered sulfur (1.31 g, 40.7 mmol) was added slowly to a stirred mixture of ^{18}O-diethyl phosphite (5.70 g, 40.7 mmol) and triethylamine (4.12 g, 40.7 mmol). After addition was complete, the mixture was heated at 45°C until all of the sulfur had dissolved (ca. 30 min.). The pale yellow syrup was dissolved in dry diethyl ether (100 mL). Next, diethyl chlorophosphate (6.94 g, 40.7 mmol) was added dropwise with stirring. Stirring was continued overnight, and the reaction was completed by heating at reflux for 2 hours. The cooled solution was filtered to remove triethylammonium chloride, washed with water (50 mL), and dried over anhydrous $MgSO_4$. The solvent was removed, and the resulting pale yellow oil was degassed under high vacuum, yielding 11.6 g. The ^{31}P NMR spectrum ($CDCl_3$, proton decoupled) showed the crude product to be greater than 95% pure (88% yield): δ = 53.7 (d, diethylphosphorothioyl group) and 14.3 (d, diethylphosphoryl group), J_{PP}= 21.6 Hz, in agreement with literature values (Harris et al., 1967).

3. ^{18}O-Bridged Pyrophosphate, Tetrasodium Salt, was prepared

from ^{18}O-bridged tetraethyl thiopyrophosphate by two methods (Scheme IVa,b). In both methods, dealkylation was adapted from the procedure of Chojnowski et al. (1978). The use of m-chloroperoxybenzoic acid in oxidation of organophosphorus compounds was described in Part 1 of this dissertation. The use of bromine to replace sulfur with oxygen in thiopyrophosphate was adapted from the procedure that Lowe et al. (1982) used in the replacement of sulfur with ^{17}O in the synthesis of adenosine-5'-[(R)$_\alpha$-^{17}O]-triphosphate.

Method A: Iodotrimethylsilane (3.52 g, 17.6 mmol) was added dropwise to a stirred solution of ^{18}O-bridged tetraethyl thiopyrophosphate (1.23 g, 4 mmol) in dry dichloromethane (3 mL) at -40°C (dry ice/acetone bath). After one hour the reaction mixture was left at room temperature for two days, followed by two hours heating at relfux. Next, the mixture was added directly to 50 mL of 1 M TAPS buffer [tris (hydroxymethyl) methylaminopropane sulfonic acid], pH 8.4. Bromine was added dropwise to the stirring mixture until a brick-red color persisted. The pH was maintained between 7-8 by dropwise addition of conc. NaOH solution. After 5 min. remaining bromine was eliminated by addition of $NaHSO_3$. The resulting aqueous solution was extracted with CH_2Cl_2 and then diluted with water to a final volume of 700 mL. This solution was applied to a DEAE Sephadex A-25 (HCO_3^-) column (2.5 cm i.d. X 40 cm long). The column was eluted with a 3 L linear gradient of 0.05 - 0.6 M triethylammonium bicarbonate buffer (pH 7.8) at 2-4°C. Fractions were assayed for acid-labile phosphate according to the procedure of Ames (1966). Pyrophosphate is eluted from the column by

0.4 M triethylammonium bicarbonate under these conditions (see Figure 7). The pooled fractions were concentrated to a syrup under vacuum using a bath temperature below 25°C. Residual water and triethylammonium bicarbonate were removed by evaporation of several 25 mL aliquots of absolute CH_3OH. The sodium salt of ^{18}O-bridged pyrophosphate was precipitated by addition of 10 - 12 equivalents of 1 M NaI in acetone and recovered by centrifugation. After washing the precipitate twice with cold acetone, it was dissolved in about 20 mL cold water. The resulting solution was adjusted to pH 11.5 with concentrated NaOH, and the product was precipitated by addition of ethanol to give white needles (0.7634 g, 42.6% yield). ^{31}P NMR analysis of this material gave a single peak identical with that for authentic pyrophosphate at two pH values (6 and 10).

Method B: m-Chloroperoxybenzoic acid (2.59 g, 12 mmol based on 80% assay) in dry CH_2Cl_2 (35 mL) was added slowly dropwise to a stirred solution of ^{18}O-bridged tetraethyl thiopyrophosphate (1.23 g, 4 mmol) in dry CH_2Cl_2 (10 mL) at 0°C. The reaction was continued at 0°C for 90 minutes. Precipitated m-chlorobenzoic acid was removed by filtration. The resulting solution was washed with 20 mL of 20% $NaHSO_3$, three 10 mL aliquots of 10% $NaHCO_3$, and 10 mL of saturated NaCl solution, in that order. The organic layer was then dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure to give an oil (0.86 g). ^{31}P NMR ($CDCl_3$, proton decoupled) showed this material to contain approximately 75% tetraethyl pyrophosphate: δ = 13.5 ppm, in agreement with the literature value (Belskii et al., 1972).

This oil was dissolved in dry CH_2Cl_2 (2 mL) and cooled to -40°C with a dry ice/acetone bath. Iodotrimethylsilane (2.80 g, 14.0 mmol) was added dropwise with stirring. Following complete addition, the reaction was continued at -20°C for 2 hours. After warming to room temperature, the solution was added to 30 mL of 1 M TAPS buffer (pH 8.4). The pH was readjusted to 7.8 after 15 minutes, and the aqueous layer was extracted with CH_2Cl_2 (20 mL) and then diluted to a final volume of 500 mL with water. This solution was applied to the DEAE Sephadex A-25 column. The product, ^{18}O-bridged tetrasodium pyrophosphate, was eluted, recovered and recrystallized as described in Method A, giving 0.3906 g (22% yield).

4. β,γ-^{18}O-ATP was prepared according to the procedure of Hoard and Ott (1965) from AMP using 3 equiv. of 1,1'-carbonyldiimidazole and 2 equiv. of ^{18}O-labeled pyrophosphate. Of the pyrophosphate used, 1.01 equiv. precipitated as the imidazolium salt, was recovered from the reaction mixture by centrifugation, and was reconverted to the sodium salt by passage through a column of DOWEX 50 WX-8 (Na^+ form). The product was purified on the DEAE Sephadex A-25 column described above using a linear gradient (1.5 L + 1.5 L, 0.1 - 0.7 M) of triethylammonium bicarbonate buffer (pH 7.8). Fractions containing ATP were pooled (see Figure 7), and the solvent and buffer were removed as described above. Precipitation of the sodium salt of β,γ-^{18}O-ATP was achieved as described for ^{18}O-bridged pyrophosphate, and washed three times with cold acetone and dried in a vacuum dessicator. The yield was 38% based on unrecovered

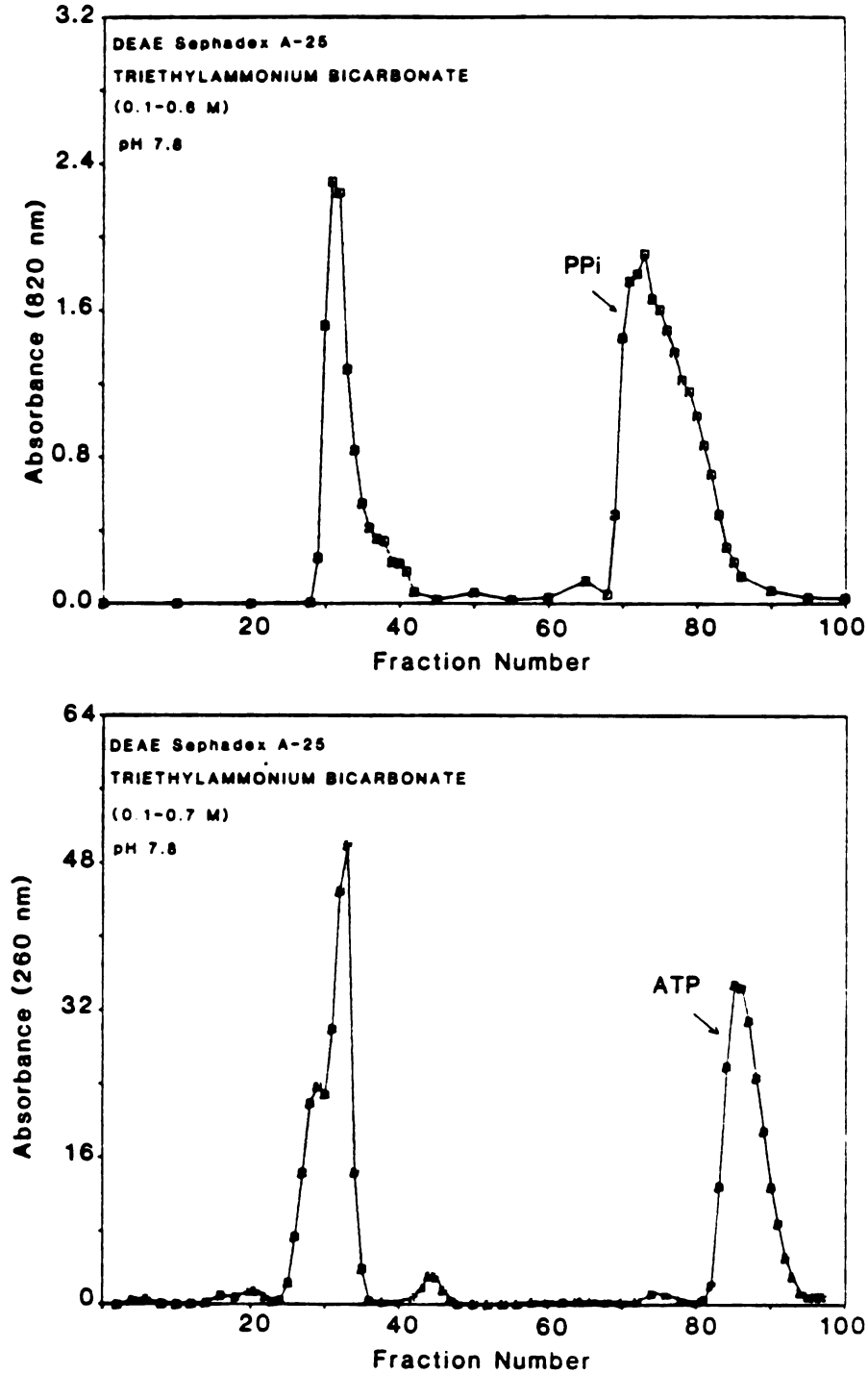


Figure 7: Representative anion-exchange elution profiles for pyrophosphate and ATP.

^{18}O-labeled pyrophosphate. This sample had an identical Rf value (0.26) when compared to authentic unlabeled ATP using polyethylenimine cellulose thin-layer chromatography with 1.2 N LiCl as eluent (Rowley and Kenyon, 1974).

5. ^{17}O-Diethylphosphite and ^{17}O-Bridged Tetraethyl Thiopyrophosphate were prepared from $H_2{}^{17}O$ using the same procedures described above for the ^{18}O analogs.

6. ^{17}O-Bridge Labeled Pyrophosphate, Tetrasodium Salt, was prepared as described for ^{18}O-bridge labeled pyrophosphate (Method A).

7. β,γ-^{17}O-ATP was prepared as described for β,γ-^{18}O-ATP.

C. Summary.

A method has been devised for synthesizing ATP highly specifically enriched with either ^{17}O or ^{18}O in the β,γ -bridge position in good yields. Based on the yields reported above, over 6 mmols of ATP (about 3.3 g, as the disodium salt) can be synthesized from a single gram of ^{17}O- or ^{18}O-enriched water, enabling numerous positional isotope exchange studies to be carried out.

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Note: For convenience, several references listed in Part 1 are repeated here.

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