# $^{17}O$ NMR Spectral Properties of Pyrophosphate, Simple Phosphonates, and Thiophosphate and Phosphonate Analogues of ATP

**John A. Gerlt,*[1,2a] Mark A. Reynolds,[2b] Peter C. Demou,[2a] and George L. Kenyon[2b]**

*Contribution from the Department of Chemistry, Yale University, New Haven, Connecticut 06511, and the Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143.* *Received October 4, 1982*

**Abstract:** The chemical shifts of the $^{17}O$ resonances associated with the bridging and nonbridging oxygens of pyrophosphate have been unambiguously assigned; this information allows the assignment of the resonances of all of the phosphoryl oxygens of ADP and ATP. The chemical shifts of the phosphoryl oxygens in pyrophosphate and several nucleotide analogues with predictable proton ionization behavior, i.e., AMPS, ATP$\gamma$S, methylphosphonate, methylenediphosphonate, and the $\beta,\gamma$-methylene analogue of ATP, have been measured as a function of pH; in each case an upfield change in chemical shift is observed upon protonation, with the magnitude of the shift change being approximately 50 ppm. These observations and those previously described (Gerlt, J. A.; Demou, P. C.; Mehdi, S. *J. Am. Chem. Soc.* **1982**, *104*, 2848) demonstrate that $^{17}O$ NMR spectroscopy can be used to quantitate the site and degree of charge neutralization in phosphate ester anions and related species.

Prior to 1980, the $^{17}O$ NMR spectral properties of the phosphoryl oxygens of inorganic and organic phosphates received little attention. However, in the past 3 years three research groups have published reports in this area.[3-7] This recent interest in the $^{17}O$ NMR properties of phosphates is largely the result of the recognition that $^{31}P$ NMR spectroscopy is unable to provide either qualitative or quantitative information about the sites and degree of charge neutralization of phosphate ester anions.[8] In biochemical systems, knowledge of the sites of coordination of nucleotides to protons, metal ions, and the cationic residues in the active sites of enzymes is essential to a complete understanding of many enyzmic reactions. Since coordination to the phosphate anions in nucleotides necessarily occurs by direct interaction with the phosphoryl oxygens, $^{17}O$ NMR spectroscopy should prove to be a useful spectroscopic technique for solving these types of problems. Although the $^{17}O$ NMR spectral properties of oxygen bonded to carbon and various metal nuclei have received considerable attention,[9,10] the advantages and limitations of $^{17}O$ NMR spectroscopy in the study of phosphates are yet to be fully defined.

With one exception, the recent $^{17}O$ NMR studies of phosphates have utilized regio- or stereospecifically enriched molecules, since these allow both convenient detection of resonances at concentrations useful in biochemical studies and also the ability to examine the properties of specific oxygens in nucleotides with more than one phosphoryl group, e.g., ATP. The natural abundance $^{17}O$ NMR study of several inorganic and organic phosphates was reported by Gerothanassis and Sheppard,[6] and the results these investigators reported are in excellent agreement with those obtained in Tsai's laboratory[3,5] and one of our own.[4,7]

In view of the limited information regarding the $^{17}O$ NMR properties of phosphates and phosphate esters and the incomplete understanding of the theory of $^{17}O$ NMR spectral parameters, one of our laboratories initiated a systematic investigation of the spectral properties of phosphate esters so that we could determine whether any useful empirical relationships exist between charge neutralization and spectral properties such as chemical shift. We recently reported the results of studies on the protonation of simple phosphate esters and the common adenine nucleotides.[7] Our data were uniformly consistent with the hypothesis that the magnitude of the charge located on a phosphoryl oxygen is a very important factor in determining the chemical shift of the associated $^{17}O$ NMR resonance: protonation of basic phosphoryl oxygens was observed to produce upfield chemical shifts of approximately 50 ppm per charge neutralized. Thus, even though the linewidths of the $^{17}O$ NMR resonances of phosphoryl oxygens can be relatively large (at least when compared to those of dipolar nuclei), charge neutralization can be easily detected and quantitated. This discovery that the chemical shift change induced by protonation is directly proportional to the magnitude of the charge neutralized suggests that $^{17}O$ NMR chemical shift information will be useful in solving otherwise difficult problems regarding charge neutralization of phosphate esters, e.g., proton binding to compounds with uncertain tautomeric structure and the $^{17}O$ NMR chemical shift changes that occur when divalent metal ions are added to samples of ADP and ATP.[5]

In this article we report additional $^{17}O$ NMR studies of biochemically important phosphates and phosphate analogues that are essential to further applications of this spectroscopic technique. We have determined the $^{17}O$ NMR spectral properties of samples of pyrophosphate with and without the bridging oxygen labeled. This information allows the resonances of oxygens in both environments to be assigned, thereby completing the assignments of all of the phosphoryl oxygens in ADP and ATP. More importantly, we have studied the $^{17}O$ NMR spectral properties of additional phosphorothioates and several phosphonates; the choice of compounds for study was based on two factors: (1) stable and predictable chemical structure and (2) the existence of anomalous $^{31}P$ NMR chemical shift behavior upon protonation. In all cases the $^{17}O$ NMR results confirmed our previous observation that protonation of basic phosphoryl oxygens induces upfield changes in chemical shift of approximately 50 ppm per charge neutralized.[7]

## Materials and Methods

$H_2{}^{17}O$ (13% $^{16}O$, 52% $^{17}O$, and 35% $^{18}O$) was purchased from Monsanto. Methylenediphosphonic acid (PCP), methylphosphonic acid, and thiophosphoryl chloride were obtained from Alfa. Unlabeled AMP and ADP and all enzymes were products of Sigma. All other chemicals used were the best grade commericially available and were used without further purification.

**Nonbridging [$^{17}O_i$]Pyrophosphate.** [$\alpha$-$^{17}O_i$]ADP (containing $^{17}O$ only in the $\alpha$-nonbridging oxygens) was prepared as previously described.[7] Oxidation of this material with periodate and subsequent base-catalyzed

(1) NIH Research Career Development Awardee (CA-00499), 1978-1983; Alfred P. Sloan Fellow, 1981-1983.
(2) (a) Yale University. (b) University of California.
(3) Tsai, M. D.; Huang, S. L.; Kozlowski, J. R.; Chang, C. C. *Biochemistry* **1980**, *19*, 3531.
(4) Coderre, J. A.; Mehdi, S.; Demou, P. C.; Weber, R.; Traficante, D. D.; Gerlt, J. A. *J. Am. Chem. Soc.* **1981**, *103*, 1870.
(5) Huang, S. L.; Tsai, M. D. *Biochemistry* **1982**, *21*, 951.
(6) Gerothanassis, I. P.; Sheppard, N. *J. Magn. Reson.* **1982**, *46*, 423.
(7) Gerlt, J. A.; Demou, P. C.; Mehdi, S. *J. Am. Chem. Soc.* **1982**, *104*, 2848.
(8) Jaffe, E. K.; Cohn, M. *Biochemistry* **1978**, *17*, 652.
(9) Klemperer, W. G. *Angew. Chem.* **1978**, *17*, 246.
(10) Harris, R. K. In "NMR and the Periodic Table"; Harris, R. K., Mann, B. E., Eds.; Academic Press: New York, 1978; Chapter 12.
(11) Richard, J. P.; Frey, P. A. *J. Am. Chem. Soc.* **1982**, *104*, 3476.

0002-7863/83/1505-6469$01.50/0 

elimination of labeled pyrophosphate were performed in 20 mM sodium carbonate, pH 10.5.[12] The nucleotide was treated with excess sodium periodate for 30 min at room temperature, and following addition of ethylene glycol the solution was heated at 50 °C for 30 min. The pyrophosphate was isolated by chromatography on DEAE-Sephadex A-25 ($HCO_3^-$) by elution with a linear gradient of triethylammonium bicarbonate, pH 7.5. By virtue of the synthetic procedures used, this sample is predicted to have an $^{17}O$ enrichment of 32% in one of the nonbridging oxygens, with no label expected in the bridging position.

**Mixture of Bridging and Nonbridging [$^{17}O_1$]Pyrophosphate.** [$\beta$-$^{17}O_1$]ADP (containing $^{17}O$ either in the $\alpha,\beta$-bridging oxygen or in the $\beta$-nonbridging oxygen) was prepared as previously described.[7] Oxidation, elimination, and isolation of the labeled pyrophosphate were carried out as described in the previous paragraph. By virtue of the synthetic procedures used, this sample is predicted to have an $^{17}O$ enrichment of 12% in the bridging position and 37% in the nonbridging positions; each molecule that is labeled contains only a single atom of $^{17}O$.

**Adenosine [$^{17}O$]Phosphorothioate ([$^{17}O$]AMPS).** The procedure described by Richard and Frey[11] for the preparation of [$^{18}O_2$]AMPS was followed, except that $H_2{}^{17}O$ was used in place of $H_2{}^{18}O$. The labeled product was isolated by chromatography on DEAE-Sephadex A-25. The isotopic composition of this sample is predicted to be 27% $^{17}O_2$ and 50% $^{17}O_1$.

**[$\alpha$-$^{17}O_1$]ATP$\gamma$S.** [$\alpha$-$^{17}O_1$]ADP was enzymatically thiophosphorylated by using the coupled enzyme system described by Trentham and Webb[12] and initial concentrations of labeled ADP and unlabeled thiophosphate of 10 and 20 mM, respectively. The progress of the reaction was monitored by HPLC with an Alltech $C_{18}$ reverse phase column and an ion-pairing eluent composed of 10% acetonitrile and 90% 100 mM sodium phosphate, pH 6.5. When the concentration of ATP$\gamma$S was maximal (about 2 h after initiation of the reaction), the ATP$\gamma$S was isolated by chromatography on DEAE-Sephadex A-25. This sample is predicted to have an $^{17}O$ enrichment of 32% in one $\alpha$-nonbridging oxygen.

**[$\beta$-$^{17}O_1$]ATP$\gamma$S.** [$\beta$-$^{17}O_1$]ADP was enzymatically thiophosphorylated as described in the previous paragraph. This sample is predicted to have an $^{17}O$ enrichment of 25% in one $\beta$-nonbridging oxygen and 12% in both the $\alpha,\beta$- and $\beta,\gamma$-bridging positions; each molecule that is labeled contains only a single atom of $^{17}O$.

**[$\gamma$-$^{17}O$]ATP$\gamma$S.** Unlabeled ADP was thiophosphorylated with labeled inorganic thiophosphate[7] by using the coupled enzyme system of Trentham and Webb[12] and initial concentrations of ADP and labeled inorganic thiophosphate (2% $^{17}O_3$, 15% $^{17}O_2$, and 43% $^{17}O_1$) of 30 and 10 mM, respectively. Approximately 45% of the product molecules should contain $^{17}O$ in the $\gamma$-nonbridging position, with 7% being $^{17}O_2$ and 38% being $^{17}O_1$.

**Methyl[$^{17}O$]phosphonic Acid.** A sample of this material, which was prepared by the hydrolysis of methylphosphonyldichloridate in $H_2{}^{17}O$, was the generous gift of Professor Barry S. Cooperman, University of Pennsylvania.

**[$^{17}O$]PCP.** Methylenediphosphonic acid was heated in $H_2{}^{17}O$ in a sealed tube for 3 days at 120 °C. The water was recovered by bulb-to-bulb distillation, yielding solid PCP, which was approximately 15% enriched with $^{17}O$ in each of the phosphonyl oxygens (see below for estimate of isotopic composition).

**[$\alpha$-$^{17}O_1$]AMP-PCP.** [$^{17}O_1$]AMP[7] was condensed with 5 equiv of unlabeled PCP according to the procedure of Hoard and Ott.[13] The product was purified by chromatography on DEAE-Sephadex A-25. This sample is predicted to an $^{17}O$ enrichment of 32% in one of the $\alpha$-nonbridging oxygens.

**[$\beta,\gamma$-$^{17}O$]AMP-PCP.** Unlabeled AMP was condensed with 5 equiv of [$^{17}O$]PCP according to the procedure of Hoard and Ott.[13] The product was purified by chromatography on DEAE-Sephadex A-25. Integration of the $^{31}P$ NMR resonances of this material relative to an internal standard of cyclic AMP revealed that the $^{17}O$ enrichment was approximately 15% in the $\alpha,\beta$-bridging position, 25% in the $\beta$-nonbridging position, and 40% in the $\gamma$ positions. On the basis of this spectrum, the $^{17}O$ enrichment in the [$^{17}O$]PCP was assigned.

**Sample Preparation.** The labeled samples were percolated through columns of Chelex-100 (tetraethylammonium) and lyophilized. Except as noted, 80 $\mu$mol of each sample was dissolved in 2 mL of 20% $D_2O$, and EGTA was added to a final concentration of 1 mM. The NMR tubes (10 mm) were soaked in a 1:1 mixture of concentrated nitric and sulfuric acids, rinsed with deionized water, and dried.

Samples to be used for $^{31}P$ NMR analyses were either percolated through columns of Chelex-100 ($Na^+$), lyophilized, and dissolved in 20% $D_2O$ containing 1 mM EGTA or directly dissolved in 20% $D_2O$ containing 1 mM EGTA. The NMR tubes (8 mm) were rendered metal free as described in the previous paragraph.

Table I. Ionization Data Obtained from $^{31}P$ NMR

| sample | shift[a] | p$K_a$ | lit. p$K_a$[b] |
|---|---|---|---|
| inorganic phosphate[c] | −2.52 | 6.7 | 7.2[e] |
| | −2.26 | 11.8 | 12.3[e] |
| pyrophosphate[c] | −3.13 | 6.0 | 6.6[e] |
| | −1.14 | 8.5 | 9.4[e] |
| methylphosphonate[c] | 3.86 | 7.6 | 7.1[f] |
| methylenediphosphonate[c] | −0.25 | 7.0 | 6.9[f] |
| | 0.70 | 11.0 | 10.3[f] |
| $\beta$-phosphorus of AMP-PCP[d] | −4.15 | 8.8 | 8.5[g] |
| $\gamma$-phosphorus of AMP-PCP[d] | 2.88 | 8.8 | |

[a] Shift in ppm observed upon protonation; negative values refer to upfield shifts and positive to downfield shifts. [b] Determined by potentiometric titration. [c] 29 °C. [d] 75 °C. [e] Reference 23. [f] Reference 24. [g] Reference 25.

**NMR Measurements.** $^{17}O$ NMR spectra at 36.6 MHz were recorded as previously described with a Bruker WH-270 NMR spectrometer with a 14085-Hz sweep width[7] except that in some experiments 1024 data points were used to acquire the free induction decay, resulting in a 0.0358-s 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 on 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 by using a spectral width of 25 kHz, resulting in a 0.0410-s recycle time; following multiplication by an exponential line-broadening 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).

$^{31}P$ NMR spectra were recorded at 32 MHz on a Varian CFT-20 NMR spectrometer equipped with a phosphorus probe. Broad-band proton decoupling was routinely employed; unless otherwise stated, spectra were obtained at ambient temperature, approximately 29 °C. Chemical shifts are measured relative to an external capillary of 85% $H_3PO_4$, with positive values being downfield of the reference.

**Data Analysis.** Values for the p$K_a$s and chemical shifts associated with species differing in state of protonation were fit to the experimental data using computer programs written by Professor W. W. Cleland, University of Wisconsin. A published program,[14] entitled WAVL, was used for analysis of monobasic titration curves, and a program for the analysis of dibasic titration curves was generously written for us by Professor Cleland. The standard error associated with the observed p$K_a$ was less than 0.1 pH unit and those associated with the chemical shifts of species differing in state of protonation were less than 0.1 ppm.

## Results and Discussion

**Phosphate and Pyrophosphate.** We have reported the $^{17}O$ NMR pH titration of inorganic phosphate over the pH range of 3 to 12;[7] for purposes of comparison, we have measured a $^{31}P$ NMR pH titration over the same pH range. The chemical shift change produced by protonation of the tri- and dianionic species are similar (2.26 and 2.52 ppm, respectively), with both protonations leading to upfield shifts. The p$K_a$s and chemical shift changes obtained from this titration are summarized in Table I. We previously reported that the $^{17}O$ NMR titration of inorganic phosphate yields similar upfield shifts when the tri- and dianionic species are protonated (13.57 and 12.37 ppm, respectively). Thus, both $^{31}P$ and $^{17}O$ NMR can be used to measure charge neutralization in inorganic phosphate.

We also titrated pyrophosphate in the pH range 3–12, and the $^{31}P$ NMR pH titration curve is shown in Figure 1. The chemical shift change produced by protonation of the tetraanionic species is significantly less that that produced by titration of the trianionic species (1.14 vs. 3.13 ppm, respectively), although both protonations do result in upfield shifts. The p$K_a$s and chemical shift

(12) Webb, M. R.; Trentham, D. R. *J. Biol. Chem.* **1980**, *255*, 1775.
(13) Hoard, D. E.; Ott, D G. *J. Am. Chem. Soc.* **1965**, *87*, 1785.
(14) Cleland, W. W. *Methods Enzymol.* **1979**, *63*, 103.

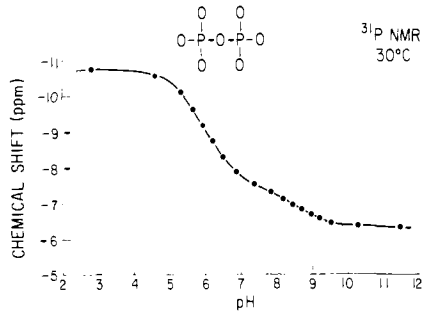



**Figure 1.** pH titration curve for pyrophosphate as determined by $^{31}$P NMR at 29 °C.

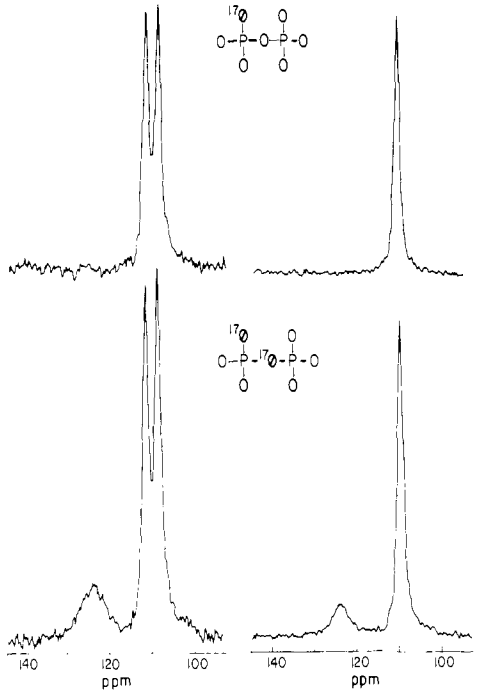



**Figure 2.** $^{17}$O NMR spectra at 36.6 MHz and 95 °C of nonbridging [$^{17}O_t$]pyrophosphate (top) and a mixture of bridging and nonbridging [$^{17}O_t$]pyrophosphate (bottom): $^{31}$P coupled spectra are on the left and $^{31}$P decoupled spectra are on the right.

changes determined from these data are also listed in Table I.

At the time this study was initiated, no synthesis of pyrophosphate labeled with an oxygen isotope only in the bridging position was available. Therefore, in order that the resonance for the bridging oxygen could be unambiguously assigned, we prepared two samples of labeled pyrophosphate, one with only the nonbridging oxygens labeled with $^{17}$O and one of a mixture of species with bridging or nonbridging oxygens labeled with $^{17}$O. The $^{17}$O NMR spectra of these samples were obtained at high pH and 95 °C with and without decoupling of the directly bonded $^{31}$P nuclei, and these are shown in Figure 2. The sample with only the nonbridging oxygens labeled (top spectra) shows a single resonance in the presence of $^{31}$P decoupling, which appears as a doublet in the absence of $^{31}$P decoupling. The sample that also contains pyrophosphate labeled in the bridging position shows an additional resonance at 124 ppm, which must be assigned to the bridging oxygen atom (bottom spectra). This assignment was recently confirmed with a sample of pyrophosphate labeled predominantly with $^{17}$O in the bridging position, which was synthesized in one of our laboratories.[15]

The resonance associated with the bridging atom in pyrophosphate is too broad to allow resolution of the one-bond $^{31}$P–$^{17}$O coupling constant in the absence of $^{31}$P decoupling. We have

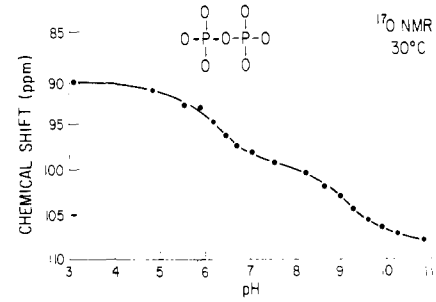



**Figure 3.** pH titration curve for the nonbridging oxygens of pyrophosphate as determined by $^{17}$O NMR at 30 °C in the presence of $^{31}$P decoupling.

obtained a reliable estimate of the value for this coupling constant by measuring the line width of this resonance in the presence and absence of $^{31}$P decoupling; the sample used for this determination was that labeled predominantly in the bridging position.[15] The difference, approximately 60 Hz, indicates that the value of the coupling constant is about 30 Hz since the bridging oxygen is coupled to two equivalent $^{31}$P nuclei; the error in this measurement is estimated to be less than 10 Hz. The one-bond $^{31}$P–$^{17}$O coupling constant for the nonbridging oxygens is about 104 Hz.

Gerothanassis and Sheppard very recently reported the natural abundance 54.2-MHz $^{17}$O NMR spectrum of pyrophosphate and observed a weak resonance at 122.8 ppm, which they attributed to the bridging oxygen.[6] This chemical shift value and the one we have unambiguously determined (124 ppm) are in excellent agreement. These authors also noted a modest increase in the one-bond $^{31}$P–$^{17}$O coupling constant as the double bond character of the phosphoryl bond increased;[6] our recent report of the $^{17}$O NMR spectral properties of inorganic phosphate and the methyl esters of phosphoric acid revealed the same correlation.[7] However, the value we have obtained for the one-bond $^{31}$P–$^{17}$O coupling constant for the bridging oxygen in pyrophosphate is about one-third that measured for anionic phosphoryl oxygens. The explanation for this observation is based on the assumption that the magnitudes of the one-bond coupling constants are expected to be dominated by Fermi contact contributions, with increasing s bond character in the $\sigma$ bond between the coupled nuclei being associated with an increase in the coupling constant.[16] Since ab initio calculations of the s bond character of P–O bonds are not yet reliable,[16] a quantitative explanation for the observed differences in coupling constants for bridging and nonbridging oxygens cannot be provided. However, as pointed out by Gerothanassis and Sheppard,[6] X-ray structure studies reveal that the bridging P–O bonds in pyrophosphate are significantly longer than those of the nonbridging bonds (1.61 vs. 1.49 Å, respectively), and our experimental data confirm their prediction that the coupling constant involving the bridging oxygen should be smaller than those observed for nonbridging oxygens.

The $^{31}$P decoupled resonances for the bridging and nonbridging oxygens in pyrophosphate differ in line width by a factor of about 3.2. By use of the nuclear quadrupolar coupling constants for P=O and P—OR in triphenyl phosphate reported by Cheng and Brown,[17] 3.8 and 9.0 MHz, respectively, and the assumption that the nuclear quadrupolar coupling constants for anionic phosphoryl oxygens can be estimated from these simply by calculating the weighted average of the contributions by P=O and P—O, the expected ratio of the nuclear quadrupolar coupling constants for the bridging and nonbridging oxygens in pyrophosphate is 1.24 and that for the line widths is 1.53. The line-width ratio we have measured is significantly larger than the predicted value, and this may reflect either that such a naive approach for calculating the values for nuclear quadrupolar coupling constants for anionic phosphoryl oxygens is not valid or that the rotational correlation times of these oxygens differ by a factor of about 2, with that of the nonbridging oxygens being the shorter. The latter explanation

(15) Reynolds, M. A., Oppenheimer, N. L., and Kenyon, G. L. *J. Am. Chem. Soc.*, in press.

(16) Gray, G. A.; Albright, T. A. *J. Am. Chem. Soc.* **1977**, *99*, 3243.
(17) Cheng, C. P.; Brown, T. L. *J. Am. Chem. Soc.* **1980**, *102*, 6418.

Table II. Ionization Data Obtained from $^{17}O$ NMR

| sample | shift[a] | $pK_a$ | lit. $pK_a$ |
|---|---|---|---|
| pyrophosphate[c] | 8.6 | 6.3 | 6.6[b,e] |
| | 8.1 | 9.2 | 9.4[b,e] |
| AMPS[c] | 22.4 | 4.9 | 5.3[f] |
| γ-oxygens of ATPγS[c] | 24.0 | 5.8 | 5.3[f] |
| methylphosphonate[c] | 18.6 | 7.7 | 7.1[b,g] |
| methylenediphosphonate[c] | 10.6 | 7.3 | 6.9[b,g] |
| | 9.0 | 11.0 | 10.3[b,g] |
| γ-oxygens of AMP-PCP[d] | 18.0 | 8.8 | 8.5[b,h] |

[a] Upfield shift in ppm observed upon protonation. [b] Determined by potentiometric titration. [c] 30 °C. [d] 75 °C. [e] Reference 23. [f] Reference 8. [g] Reference 24. [h] Reference 25.

is in keeping with the expectation that rotation about the P–O bridging bonds would be associated with decreased line widths.[7] In either case the larger line width observed for the bridging oxygen in pyrophosphate is expected on the basis of the reported nuclear quadrupolar coupling constants for P=O and P—O bonds.

The $^{17}O$ NMR pH titration behavior of the nonbridging phosphoryl oxygens in pyrophosphate (using a mixture of bridging and nonbridging labeled species) was assessed at 30 °C, and the titration curve is shown in Figure 3. The upfield chemical shift changes produced by protonation are similar for the tetra- and trianionic species (8.09 and 8.65 ppm, respectively). The titration data are summarized in Table II. The equal changes in $^{17}O$ NMR chemical shift are consistent with equal amounts of charge being neutralized by the addition of each proton, in contrast to the impression that is derived from the $^{31}P$ NMR pH titration data.

The magnitude of the change in $^{17}O$ NMR chemical shift associated with protonation of pyrophosphate is one-half that previously reported for monomethyl phosphate, AMP, the β-nonbridging oxygens of ADP, and the γ-nonbridging oxygens of ATP; this is precisely the result to be expected if the chemical shift change is directly proportional to the magnitude of the charge neutralized on each oxygen by protonation ($^1/_6$ for pyrophosphate and $^1/_3$ for monoesters and the terminal phosphoryl groups of anhydrides). This titration curve, therefore, provides a very important and convincing confirmation of our hypothesis that the chemical shifts of weakly acidic phosphoryl oxygens are influenced significantly by the charge on the oxygen.[7] In addition, the chemical shift induced by charge neutralization in pyrophosphate (50 ppm per full negative charge) is in excellent agreement with the values we previously reported for inorganic phosphate (52 ppm), monomethyl phosphate (46 ppm), AMP (44 ppm), the β-nonbridging oxygens of ADP (48 ppm), and the γ-nonbridging oxygens of ATP (48 ppm).

**Complete Chemical Shift Assignments of ADP and ATP.** In our previous report of the $^{17}O$ NMR spectral properties of nucleotides,[7] we presented spectra of ATP samples that were enriched in the α,β- and β,γ-bridging oxygens (as well as in nonbridging positions) and revealed the presence of broad resonances at 120–125 ppm in addition to the more upfield resonances for nonbridging oxygens. Given the chemical shift of the bridging oxygen atom in pyrophosphate, we believe that we now can confidently attribute these resonances to the bridging oxygens in ATP.[18]

Our published 36.6-MHz spectra of a sample of ADP labeled in the bridging oxygen (as well as in the β-nonbridging oxygens) did not reveal the presence of a broad resonance that could be attributed to the bridging oxygen. We have recorded 67.8-MHz $^{17}O$ spectra of this sample as a function of temperature, and in these spectra a broad resonance at 117 ppm can be observed (Figure 4). On the basis of our spectra of pyrophosphate, this resonance can be assigned to the bridging oxygen in ADP. Our inability to detect this signal previously may be attributed either to the higher resolution achieved at the higher field (even in the absence of $^{31}P$ decoupling) or the increased sensitivity that results from operating at higher field. As expected, the line widths of the resonances for ADP were identical at 36.6 and 67.8 MHz (when corrected for the presence of $^{31}P$ coupling at 67.8 MHz), confirming our earlier expectation that an increase in field strength would be accompanied by an increase in resolution.

(18) The assignment of the chemical shift of the resonance for the β,γ-bridging oxygen in ATP has recently been confirmed with a sample of ATP labeled predominantly in this position. This sample was prepared from pyrophosphate labeled predominantly in the bridging oxygen.[15]

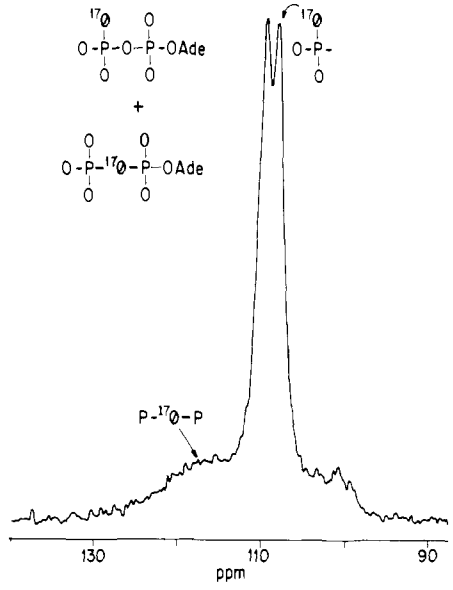



Figure 4. $^{17}O$ NMR spectrum at 67.8 MHz, 95 °C, and pH 9 of [β-$^{17}O_1$]ADP that contains a labeled oxygen in both the β,γ-bridging and β-nonbridging positions; this spectrum was obtained without $^{31}P$ decoupling. The resonances at about 100 ppm are associated with small amounts of labeled inorganic phosphate and AMP, which were formed by hydrolysis.[7]

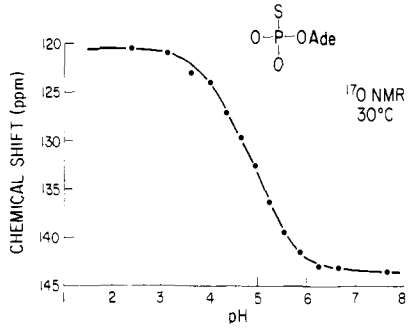



Figure 5. pH titration curve for AMPS as determined by $^{17}O$ NMR at 30 °C in the presence of $^{31}P$ decoupling.

**AMPS and ATPγS.** Jaffe and Cohn have published $^{31}P$ NMR pH titrations of samples of AMPS and ATPγS,[8] with the most important observation being that protonation of the thiophosphoryl groups resulted in downfield shifts of the thiophosphoryl resonance in contrast to the upfield shifts observed for the protonation of the analogous phosphoryl groups in AMP and ATP. Furthermore, small but significant upfield shifts were observed for the resonances of the α- and β-phosphorus atoms of ATPγS. We have prepared an enriched sample of AMPS and three samples of ATPγS so that comparable $^{17}O$ NMR pH titrations could be performed.

The $^{17}O$ NMR resonance for AMPS shifts upfield 22.4 ppm upon protonation at 30 °C (Figure 5); a similar upfield change in chemical shift for AMPS (26 ppm) was reported recently by Huang and Tsai.[5] The $pK_a$ and chemical shift parameters obtained from this titration curve are included in Table II.

The $^{17}O$ NMR pH titration curves for the three samples of ATPγS measured at 30 °C are shown in Figure 6. The behavior is similar to that previously reported by us for titrations of ATP samples labeled in each of the phosphoryl groups,[7] with the only difference being the larger magnitude of the upfield shift exhibited by the oxygens of the thiophosphoryl group (24 ppm for ATPγS vs. 16 ppm for ATP). This change in $^{17}O$ NMR chemical shift

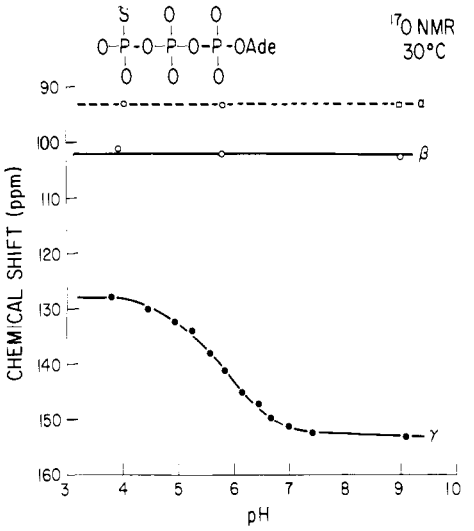



**Figure 6.** pH titration curves for the nonbridging oxygens in ATPγS as determined by $^{17}O$ NMR at 30 °C in the presence of $^{31}P$ decoupling.

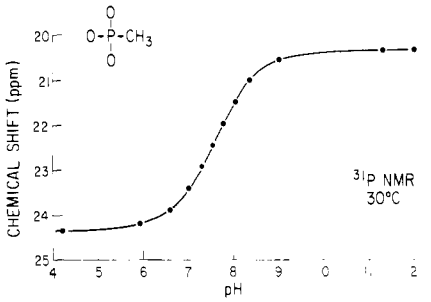



**Figure 7.** pH titration curve for the methylphosphonate as determined by $^{31}P$ NMR at 29 °C in the presence of $^{1}H$ decoupling.

is nearly identical with that found for AMPS. The resonances for the α- and β-oxygen atoms of ATPγS are unaffected by pH, as were the analogous resonances in ATP. The p$K_a$ and spectral parameters fitted to this titration curve are listed in Table II.

The magnitudes of the upfield shifts observed for the thiophosphoryl oxygens of AMPS and ATPγS are larger than those we reported for the protonation of the tri- and dianionic species of thiophosphate (18.3 ppm).[7] This increase in sensitivity to protonation can be explained by the chemical shift change being proportional to the charge neutralized on each oxygen, assuming that very little charge is delocalized on the sulfur in inorganic thiophosphate[19] or in the nucleoside phosphorothioates. Thus, the charge neutralized per oxygen by protonation is $^1/_2$ for AMPS and ATPγS and $^1/_3$ for thiophosphate. The chemical shift changes observed for AMP and ATPγS provide additional evidence for our hypothesis regarding the relationship between chemical shift changes and extent of charge neutralization,[7] since the chemical shifts induced per charge neutralized are 45 ppm for AMPS and 48 ppm for ATPγS.

It is interesting to note that in the $^{31}P$ NMR pH titration curves for ATP and ATPγS the resonances associated with the α- and β-phosphorus nuclei experience small but significant upfield shifts upon protonation of the molecule;[8] no changes in chemical shift are observed for the analogous $^{17}O$ NMR resonances, implying that the $^{31}P$ NMR behavior is not due to direct charge neutralization of these phosphate anions but rather to indirect electronic or conformational effects.

**Phosphonates.** Although two recent reports in the literature described the $^{31}P$ NMR pH titration behavior of AMP-PCP,[20,21] we are unaware of similar titration studies on either methylphosphonate or PCP, the simplest phosphonic acid analogues of inorganic phosphate and pyrophosphate. The $^{31}P$ NMR pH titration curve for methylphosphonate is shown in Figure 7 and that for PCP is shown in Figure 8. Protonation of the dianionic species of methylphosphonate is accompanied by a downfield shift of 3.86 ppm for the $^{31}P$ NMR resonance; qualitatively this behavior is analogous to that observed for inorganic thiophosphate but not inorganic phosphate since protonation causes a downfield shift of the $^{31}P$ NMR resonance. In view of the large downfield shift found for methyl phosphonate, the results for PCP are interesting in that protonation of the tetraanionic species results in a small downfield change in chemical shift (0.70 ppm) whereas protonation of the trianionic species results in a small upfield change in chemical shift (0.25 ppm). The p$K_a$ and chemical shift data derived from these titration curves are included in Table I.

The behavior for the simple phosphonates can be compared to those reported for the $^{31}P$ NMR pH titration of AMP-PCP,[20,21] which revealed an upfield chemical shift change of about 4 ppm for the β-phosphorus resonance and a downfield chemical shift change of about 3 ppm for the γ-phosphorus resonance (with very little change for the α-phosphorus resonance). Since the $^{17}O$ NMR titrations of AMP-PCP to be described later in this section were performed at 75 °C, the p$K_a$ and chemical shift data for a $^{31}P$ NMR pH titration at this temperature were measured; these are included in Table I. No significant differences in titration behavior were observed at the elevated temperature. Consideration of all of the available $^{31}P$ NMR pH titration data for phosphonates demonstrates that $^{31}P$ NMR is clearly not a straightforward

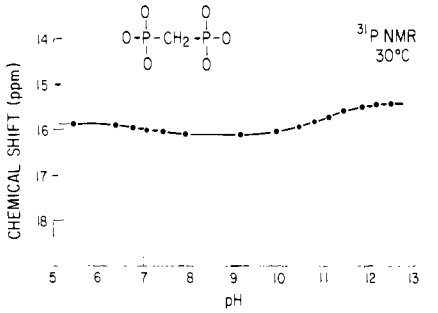



**Figure 8.** pH titration curve for PCP as determined by $^{31}P$ NMR at 29 °C in the presence of $^{1}H$ decoupling.

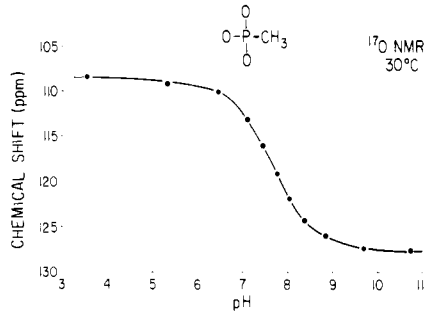



**Figure 9.** pH titration curve for methylphosphonate as determined by $^{17}O$ NMR at 30 °C in the presence of $^{31}P$ decoupling; the concentration of methyl[$^{17}O$]phosphonate was approximately 10 mM.

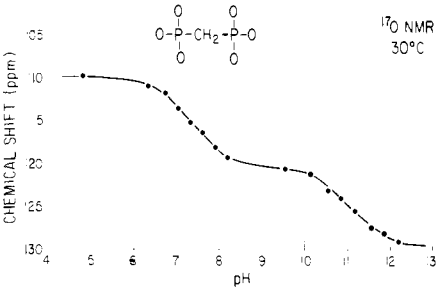



**Figure 10.** pH titration curve for PCP as determined by $^{17}O$ NMR at 30 °C in the presence of $^{31}P$ decoupling.

(19) Steger, E.; Martin, K. *Z. Anorg. Allg. Chem.* **1963**, *323*, 108.
(20) Schliselfeld, L. H.; Burt, C. T.; Labotka, R. J. *Biochemistry* **1982**, *21*, 317.
(21) Vogel, H. J.; Bridger, W. A. *Biochemistry* **1982**, *21*, 394.

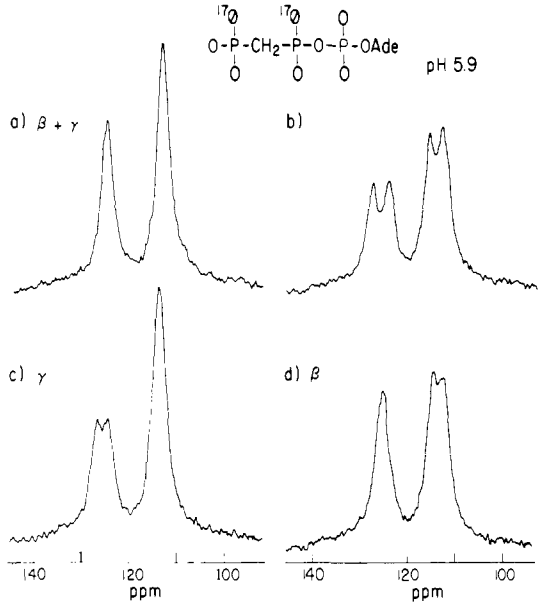



**Figure 11.** $^{17}O$ NMR spectra at 36.6 MHz, 75 °C, and pH 5.9 of [β,γ-$^{17}O$]AMP-PCP used to assign the resonances. The spectra were taken with the following $^{31}P$ decoupling conditions: (a) decoupling of both the β- and γ-nuclei; (b) no decoupling; (c) decoupling of only the γ-nucleus; and (d) decoupling of only the β-nucleus.

technique for providing information about the site and degree of charge neutralization.

The $^{17}O$ NMR pH titration data for methyl phosphonate and PCP obtained at 30 °C are shown in Figures 9 and 10, respectively. Titration of the dianionic species of methylphosphonate results in an upfield change in chemical shift of 18.6 ppm, or about 56 ppm per charge neutralized. Titration of either the tetra- or trianionic species of PCP results in similar upfield changes in chemical shift (9.0 and 10.6 ppm, respectively), a marked contrast to the $^{31}P$ NMR behavior shown in Figure 9; the chemical shift change per charge neutralized is about 58 ppm. The p$K_a$s and chemical shift data for methylphosphonate and PCP are summarized in Table II.

Confident interpretation of the titration data for [β,γ-$^{17}O$]-AMP-PCP requires unambiguous assignment of the two resonances that are observed in the $^{17}O$ NMR spectrum. We chose to make these assignments in a rigorous fashion, although the final result is, in fact, predictable on the basis of the different amount of label present in the β- and γ-nonbridging positions by virtue of the synthesis employing the symmetrical PCP molecule. At low pH, the $^{31}P$ NMR chemical shifts of the resonances associated with β- and γ-phosphorus nuclei are sufficiently different (even though these resonances are spin–spin coupled to $^{17}O$ and also broadened by the directly bonded $^{17}O$) that it is possible to decouple selectively these nuclei and observe the effect on the directly bonded $^{17}O$ nuclei, thereby allowing the unambiguous assignment of the $^{17}O$ resonances. In Figure 11 we present spectra of the β,γ-labeled material at 75 °C and pH 5.9 which were obtained under the following decoupling conditions: spectrum a, broad-band decoupling of both the β- and γ-phosphorus nuclei; spectrum b, no $^{31}P$ decoupling; spectrum c, $^{31}P$ decoupling of only the γ-phosphorus nucleus; and spectrum d, $^{31}P$ decoupling of only the β-phosphorus nucleus. Clearly the resonance at 113 ppm, which has the greater intensity, can be assigned to the γ-oxygens, and the resonance at 125 ppm can be assigned to the β-oxygens.

Having established the chemical shift assignments, we performed $^{17}O$ NMR pH titrations of both the α- and β,γ-labeled samples of AMP-PCP at 75 °C (to give increased resolution in the doubly labeled sample; at higher temperatures AMP-PCP is not sufficiently stable to allow a complete titration curve to be obtained), and the data are shown in Figure 12. The behavior is similar to that of ATP and ATPγS since only the resonance associated with the terminal oxygens is sensitive to protonation; the upfield shift produced is 18.0 ppm. This change in chemical

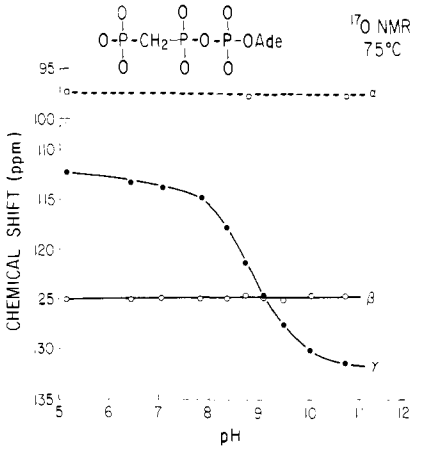



**Figure 12.** pH titration curves for the nonbridging oxygens in AMP-PCP as determined by $^{17}O$ NMR at 75 °C in the presence of $^{31}P$ decoupling.

shift is essentially the same as that found for methyl phosphonate (18.6 ppm) and twice that measured for PCP (average of 9.8 ppm). These chemical shift changes resulting from protonation are consistent with the hypothesis that the chemical shift change accompanying charge neutralization is directly protonational to the charge neutralized[7] ($^1/_6$ for PCP and $^1/_3$ for methyl phosphonate and AMP-PCP). The p$K_a$ and chemical shift data for the behavior of AMP-PCP are listed in Table II.

## Summary

The $^{17}O$ NMR pH titration data reported in this and a previous paper[7] convincingly demonstrate that the protonation of a phosphoryl oxygen is accompanied by an upfield shift of the associated $^{17}O$ NMR resonance of approximately 50 ppm per charge neutralized. This relationship is valid for phosphates, phosphorothioates, and phosphonates; for these types of compounds there is no ambiguity as to the tautomeric structure of the molecule, i.e., the magnitude of the charge on the phosphoryl oxygens can be readily assigned. Thus, in contrast to the problems associated with the interpretation of $^{31}P$ NMR chemical shift changes arising from charge neutralization, changes in $^{17}O$ NMR chemical shift can be reliably interpretted. This conclusion should encourage the application of $^{17}O$ NMR spectroscopy to problems of chemical and biochemical importance. In the following paper we have used the relationship between phosphoryl oxygen charge and chemical shift as part of an extensive heteronuclear NMR study of the tautomeric structure of the β,γ-imido analogue of ATP in solution.[22]

**Acknowledgment.** We thank Professor Sunney I. Chan and Dr. Luciano Mueller for their assistance in obtaining the 67.8-MHz $^{17}O$ NMR spectra and Professor Barry S. Cooperman for the gift of methyl [$^{17}O$]phosphonate. We are grateful to Professor Perry A. Frey for providing us with a preprint of ref 11. This research was supported by grants from the National Institutes of Health (GM-22350 to J.A.G. and AM-17323 to G.L.K.). The high-field NMR spectrometers used in this research are supported by grants from the National Science Foundation (Bruker WH-270 in the Southern New England Regional NMR Facility, NSF CHE-791620, and Bruker WM-500 in the Southern California Regional NMR Facility, NSF CHE-7916324).

**Registry No.** $^{17}O$, 13968-48-4; inorganic phosphate, 14265-44-2; pyrophosphate, 14000-31-8; methylphosphonate, 993-13-5; PCP, 1984-15-2; AMP-PCP, 3469-78-1; AMPS, 19341-57-2; ATPYS, 35094-46-3; ADP, 58-64-0; ATP, 56-65-5.

(22) Reynolds, M. A.; Gerlt, J. A.; Demou, P. C.; Oppenheimer, N. J.; Kenyon, G. L. *J. Am. Chem. Soc.*, following paper in this issue.
(23) Jencks, W. P.; Regenstein, J. In "Handbook of Biochemistry and Molecular Biology, 3rd ed.; Physical and Chemical Data"; Fasman, G. D., Ed.; CRC Press: Cleveland, OH, 1976; Vol. I, p 305.
(24) Freedman, L. D.; Doak, G. O. *Chem. Rev.* **1957**, *57*, 479.
(25) Myers, T. C.; Nakamura, K.; Flesher, J. W. *J. Am. Chem. Soc.* **1963**, *85*, 3292.

# $^{15}N$ and $^{17}O$ NMR Studies of the Proton Binding Sites in Imidodiphosphate, Tetraethyl Imidodiphosphate, and Adenylyl Imidodiphosphate

**Mark A. Reynolds,[1a] John A. Gerlt,*[1b,2] Peter C. Demou,[1b] Norman J. Oppenheimer,[1a,3] and George L. Kenyon*[1a]**

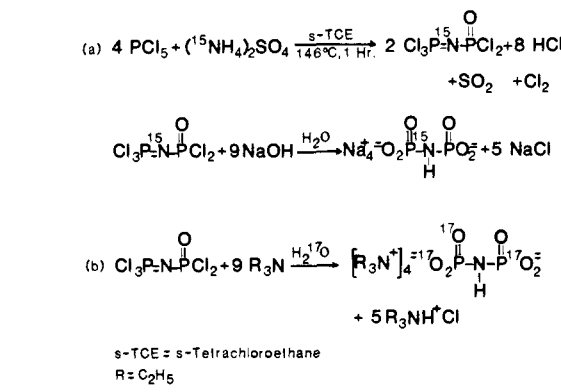
*Contribution from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143, and the Department of Chemistry, Yale University, New Haven, Connecticut 06511.* *Received October 4, 1982*

**Abstract:** $^{15}N$- and $^{17}O$-enriched samples of imidodiphosphate (PNP), its tetraethyl ester, and 5'-adenylyl imidodiphosphate (AMP-PNP) have been prepared. The $^{15}N$ NMR spectra of both PNP and AMP-PNP reveal the presence of approximately 70-Hz $^{1}H-^{15}N$ coupling constants for the fully ionized samples, demonstrating an imido tautomeric structure in both cases. For AMP-PNP this coupling persists in the presence of a stoichiometric amount of $Mg^{2+}$. The $^{17}O$ NMR chemical shifts of the resonances associated with the phosphoryl oxygens have been assigned. The effect of pH on the resonances for PNP and AMP-PNP is similar to that observed for ATP (Gerlt, J. A.; Demou, P. C.; Mehdi, S. *J. Am. Chem. Soc.* **1982**, *104*, 2848) and its thiophosphate and phosphonate structural analogues (Gerlt, J. A.; Reynolds, M. A.; Demou, P. C.; Kenyon, G. L. *J. Am. Chem. Soc.*, preceding paper in this issue), indicating that protonation of the tetraanion of PNP occurs exclusively on the oxygens and that protonation of the tetraanion of AMP-PNP occurs exclusively on the $\gamma$-phosphoryl oxygens. Although $^{17}O$ NMR demonstrates that protonation of the monoanion of the tetraethyl ester of PNP occurs predominantly on nitrogen, the corresponding $^{15}N$ NMR chemical shift change was only 2.50 ppm. Thus, $^{15}N$ NMR chemical shift changes cannot be used reliably to ascertain the sites of protonation in imidodiphosphates.

5'-Adenylyl imidodiphosphate (AMP-PNP, Ib) was synthesized in 1971 by Yount and his co-workers and has been used widely in enzymological studies by virtue of its close structural similarity to ATP. Since the P-N-P bridge in AMP-PNP is relatively inert to hydrolysis both in basic solution and in the presence of enzymes that catalyze the hydrolysis or transfer of the $\gamma$-phosphoryl group of ATP, this compound has proven to be a useful competitive inhibitor of kinases and adenosine triphosphatases. The ability of enzymes to effectively bind AMP-PNP has been rationalized by its being isosteric with ATP, since the crystal structures for imidodiphosphate (PNP) and pyrophosphate show that the bond angles and lengths of the P-N-P and P-O-P linkages are nearly identical.[5] However, the solution structures of PNP and AMP-PNP have not been examined, and the possibility does exist that if the $pK_a$ of the N-H proton in the bridge were sufficiently low the proton could reside on oxygen rather than nitrogen (Figure 1a);[6] the stability of these imino structures could be explained by the presence of intramolecular hydrogen bonding as illustrated in Figure 1b. In addition, since enzymatic reactions that utilize ATP as a substrate almost always require it in the form of its complex with $Mg^{2+}$, coordination of AMP-PNP with $Mg^{2+}$ could stabilize the imino tautomers; direct coordination of $Mg^{2+}$ with the nitrogen of the P-N-P bridge in AMP-PNP has been suggested on the basis of $^{31}P$ NMR studies.[7]

The $^{31}P$ NMR spectral properties of AMP-PNP as a function of both pH and $Mg^{2+}$ concentration have been reported, and these demonstrate a behavior markedly different from that of ATP.[7,8]

Scheme I




For example, upon protonation the resonance associated with the $\beta$-phosphorus of AMP-PNP shifts upfield more than the resonance associated with the $\gamma$-phosphorus; protonation of ATP leads to a larger upfield shift for the resonance associated with the $\gamma$-phosphorus. These data may suggest a fundamentally different ionization behavior for AMP-PNP as compared to ATP and could support the existence of the hypothetical imino structures shown in Figure 1b. However, Jaffe and Cohn have recently summarized a large body of experimental data which lead to the conclusion that chemical shift changes observed in $^{31}P$ NMR spectroscopy cannot be used reliably to identify sites of protonation in polyphosphates.[9]

To resolve the problem of the structure of the P-N-P bridge in PNP and AMP-PNP in aqueous solution, we have utilized both $^{15}N$ and $^{17}O$ NMR to ascertain the sites of proton binding. We have synthesized $^{15}N$- and $^{17}O$- enriched samples of PNP and its tetraethyl ester and of AMP-PNP. Our results provide strong evidence for the existence of the imido tautomer of the P-N-P bridge in both PNP and AMP-PNP. Our experimental observations on the tetraethyl ester of PNP indicate that, in contrast to other nitrogen acids, the $^{15}N$ NMR chemical shift of the bridging nitrogen is surprisingly insensitive to ionization of the directly bonded proton.

(1) (a) University of California. (b) Yale University
(2) NIH Research Career Development Awardee (CA-00499), 1978-83; Alfred P. Sloan Foundation Fellow, 1981-83.
(3) NIH Research Career Development Awardee (CA-00587), 1979-1984.
(4) Youni, R. G.; Babcock, D.; Ballantyne, W.; Ohala, D. *Biochemistry 1971*, **10**, 2484.
(5) Larsen, M.; Willei, R.; Young, R. G. *Science (Washington, D.C.)* **1969**, *166*, 1510.
(6) The predominance of an imino tautomer in a P(O)-N(H)-P(O) bridge system was proposed for the tetramethyl and tetraethyl esters of PNP by using infrared spectroscopy: (a) Kabachnik, M. K.; Gilyarov, V. A.; Popov, E. M. *Izv. Akad. Nauk SSSR, Ser. Khim.* **1961**, *6*, 1022; (b) Kireev, V. V.; Kolesnikov, G. C.; Titov, S. S. *Zh. Obshch. Khim.* **1970**, *40*, 2105. In addition, these esters have been found to be reasonably acidic; the $pK_a$ values for the tetramethyl and tetraethyl esters of PNP have been measured as 2.6 and 3.7, respectively: (c) Riesel, L.; Pich, G.; Ruby, C. *Z. Anorg. Allg. Chem.* **1977**, *430*, 227.
(7) Tran-Dinh, S.; Roux, M. *Eur. J. Biochem.* **1977**, *76*, 245.
(8) Tran-Dinh, S.; Roux, M.; Ellenberger, M. *Nucleic Acids Res.* **1975**, *2*, 1101.
(9) Jaffe, E. K.; Cohn, M. *Biochemistry* **1978**, *17*, 652.

0002-7863/83/1505-6475$01.50/0 