^{17}O NMR Spectral Properties of Simple Phosphate Esters and Adenine Nucleotides

John A. Gerlt,*[1] Peter C. Demou, and Shujaath Mehdi

Contribution from the Department of Chemistry, Yale University, New Haven, Connecticut 06511. *Received March 9, 1981*

Abstract: The ^{17}O NMR chemical shifts and one-bond ^{31}P–^{17}O coupling constants of the phosphoryl oxygens in inorganic phosphate, methyl phosphate, dimethyl phosphate, trimethyl phosphate, inorganic thiophosphate, AMP, ADP, and ATP have been measured at 95 °C in 20% D_2O as solvent. The chemical shifts of the resonances of the weakly acidic phosphoryl oxygens of these compounds are dependent on pH at 30 °C, with protonation of the oxygen anions leading to upfield shifts; the magnitude of the charge on the phosphoryl oxygen appears to be an important factor in determining the chemical shift of the associated resonance. An evaluation of the temperature dependence of the line widths of several of the labeled compounds suggests that ^{17}O NMR spectroscopy may be able to provide useful information about the electronic structure and molecular motion of phosphate esters.

^{31}P NMR spectroscopy has been applied successfully to the solution of chemical and biochemical problems involving phosphate esters and nucleotides. For example, Cohn and co-workers have demonstrated that ^{31}P NMR spectroscopy provides the unique ability to measure the equilibrium constant between reactants and products bound in the active sites of enzymes and directly determine the rates of interconversion of bound reactants and products.[2] However, experimental data summarized recently by Jaffe and Cohn have lead to the unfortunate conclusion that ^{31}P NMR spectroscopy does not provide an unambiguous probe of charge neutralization of phosphoryl oxygens.[3] The evidence for this conclusion is derived from several observations regarding the effect of protonation on the ^{31}P NMR resonances of various phosphate esters and structural analogues. Although the resonance for the γ-phosphate of ATP is shifted (upfield) the most by protonation of the most basic phosphoryl oxygen in the nucleotide, analogous behavior is not observed for two ATP analogues: when the β,γ-bridging oxygen of ATP is replaced by a methylene group or an imido group, the resonance for the β-phosphorus nucleus is shifted (upfield) more than the resonance for the γ-phosphorus nucleus (downfield shift for the methylene analogue and upfield for the imido analogue). Since it can be safely assumed that the most basic oxygens in ATP and the analogues are those of the terminal position, these observations demonstrate that ^{31}P NMR chemical shifts cannot be used with confidence to determine the sites of charge neutralization in nucleotides. In addition, results obtained for the protonation of thiophosphates reveal a striking difference when compared to those obtained with phosphates: protonation of phosphates leads to upfield changes in chemical shift whereas protonation of thiophosphates leads to downfield changes in chemical shift.

A precise explanation for these data cannot be provided since it is presently impossible to quantitatively interpret ^{31}P NMR chemical shifts. Gorenstein and co-workers have provided the results of theoretical calculations and supporting experimental data that demonstrate that ^{31}P NMR chemical shifts are sensitive to changes in both O–P–O bond angles and P–O torsional angles;[4,5] thus, the behavior of the ^{31}P NMR chemical shifts of ATP and its analogues may be least partially explained by changes in phosphate ester conformation.

^{31}P NMR spectroscopy is necessarily an indirect probe of the environment of the phosphoryl oxygens of phosphate esters, since intermolecular interactions occur via coordination to the oxygens and not to the phosphorus. A priori, it might be anticipated that ^{17}O NMR spectroscopy would be capable of providing useful information about the environment of the phosphoryl oxygens in phosphate esters and nucleotides. However, until recently, ^{17}O NMR has found limited application in the study of phosphate esters. Several reasons may explain the paucity of ^{17}O NMR spectral data: (1) the low natural abundance of ^{17}O (0.037%) requires that enriched materials be synthesized and studied; (2) the quadrupolar nature of ^{17}O ($I = {}^5/_2$) may be expected to produce resonances which are so broad that little, if any, quantitative data can be obtained;[6,7] and (3) high-field NMR spectrometers that are equipped for observing ^{17}O while simultaneously decoupling directly bonded ^{31}P nuclei are not routinely available. Despite these potential problems, Tsai and co-workers reported the first ^{17}O NMR spectra of adenine nucleotides and observed that the resonances were broad (>400 Hz);[8] at the low field used (1.9 T) and in the absence of ^{31}P decoupling, the spectra suggested that direct study of ^{17}O NMR resonances would provide limited useful information, and Tsai indicated that a more useful application of ^{17}O-labeled phosphate esters would be to use the quadrupolar relaxation effect of ^{17}O on directly bonded ^{31}P nuclei (extensive line broadening) to indirectly monitor changes in the environment of the ^{17}O nucleus.

Tsai's proposal of using ^{31}P NMR spectroscopy to monitor physical changes at directly bonded ^{17}O nuclei is based upon the prediction that the product of the ^{17}O and ^{31}P NMR line widths of directly bonded ^{17}O and ^{31}P nuclei should be a constant when molecular motion is rapid; this hypothesis has not been confirmed experimentally, and such methodology would be insensitive to changes in ^{17}O NMR chemical shifts (and one-bond ^{31}P–^{17}O coupling constants). These spectral parameters could be expected to provide useful information about the chemical and physical properties of the phosphoryl oxygens. We, therefore, have sought to determine whether "high resolution" ^{17}O NMR spectra of the phosphoryl oxygens in phosphate esters and nucleotides can be obtained and whether the chemical shifts, line widths, and ^{31}P–^{17}O coupling constants can be related to chemical structure and chemical and physical variations in environment.

The fact that ^{17}O is a quadrupolar nucleus suggested to us that higher resolution than that which Tsai reported could be easily obtained. The predominant relaxation mechanism for ^{17}O is quadrupolar in nature, with the line width of the resonance being directly related to the square of the nuclear quadrupolar coupling constant, e^2qQ/h, and to the rotational correlation time of the ^{17}O nucleus, τ_c[5]:

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

(1) NIH Research Career Development Awardee, 1978–1983; Alfred P. Sloan Fellow, 1981–1983.
(2) Cohn, M.; Rao, B. D. N. *Bull. Magn. Res.* **1979**, *1*, 38.
(3) Jaffe, E. K.; Cohn, M. *Biochemistry* **1978**, *17*, 652.
(4) Gorenstein, D. G.; Kar, D.; Luxon, B. A.; Momii, R. K. *J. Am. Chem. Soc.* **1976**, *98*, 1668.
(5) Gorenstein, D. G.; Findlay, J. B.; Momii, R. K.; Luxon, B. A.; Kar, D. *Biochemistry* **1976**, *15*, 3796.
(6) Klemperer, W. G. *Angew. Chem.* **1978**, *17*, 246.
(7) Harris, R. K. In "NMR and Periodic Table"; Harris, R. K., Mann, B. E., Eds.; Academic Press: New York, 1978; Chapters 1 and 2.
(8) Tsai, M. D.; Huang, S. L.; Kozlowski, J. R.; Chang, C. C. *Biochemistry* **1980**, *19*, 3531.

0002-7863/82/1504-2848$01.25/0

where eq is the field gradient at the nucleus due to the electrons, eQ is the field gradient due to the nucleus, and η is an asymmetry parameter (which can vary from 0 to 1). This expression for line width is valid only for rapid molecular motion and, given such molecular motion, is independent of the field strength. Thus, for small molecules, resolution can be increased simply by increasing the magnetic field. In addition, resolution can be increased by increasing T_2, the spin–spin relaxation time. Since the nuclear quadrupolar coupling constant should be determined only by the molecular structure, the simplest method to increase T_2 is decrease τ_c. For spherical molecules the Stokes equation predicts that τ_c should be directly proportional to both molecular mass, $4\pi a^3/3$, where a is the molecular radius, and solvent viscosity, η', and indirectly proportional to temperature:

$$\tau_c = \frac{4\pi a^3 \eta'}{3kT} \qquad (2)$$

Thus, a decrease in the viscosity of the solvent, an increase in the temperature, or, more practically, a combination of the two is expected to decrease τ_c and increase the resolution. Also, since the ^{17}O NMR spectrum of trimethyl phosphate which Tsai published[8] and data reported by earlier investigators[9,10] suggest that the ^{31}P–^{17}O coupling constants are large, heteronuclear ^{31}P decoupling would be expected to improve resolution. Therefore, it can be expected that by using the highest possible magnetic field, high sample temperature with the accompanying low solvent viscosity, and heteronuclear ^{31}P decoupling, "high resolution" and potentially useful ^{17}O NMR spectra should be realized.

In a previous communication, this laboratory reported the first application of these considerations in a demonstration of one type of information that can be obtained from "high-resolution" ^{17}O NMR spectra.[11] Using a magnetic field of 6.3 T, water as solvent, sample temperatures of 95 °C, and decoupling of the directly bonded ^{31}P nuclei, we were able to determine that the diastereomers of cyclic 5′-deoxyadenosine 3′,5′-[^{17}O,^{18}O]phosphate have different ^{17}O NMR spectral parameters. The chemical shifts were sufficiently different that two resonances could be resolved in a racemic mixture of the diastereomers. Also, both the line widths and the ^{31}P–^{17}O coupling constants were observed to be significantly different.

Given these encouraging results, we have now investigated the ^{17}O NMR spectral properties of a number of phosphate esters and nucleotides, including ATP. Our spectra demonstrate that the magnitude of the charge on the phosphoryl oxygen is important in determining the ^{17}O chemical shift, with the chemical shifts of weakly acidic phosphoryl oxygens being significantly dependent on pH. We have found that it is possible to resolve the nonbridging phosphoryl oxygens in ADP and ATP. Unlike the situation found by Jaffe and Cohn for the ^{31}P chemical shifts of phosphate and thiophosphate, the ^{17}O NMR chemical shifts of these materials have similar dependencies on pH, suggesting that ^{17}O NMR may prove useful in evaluating the extent of charge neutralization of phosphoryl oxygens. Finally, an evaluation of the line widths of the resonances of various phosphoryl oxygens and their dependency on solvent viscosity and temperature suggests that ^{17}O NMR spectroscopy may be able to provide useful information about electronic structure and molecular motion. A brief account of some of our preliminary results has appeared.[12]

Experimental Section

$H_2{}^{17}O$ (13% ^{16}O, 52% ^{17}O, 35% ^{18}O) was obtained from Monsanto. All other chemicals were the best grades commercially available and were used without additional purification.

[^{17}O]POCl$_3$ was prepared by the reaction of $H_2{}^{17}O$ with PCl_5 according to the method described by Abbott et al.[13]

Trimethyl [^{17}O]phosphate was prepared by the reaction of [^{17}O]$POCl_3$ with excess methanol. After evaporation of the solvent and HCl, the isotopic composition was determined by gas chromatography/mass spectral analysis: 18% ^{16}O, 49% ^{17}O, 33% ^{18}O.

Dimethyl [^{17}O]phosphate (sodium salt) was prepared by the reaction of trimethyl [^{17}O]phosphate with excess NaI in refluxing acetone.[14] The precipitated product was collected and washed with acetone.

Monomethyl [^{17}O]phosphate (monosodium salt) was prepared by the reaction of dimethyl [^{17}O]phosphoric acid with excess NaI in refluxing acetone.[14] The precipitated product was collected and washed with acetone.

Inorganic [^{17}O]phosphate was prepared by the reaction of [^{17}O]$POCl_3$ with unlabeled water. After lyophilization of the solvent and HCl, the labeled phosphoric acid was dissolved in water, neutralized with ammonium hydroxide, and lyophilized.

[^{17}O]AMP was prepared by the reaction of [^{17}O]$POCl_3$ with adenosine dissolved in triethyl phosphate as described by Eckstein and Goumet.[15] The resulting barium salt was converted to the bis(triethylammonium) salt by treatment of an aqueous solution with excess triethylammonium bicarbonate and triethylamine followed by centrifugation to remove precipitated barium carbonate.

[α-^{17}O]ADP was prepared according to the procedure of Hoard and Ott[16] from [^{17}O]AMP and 5 equiv of unlabeled inorganic phosphate. The product was purified by chromatography on DEAE-Sephadex A-25 (HCO_3^-) by elution with a linear gradient of triethylammonium bicarbonate, pH 7.5. This sample is predicted to have a ^{17}O enrichment of 32% in the α nonbridging oxygens. Each molecule that is labeled contains only a single atom of ^{17}O.

[β-^{17}O]ADP was prepared according to the procedure of Hoard and Ott[16] from AMP and 2 equiv of inorganic [^{17}O]phosphate. The product was purified by chromatography on DEAE-Sephadex A-25. This sample is predicted to have a ^{17}O enrichment of 12% in the bridging oxygen and 37% in the β nonbridging oxygens.

[α-^{17}O]ATP was prepared according to the procedure of Hoard and Ott[16] from [^{17}O]AMP and 5 equiv of unlabeled pyrophosphate. The product was purified by chromatography on DEAE-Sephadex A-25. This sample is predicted to have a ^{17}O enrichment of 32% in the α nonbridging oxygens.

[β-^{17}O]ATP was prepared according to the procedure of Hoard and Ott[16] from [β-^{17}O]ADP and 5 equiv of unlabeled inorganic phosphate. The product was purified by chromatography on DEAE-Sephadex A-25. This sample was predicted to have a ^{17}O enrichment of 12% in the α,β-bridging oxygen and 25% in the β-nonbridging oxygens.

[γ-^{17}O]ATP was prepared according to the procedure of Hoard and Ott[16] from ADP and 2 equiv of inorganic [^{17}O]phosphate. The product was purified by chromatography on DEAE-Sephadex A-25. This sample is predicted to have a ^{17}O enrichment of 12% in the β,γ-bridging oxygen and 37% in the γ-nonbridging oxygens.

Inorganic [^{17}O]thiophosphate was prepared by the reaction of thiophosphoryl chloride with equal amounts of 26% enriched $H_2{}^{17}O$ and dry pyridine. After evaporation of the solvent, the residue was dissolved in dilute ammonium hydroxide solution and the solvent was evaporated; this process was repeated twice. The residue was dissolved in water and the solvent was removed by lyophilization to yield the ammonium salt of thiophosphate; this sample was not further purified by chromatography to avoid hydrolysis of the P–S bond. By ^{31}P NMR spectroscopy, this sample appeared to be contaminated with no more than 5% inorganic phosphate. Approximately 60% of the molecules of thiophosphate are predicted to contain ^{17}O, with 43% being $^{17}O_1$, 15% being $^{17}O_2$, and 2% being $^{17}O_3$.

Sample Preparation. The sodium salts of enriched inorganic [^{17}O]-phosphate and its methyl esters were dissolved in 20% D_2O and treated with Chelex-100 (Na^+); the resin was removed by filtration and washed with additional solvent. The total volume of each sample was about 2.5 mL, and the final concentration of solute was about 40 mM.

The ^{17}O-enriched thiophosphate was percolated through Chelex-100 (Na^+) and lyophilized. Approximately 80 μmol was dissolved in 2 mL of 20% D_2O.

The tetraethylammonium salts of the ^{17}O-enriched samples of nucleotides were percolated through columns of Chelex-100 (tetraethylammonium) and lyophilized. Eighty micromoles of each sample was dissolved in 2 mL of 20% D_2O.

EGTA was added to each sample to a final concentration of 1 mM.

(9) Grossman, G.; Gruner, M.; Seifert, G. *Z. Chem.* **1976**, *16*, 362.
(10) Gray, G. A.; Albright, T. A. *J. Am. Chem. Soc.* **1977**, *99*, 3243.
(11) Coderre, J. A.; Mehdi, S.; Demou, P. C.; Weber, R.; Traficante, D. D.; Gerlt, J. A. *J. Am. Chem. Soc.* **1981**, *103*, 1870.
(12) Gerlt, J. A.; Demou, P. C.; Mehdi, S. *Nucleic Acids Res., Spec. Publ.* **1981**, *9*, 11.
(13) Abbott, S. J.; Jones, S. R.; Weinmann, S. A.; Bockhoff, F. M.; McLafferty, F. W.; Knowles, J. R. *J. Am. Chem. Soc.* **1979**, *101*, 4323.
(14) Kluger, R.; Wasserstein, P. *J. Am. Chem. Soc.* **1973**, *95*, 1071.
(15) Eckstein, F.; Goumet, M. In "Nucleic Acid Chemistry, Part Two"; Townsend, L. B.; Tipson, R. S., Eds.; Wiley: New York, 1978, p 861.
(16) Hoard, D. E.; Ott, D. G. *J. Am. Chem. Soc.* **1965**, *87*, 1785.

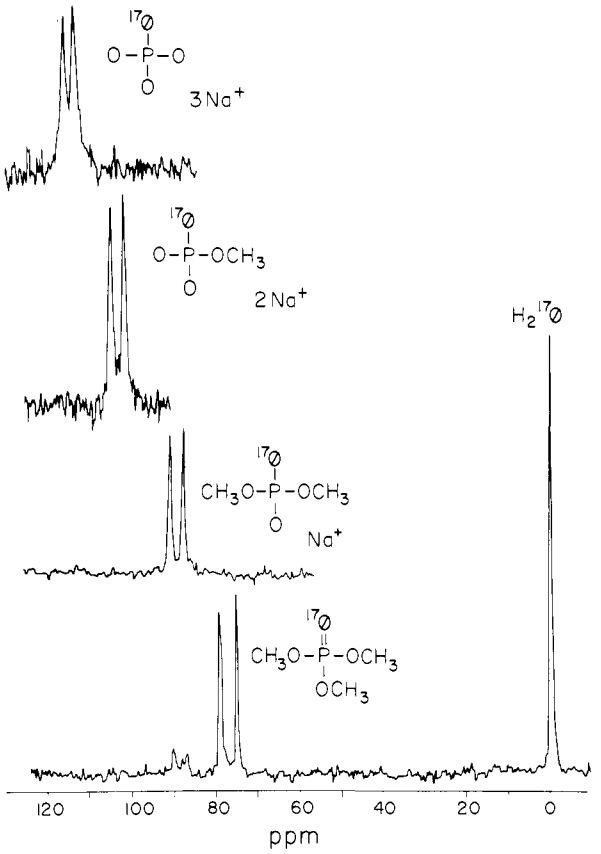

Figure 1. Phosphorus-coupled ^{17}O NMR spectra at 36.6 MHz and 95 °C of ^{17}O-enriched samples of inorganic phosphate, methyl phosphate, dimethyl phosphate, and trimethyl phosphate.

The NMR tubes (10 mm) were soaked in a 1:1 mixture of concentrated nitric and sulfuric acids and rinsed with deionized water.

NMR Measurements. ^{17}O NMR spectra were recorded at 36.6 MHz with a Bruker HX-270 NMR spectrometer; the tunable multinuclear probe was equipped to perform broad-band ^{31}P decoupling. A spectral width of 14085 Hz and 4096 data points were used to acquire the free induction decay; no delay was used between pulses resulting in a 0.1434-s recycle time. Ten thousand transients were usually collected. A line-broadening factor varying between 2 and 10 Hz was applied to each total free induction decay prior to Fourier transformation. Chemical shifts are measured relative to natural abundance $H_2{}^{17}O$.

Data Analysis. The titration data describing the effect of pH on ^{17}O NMR chemical shifts were fitted to equations describing the ionization of mono- or dibasic acids with use of computer programs prepared by Cleland. For monobasic acids, the published program WAVL was used;[17] for dibasic acids, a program generously written for us by Professor Cleland was used. The output of these programs provided the observed pK_a and the chemical shifts for the species differing in state of protonation. In general, the standard error associated with the pK_a was less than 0.1 pH unit and those associated with the chemical shifts were less than 0.2 ppm.

Results

^{17}O NMR Chemical Shifts and One-Bond ^{31}P–^{17}O Coupling Constants. The phosphorus-coupled ^{17}O NMR spectra of the enriched samples of inorganic phosphate and its methyl esters obtained at 95 °C are shown in Figure 1; the pHs of the samples used to obtain these spectra were such that the ionizable compounds would be present in their most highly ionized state. Upon irradiation of the directly bonded phosphorus nuclei, each ^{17}O resonance collapsed to a singlet (data not shown). Chemical shifts and one-bond ^{31}P–^{17}O coupling constants for these spectra are summarized in Table I.

The phosphorus-coupled ^{17}O NMR spectrum of a mixture of enriched thiophosphate and phosphate obtained at 95 °C is shown in Figure 2. Even through the pH of the sample used to obtain this spectrum was 12.3, the inorganic phosphate observed arose by hydrolysis of the enriched thiophosphate under the conditions of data collection. Upon irradiation of the directly bonded phosphorus nucleus, the ^{17}O resonance of the labeled thiophosphate collapsed to a singlet (data not shown). The chemical shift and one-bond ^{31}P–^{17}O coupling constant for thiophosphate are also included in Table I.

(17) Cleland, W. W. *Methods Enzymol.* **1979**, *63*, 103.

Table I. ^{17}O NMR Spectral Data at 95 °C

sample	chemical shift[a]	J_{P-O}[b]
[^{17}O]phosphate	113.3	91
methyl [^{17}O]phosphate	101.7	104
dimethyl [^{17}O]phosphate	88.1	117
trimethyl [^{17}O]phosphate	76.6	133
[^{17}O]thiophosphate	158.0	119
[^{17}O]AMP	100.1	98
[α-^{17}O]ADP	97.8	119
[β-^{17}O]ADP	110.5	112
[α-^{17}O]ATP	97.9	105
[β-^{17}O]ATP	105.0	119
[γ-^{17}O]ATP	110.0	112
(R_P)-[^{17}O,^{18}O]cdAMP (axial ^{17}O)[c]	92.8	130
(S_P)-[^{17}O,^{18}O]cdAMP (equatorial ^{17}O)[c]	91.2	102

[a] In ppm from $H_2{}^{17}O$, with a positive value indicating downfield; estimated error, ±0.2 ppm. [b] In Hz; estimated error, ±7 Hz. [c] Reference 11.

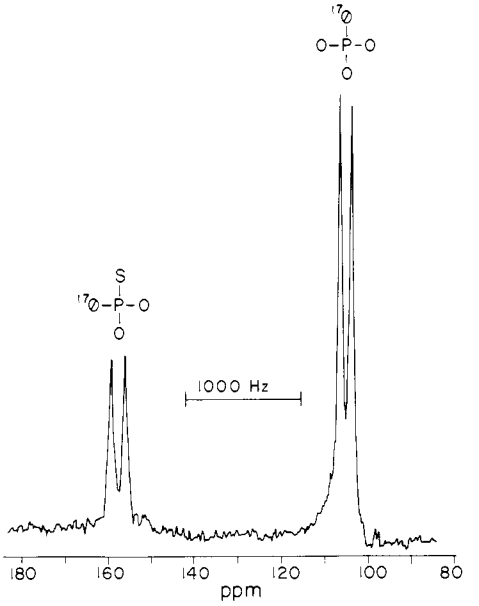

Figure 2. Phosphorus-coupled ^{17}O NMR spectrum at 36.6 MHz and 95 °C of a mixture of inorganic [^{17}O]thiophosphate and phosphate.

The phosphorus-coupled and -decoupled ^{17}O NMR spectra of the enriched samples of AMP, ADP, and ATP obtained at 95 °C are shown in Figures 3–5, respectively; chemical shifts and one-bond ^{31}P–^{17}O coupling constants for the acyclic nucleotides and those previously reported for the diastereomers of cyclic [^{17}O,^{18}O]dAMP are also summarized in Table I. The ATP samples which have a bridging oxygen atom labeled appear to have a broad resonance at about 120 ppm in addition to the relatively narrow resonance associated with the more upfield nonbridging oxygen atom; the assignment of the narrow resonances as the nonbridging oxygen atoms is based on the observation that these resonances are doublets (and not triplets or doublets of doublets) in the absence of phosphorus decoupling. At 30 °C the resonances of all of the nucleotides are too broad to resolve the one-bond ^{31}P–^{17}O coupling (spectra not shown).

The chemical shift differences measured for the individual samples of ADP and of ATP allow resolution and assignment of the resonances in mixtures of either the two enriched ADP samples or the three enriched ATP samples at 95 °C; the ^{31}P-decoupled

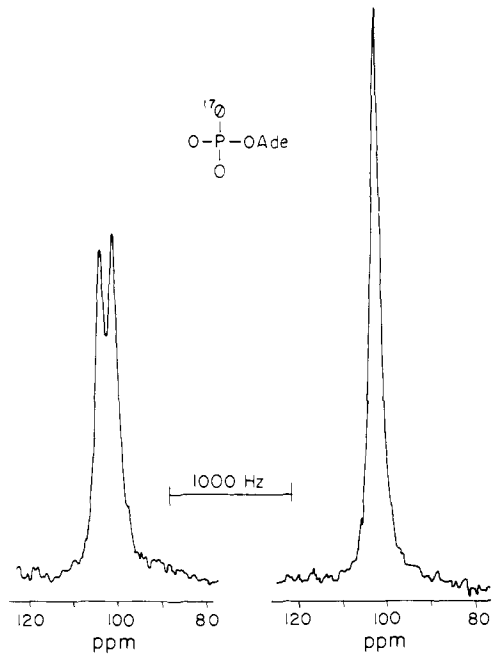

Figure 3. ^{17}O NMR spectra at 36.6 MHz and 95 °C of [^{17}O]AMP: (left) phosphorus-coupled spectrum; (right) phosphorus-decoupled spectrum.

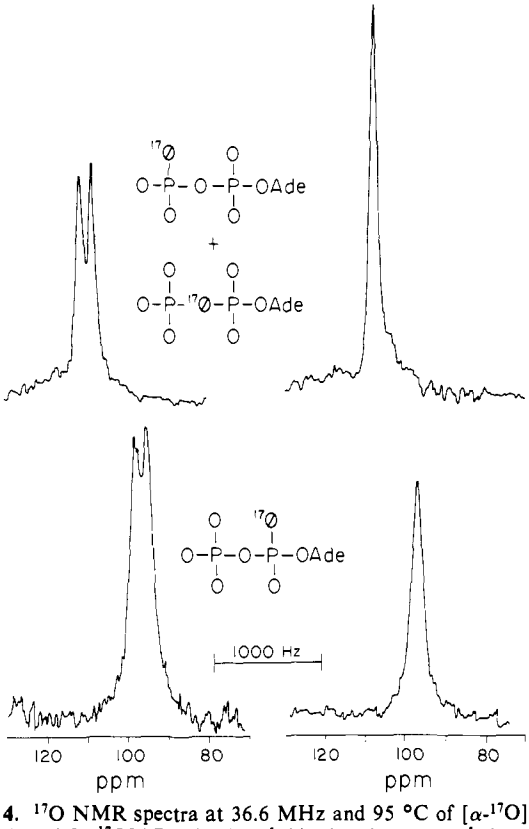

Figure 4. ^{17}O NMR spectra at 36.6 MHz and 95 °C of [α-^{17}O]ADP (bottom) and [β-^{17}O]ADP (top): (left) phosphorus-coupled spectra; (right) phosphorus-decoupled spectra.

spectra of equimolar mixtures of the ADP and ATP samples obtained at 95 °C are shown in Figures 6 and 7, respectively.

Effect of pH on ^{17}O NMR Chemical Shifts. The chemical shifts of weakly acidic phosphoryl oxygens were observed to be dependent on pH. In the pH range 2–13, dimethyl phosphate does not undergo any significant change in state of protonation, and its chemical shift was observed to be independent of pH in this range. However, when a sample of enriched monomethyl phosphate was titrated with acid, an upfield shift of 15.3 ppm was easily observed

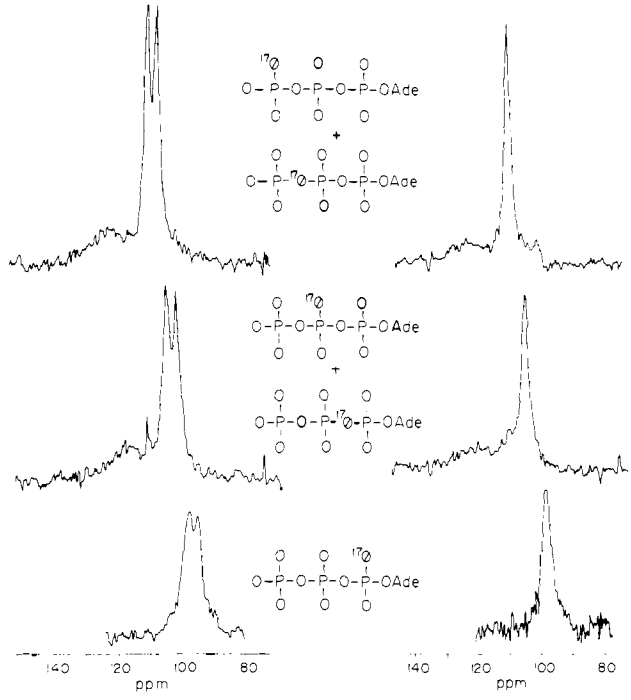

Figure 5. ^{17}O NMR spectra at 36.6 MHz and 95 °C of [α-^{17}O]ATP (bottom), [β-^{17}O]ATP (middle), and [γ-^{17}O]ATP (top): (left) phosphorus-coupled spectra; (right) phosphorus-decoupled spectra.

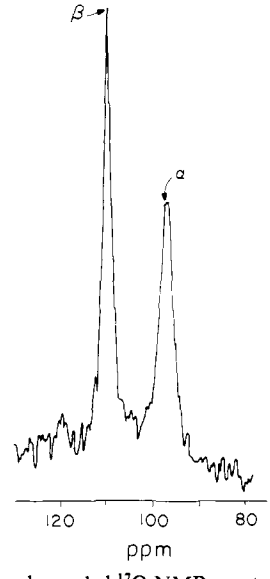

Figure 6. Phosphorus-decoupled ^{17}O NMR spectrum at 36.6 MHz and 95 °C of an equimolar mixture of ^{17}O-labeled ADP samples.

Table II. Ionization Data Obtained from ^{17}O NMR at 30 °C

sample	shift[a]	pK_a	lit. pK_a
[^{17}O]phosphate	12.4	6.7	7.2[b]
	13.6	11.7	12.3[b]
methyl [^{17}O]phosphate	15.3	6.4	6.3[b]
[^{17}O]thiophosphate	18.4	5.4	5.4[c]
	18.3	10.2	10.1[c]
[^{17}O]AMP	14.8	6.4	6.3[b]
[β-^{17}O]ADP	15.8	6.6	6.3[b]
[γ-^{17}O]ATP	15.6	6.9	6.5[b]

[a] Upfield shift in ppm observed upon protonation. [b] Reference 28. [c] Reference 29.

upon protonation of the dibasic species. When a sample of enriched phosphate was titrated with acid, an upfield shift averaging 13.0 ppm was observed upon protonation of either the tribasic or dibasic species. The titration curves obtained at 30 °C are

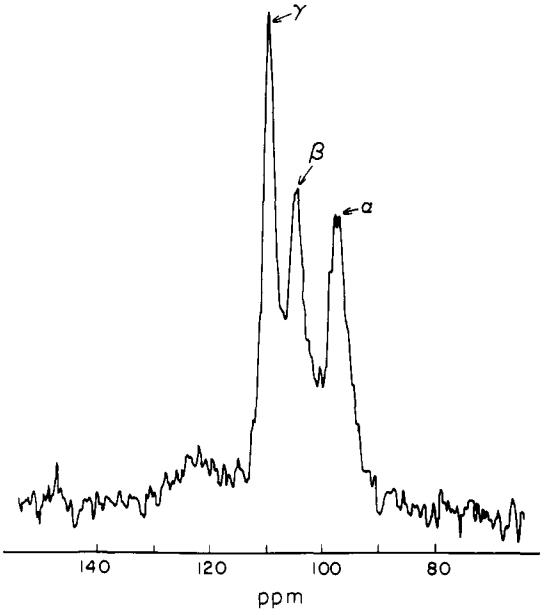

Figure 7. Phosphorus-decoupled ^{17}O NMR spectrum at 36.6 MHz and 95 °C of an equimolar mixture of ^{17}O-labeled ATP samples.

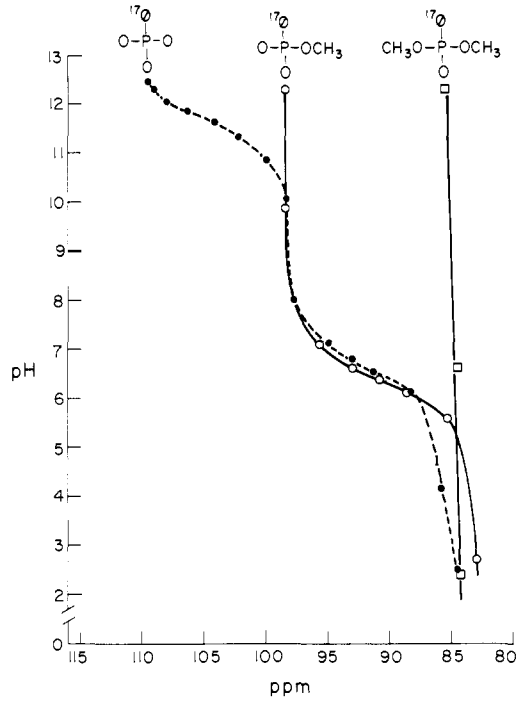

Figure 8. Titration curves for inorganic [^{17}O]phosphate, methyl [^{17}O]-phosphate, and dimethyl [^{17}O]phosphate obtained at 30 °C.

presented in Figure 8. The pK_as and changes in chemical shift resulting from protonation are listed in Table II; for comparison, literature values for the pK_as determined by potentiometric titrations are also included in Table II.

The titration curve obtained for inorganic thiophosphate is compared with that for inorganic phosphate in Figure 9. When thiophosphate was titrated with acid at 30 °C, an upfield shift averaging 18.4 ppm was observed upon protonation of either the tribasic or dibasic species. The pK_as and changes in chemical shift resulting from protonation are also included in Table II.

The titration curves for AMP, ADP, and ATP obtained by titration of the fully ionized species with acid at 30 °C are shown in Figures 10–12, respectively. The magnitude of the upfield chemical shift change produced by protonation of the terminal phosphoryl groups of these nucleotides averaged 15.4 ppm. The experimentally determined pK_as and changes in chemical shift resulting from protonation are listed in Table II.

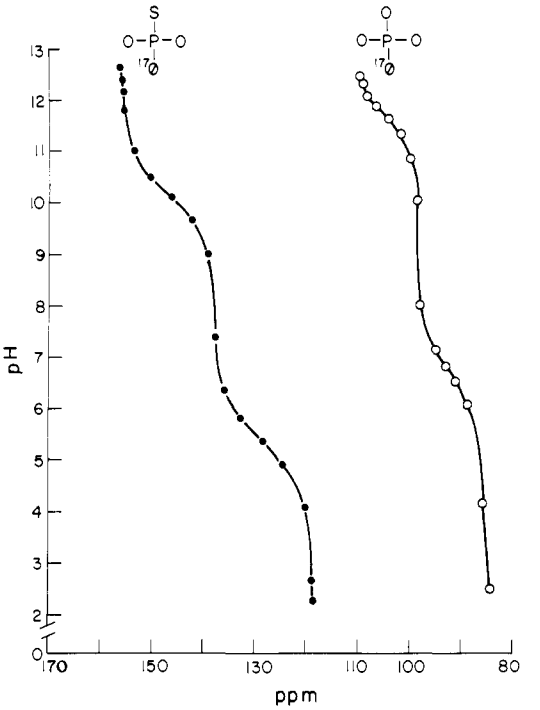

Figure 9. Titration curves for inorganic [^{17}O]thiophosphate and phosphate obtained at 30 °C.

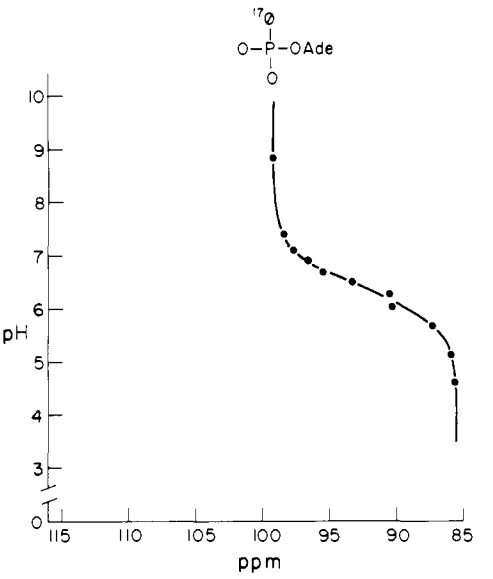

Figure 10. Titration curve for [^{17}O]AMP obtained at 30 °C.

^{17}O NMR Line Widths. If eq 1 and 2 are valid, the line widths of the ^{17}O NMR resonances of phosphoryl oxygens are expected to vary linearly with the ratio of solvent viscosity to temperature. Line widths have been measured as a function of temperature for aqueous solutions of several of the enriched compounds. In Figure 13, the line widths of sodium dimethyl phosphate and disodium monomethyl phosphate are plotted as functions of η'/T; for both compounds the data can be fitted to linear equations which extrapolate through the origin. This behavior is in accord with the prediction of eq 1 and 2. In Figure 14, the line widths of the diastereomers of cyclic [^{17}O,^{18}O]dAMP are plotted as a function of η'/T; in Figure 15, the line widths of the samples of [α-^{17}O]- and [β-^{17}O]ADP are plotted as a function of η'/T. In contrast to the behavior observed for dimethyl and monomethyl phosphates, the data for the nucleotides clearly do not follow a linear dependence on η'/T.

The data plotted in Figures 13–15 allow several important observations regarding ^{17}O line widths of phosphoryl oxygens: (1) the line width of dimethyl phosphate is less than that of mono-

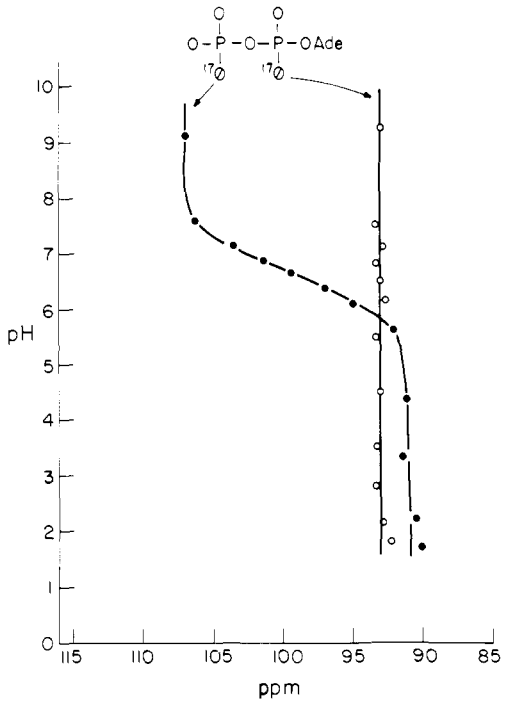

Figure 11. Titration curves for the samples of [^{17}O]ADP obtained at 30 °C.

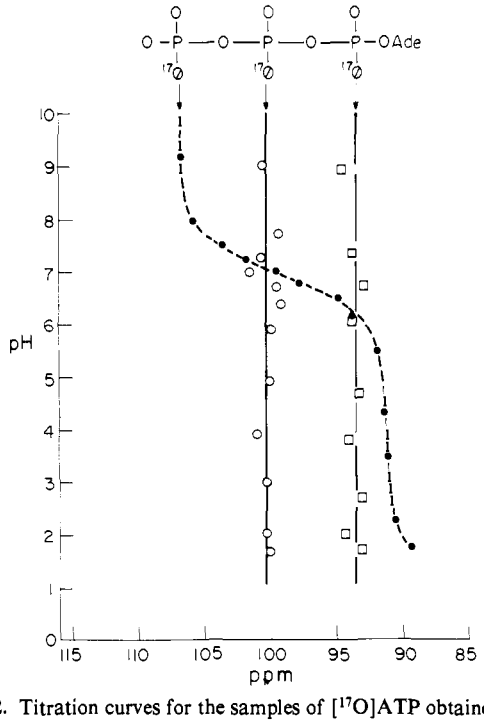

Figure 12. Titration curves for the samples of [^{17}O]ATP obtained at 30 °C.

methyl phosphate; (2) the line widths of the phosphodiester phosphoryl oxygens in the conformationally rigid cyclic dAMP are not identical, with the axially positioned ^{17}O nucleus having the smaller line width; and (3) the line widths for the α- and β-phosphoryl oxygens of ADP differ, with that for the terminal phosphoryl oxygens being smaller than that for the internal phosphoryl oxygens.

Discussion

General Comments Concerning Line Widths. The relatively narrow ^{17}O NMR resonances we have observed for phosphoryl oxygens can be rationalized by the reported values for the nuclear quadrupolar coupling constants experimentally established for the oxygen atoms in solid triphenyl phosphate. Cheng and Brown

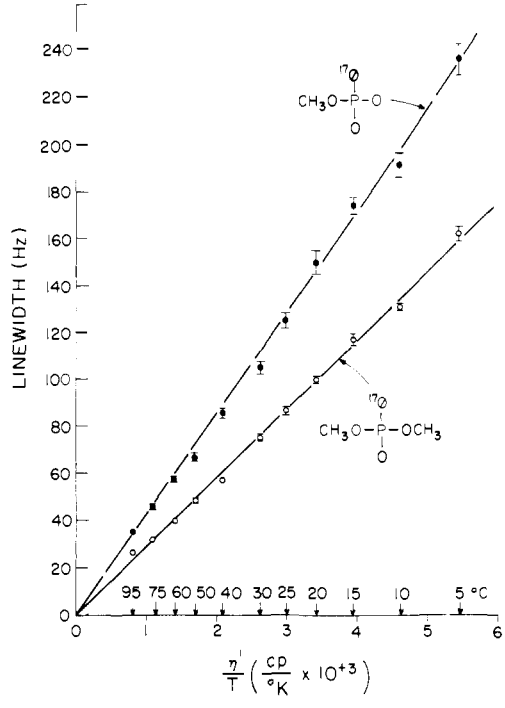

Figure 13. Dependence of ^{17}O NMR line width on temperature for aqueous solutions of methyl and dimethyl phosphates.

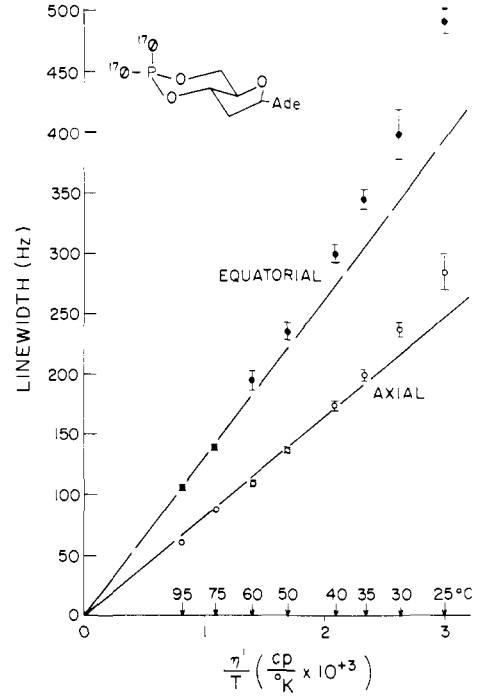

Figure 14. Dependence of ^{17}O NMR line widths on temperature for aqueous solutions of the diastereomers of cyclic [^{17}O,^{18}O]dAMP.

reported that the phosphoryl oxygen in triphenyl phosphate has an associated nuclear quadrupolar coupling constant of 3.8 MHz whereas the ester oxygens have nuclear quadrupolar coupling constants of 9.0 MHz.[18] Since, according to eq 1, the line widths of ^{17}O resonances are directly proportional to the square of the nuclear quadrupolar coupling constant, it can be anticipated that the phosphoryl oxygens would have smaller line widths than those of ester oxygens. This dependence of line widths on nuclear quadrupolar coupling constants is dramatically illustrated by the ^{17}O NMR spectrum of trimethyl phosphate which Tsai and co-workers recently published;[8] the line widths of the resonances were

(18) Cheng, C. P.; Brown, T. L. *J. Am. Chem. Soc.* **1980**, *102*, 6418.

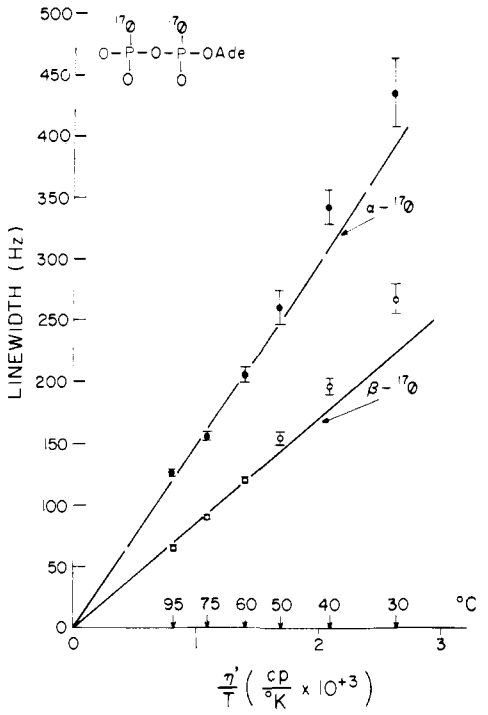

Figure 15. Dependence of ^{17}O NMR line width on temperature for aqueous solutions of the ^{17}O-labeled ADP samples.

such that the one-bond ^{31}P–^{17}O coupling constant for the phosphoryl oxygen was well resolved whereas that for the ester oxygens could not be resolved. In the samples of ^{17}O-enriched materials we prepared, only those samples of ADP and ATP that have bridging labeled oxygens are expected to have broad resonances, and in these samples we may have detected extremely broad resonances that might be associated with these oxygens and that may be predicted to have large nuclear quadrupolar coupling constants.

The equation for line width (eq 1) also contains a factor which expresses the effect of field gradient asymmetry on the line width. The asymmetry parameter, η, can vary between 0 and 1, with nuclei in axially symmetric environments having $\eta = 0$ (nuclei which are in spherically symmetric environments have no field gradient asymmetry).[7] Thus, even though this factor cannot deviate significantly from unity, phosphoryl oxygens can be anticipated to experience little line broadening from field gradient asymmetry.

Effect of Charge on Chemical Shift. The spectra shown in Figure 1 suggest that the magnitude of the negative charge on phosphoryl oxygens is of importance in determining the ^{17}O NMR chemical shift. In their most highly ionized forms, inorganic phosphate, methyl phosphate, dimethyl phosphate, and trimethyl phosphate may naively be considered to have phosphoryl oxygen negative charges of $^3/_4$, $^2/_3$, $^1/_2$, and 0 per oxygen atom, respectively. The spectra indicate that as the charge per oxygen decreases, the resonance moves upfield.[19] This dependence of chemical shift on phosphoryl oxygen charge is further demonstrated by the effect of protonation on the chemical shifts of weakly acidic phosphoryl oxygens. The titration curves presented in Figure 8 indicate that as the phosphoryl oxygen charge is made more positive, the resonance shifts upfield. The pK_a values obtained from the ^{17}O NMR data are in good agreement with those obtained by potentiometric titration (Table II).

The relative magnitudes of the upfield shifts induced by protonation of the phosphoryl oxygens in inorganic phosphate and methyl phosphate additionally suggest that the charge on the phosphoryl oxygen may be quantitatively related to the changes in chemical shift produced by charge neutralization. The upfield shift observed upon protonation of either the tribasic or dibasic species of inorganic phosphate (approximately 13 ppm) is less than that observed for the protonation of the dibasic form of methyl phosphate (approximately 15.3 ppm); in the former case, the charge neutralized by protonation is $^1/_4$ per phosphoryl oxygen, whereas in the latter case the charge neutralized is $^1/_3$ per phosphoryl oxygen. At least qualitatively, therefore, the changes in chemical shift produced by protonation can be related to the change in anionic charge on the phosphoryl oxygens. Interestingly, our ^{17}O NMR data and those available in the literature are consistent with the hypothesis that the change in chemical shift induced by charge neutralization (e.g., protonation) is quantitatively proportional to the change in charge. Protonation of carboxylate anions produces upfield shifts in the ^{17}O NMR chemical shift of the carboxylate oxygens, with the values reported being 21.3 ppm for formate anion[20] and 23.6 ppm for acetate anion;[21] in each case, the charge neutralized is $^1/_2$ per oxygen. Thus, the data now available for the protonation of weakly acidic oxygen atoms indicate that the upfield chemical shift per charge neutralized is approximately constant, with the data for inorganic phosphate, methyl phosphate, and carboxylates giving values of 52, 46, and 44 ppm, respectively; the change in ^{17}O NMR chemical shift of hydroxide induced by protonation is of the same order.[22] Although the available data are limited, these observations could suggest that changes in ^{17}O NMR chemical shifts produced by charge neutralization may be used to directly measure changes in charge density on oxygens. If further experimentation supports this hypothesis, ^{17}O NMR studies may be of considerable use in determining sites and degree of charge neutralization of phosphoryl oxygens both in small molecules and perhaps even those associated with macromolecules.

As previously noted, Jaffe and Cohn have concluded that ^{31}P NMR chemical shifts cannot be used with confidence to determine sites and extent of charge neutralization in phosphate esters and nucleotides.[3] One experimental observation that forced this conclusion was the dependence of ^{31}P NMR chemical shifts on pH for inorganic phosphate and thiophosphate: protonation of phosphate causes an upfield shift of the ^{31}P resonance whereas protonation of thiophosphate causes a downfield shift of the ^{31}P resonance. To determine whether a similar difference in behavior would be observed when the titrations of phosphate and thiophosphate were monitored by ^{17}O NMR spectroscopy, we prepared a sample of labeled thiophosphate. As demonstrated in Figure 9, the ^{17}O NMR resonances of enriched phosphate and thiophosphate both shift upfield upon protonation, with the pK_a values determined from the data (Table II) being in excellent agreement with values obtained from potentiometric titrations. This observation additionally suggests that ^{17}O NMR spectroscopy can be used profitably to unambiguously determine sites of charge neutralization. Clearly, additional examples of upfield shifts of resonances of thiophosphates arising from protonation must be established and the syntheses and titrations of ^{17}O-enriched samples of various thionucleotides are under way in this laboratory. Quantitatively, protonation of thiophosphate produces a larger upfield shift (approximately 18.4 ppm) than does protonation of

(19) A referee has pointed out that charge neutralization, i.e., a decrease in the electron density surrounding the nucleus, is not expected to lead to upfield shifts of resonances, since the resulting effect on both the diamagnetic and paramagnetic contributions to the chemical shift is predicted to cause downfield shifts of resonances (less shielding). Since we have repeatedly verified that we have not inadvertently reversed our spectra, our experimental observations point to an inadequacy in the understanding of the origin of chemical shifts. Given this inadequacy in the existing theory, our experimental investigation of the ^{17}O NMR spectral properties of phosphate esters and nucleotides is necessarily directed toward determining whether useful *empirical* relationships exist between charge neutralization and any spectral parameters, since, as pointed out in this article, such relationships do not exist for ^{31}P NMR spectral parameters. The data we report in this article suggest that the ^{17}O chemical shift may prove to be an informative spectral parameter, and the interpretations we make in this discussion are intended to point out the potential utility of this spectral parameter, even though a precise theoretical explanation for the behavior we have observed does not yet exist.

(20) Weaver, J. E.; Tolbert, B. M.; LaForce, R. D. *J. Chem. Phys.* **1955**, *23*, 1956.
(21) Rueben, J. *J. Am. Chem. Soc.* **1969**, *91*, 5725.
(22) Luz, Z.; Yagil, G. *J. Phys. Chem.* **1966**, *70*, 554.

phosphate; this can be most easily rationalized by the previously reported determination that in the thiophosphate the P–S bond is largely a double bond that does not participate in resonance delocalization of the negative charges found on the phosphoryl oxygens.[23] The upfield chemical shift per charge neutralized, 56 ppm, is similar to that observed for inorganic phosphate, methyl phosphate, and carboxylates.

The relative chemical shifts for the various enriched nucleotides may be rationalized on the basis of the charge density at the various phosphoryl oxygens. The chemical shifts of the phosphoryl oxygens of AMP and methyl phosphate are nearly identical in the pH range 2–9, as would be expected considering the chemical similarity of these compounds. The chemical shift data for the samples of ADP and of ATP are qualitatively consistent with the relative chemical shifts found for methyl and dimethyl phosphates; i.e., the oxygens of monoester-like phosphoryl oxygens are downfield of those of the diester-like phosphoryl oxygens. However, quantitative differences are apparent, since the terminal phosphoryl oxygens of ADP and ATP have chemical shifts more downfield than those found for methyl phosphate or AMP; in addition, the chemical shifts of the internal phosphoryl oxygens of the nucleotides are also downfield of that found for dimethyl phosphate, and the chemical shifts of the α- and β-phosphoryl oxygens of ATP differ significantly. Although calculations of the electronic structures of phosphate esters and nucleotides have been reported, these do not appear to provide a quantitative explanation for the chemical shifts observed for the phosphoryl oxygens in ADP and ATP. The atomic charges assigned to the phosphoryl oxygens in ADP and ATP by Boyd and Lipscomb[24] are not sufficiently different to explain the observed differences in ^{17}O NMR chemical shifts. However, if it is true that phosphoryl oxygen charge density is important in determining ^{17}O NMR chemical shifts, as suggested by our data, the chemical shifts we have observed may indicate that the charge densities on the phosphoryl oxygens in the nucleoside di- and triphosphates are greater than those found for simple phosphate esters, e.g., AMP and methyl and dimethyl phosphates. This effect may be due to the donation of electron density from the electron-rich phosphoryl functional groups to adjacent phosphorus atoms; such an explanation would explain the more downfield chemical shift of the β-phosphoryl oxygens of ATP relative to the α-phosphoryl oxygens. Our ^{17}O NMR spectral data may be a direct measure of the charge density on the phosphoryl oxygens of nucleoside di- and triphosphates and provide an impetus for renewed theoretical attempts to calculate the charge densities in these biologically important molecules.

The titraton data for the nucleotides provide the first unambiguous (but not unexpected) demonstration that the terminal phosphoryl oxygens of ADP and ATP are the most basic in the molecules. ^{17}O NMR spectroscopy provides a microscopic probe of the ionization behavior of polyelectrolytes[6] such as nucleotides, and these types of determinations emphasize the types of unique data that are available only through this spectroscopic probe. The titration data also demonstrate that the change in chemical shift induced by protonation is again consistent with the hypothesis that charge density is important in determining ^{17}O NMR chemical shifts, since for each nucleotide the upfield shift induced by protonation is approximately 47 ppm per charge neutralized.

Dependence of Line Widths on Temperature. Although the initial part of this section considered the fact that the line widths of phosphoryl oxygens are sufficiently small that the resonances can be relatively easily detected, it is important to note that line-width data can potentially provide important chemical and physical information. The data in Figure 13 demonstrate that the line widths of methyl and dimethyl phosphates are in accord with eq 1, since the line width of dimethyl phosphate is smaller than that observed for methyl phosphate and both are directly proportional to η'/T. The data of Cheng and Brown indicate that increasing double bond character in P–O bonds is associated with a decrease in the nuclear quadrupolar coupling constant,[17] thus it can be expected that dimethyl phosphate would have a smaller line width than methyl phosphate, and this is experimentally realized. The fact that the data for both methyl and dimethyl phosphates can be fitted to linear equations demonstrates that the molecular motion of these molecules is isotropic, an assumption on which the Stokes equation (eq 2) is based. In principle it is possible to obtain values for either the nuclear quadrupolar coupling constant or the rotational correlation times for these molecules from the data in Figure 13, if the other parameter is known. Future work in this laboratory will include evaluation of these quantities.

Examination of the line-width data in Figure 14 allows two important observations to be made about the physical and chemical properties of cyclic dAMP: (1) the line widths of the phosphoryl oxygens of the conformationally rigid diester cyclic dAMP are not identical, and (2) the Stokes equation (eq 2) is apparently not obeyed by this solute. The first observation can be most easily explained in terms of different nuclear quadrupolar coupling constants for these oxygens, since it can be expected that the phosphoryl oxygens should experience the same rotational motion. The hypothesis that the nuclear quadrupolar coupling constants are different implies that the bond orders of these P–O bonds are different, with the axial P–O bond having more double bond character than the equatorial P–O bond. This explanation is in accord with chemical data which has been reported independently by the laboratories of Verkade[25] and Gorenstein.[26] These workers found that the reaction of conformationally rigid cyclic phosphodiesters with diazomethane leads to preferential reaction with either the axial or equatorial phosphoryl oxygen, depending on the solvent. In aqueous solution, Gorenstein observed that a rigid six-membered ring phosphodiester was methylated exclusively on the equatorial phosphoryl oxygen, which is in accord with our observation that the equatorial P–O bond in cyclic dAMP apparently has more single bond character than the axial P–O bond. Clearly, our observation demands more experimental exploration, e.g., an examination on the line-width dependence on solvent, but the ability to experimentally determine P–O bond character in solution clearly would be of use in a variety of chemical and biochemical problems. The observation that the Stokes equation is apparently not obeyed by cyclic dAMP may be surprising considering the relatively small size of this solute molecule, but examination of a molecular model reveals that the molecule is certainly not spherical. ^{17}O NMR spectral data may provide a sensitive method for determining whether solute molecules undergo isotropic or anisotropic motion in solution.

Examination of the line-width data in Figure 15 similarly allows deduction of important properties of the ADP molecule: (1) the line width of the β-phosphoryl oxygens is smaller than that of the α-phosphoryl oxygens; (2) the Stokes equation is also not obeyed by this solute. As previously discussed, the line width of methyl phosphate is expected and observed to be greater than that of dimethyl phosphate; if these are considered as models for the β- and α-phosphoryl oxygens, respectively, of ADP, some other factor is clearly affecting the line widths of the phosphoryl oxygens in ADP. The most straightforward explanation for the observed behavior is that the rotational correlation times of the α- and β-phosphoryl oxygens of ADP are significantly different, with the β-phosphoryl oxygens having a smaller correlation time. This is easily understood since the terminal phosphoryl group in ADP can experience rotation about the bridging P–O bonds that is unavailable to the internal phosphoryl group. Without an independent measure of the nuclear quadrupolar coupling constant for the phosphoryl oxygens of nucleotides, it is presently impossible to quantitatively evaluate the differences in rotational correlation time. However, this observation does indicate that a potentially valuable application of ^{17}O NMR spectroscopy is the direct evaluation of rotational correlation times, since the predominant relaxation mechanism for ^{17}O is quadrupolar; estimation of

(23) Steger, E.; Martin, K. *Z. Anorg. Allg. Chem.* **1963**, *323*, 108.
(24) Boyd, D. B.; Lipscomb, W. N. *J. Theor. Biol.* **1969**, *25*, 403.
(25) Hong, A. P.; Lee, J. B.; Verkade, J. G. *J. Am. Chem. Soc.* **1976**, *98*, 6547.
(26) Gorenstein, D. G.; Rowell, R. *J. Am. Chem. Soc.* **1980**, *102*, 6166.

correlation times from the relaxation times of dipolar nuclei is usually complicated by multiple relaxation mechanisms.

Summary

In summary, we believe that the ^{17}O NMR spectral data reported in this paper illustrate the great potential of this spectroscopic technique in providing otherwise inaccessible information about the chemical and physical properties of phosphate esters and nucleotides. Although it is presently uncertain whether direct examination of the ^{17}O NMR spectral properties of nucleotides bound to enzymes will be possible (since the line widths are proportional to rotational correlation times), further characterization of ^{17}O NMR spectral properties of phosphate esters and development of techniques for improved resolution of resonances of quadrupolar nuclei[27] may ultimately allow such experiments to be performed.

Note Added in Proof. Since the manuscript was accepted for publication, we have made several additional observations regarding the ^{17}O NMR spectral properties of phosphate esters and nucleotides which are relevant to the content of the article.

(1) We have assigned the ^{17}O NMR resonances of the bridging and nonbridging phosphoryl oxygens in pyrophosphate, with the chemical shift of the bridging oxygen being 123 ppm. This chemical shift is in very good agreement with those associated with the broad resonances observed in the spectra of the ATP samples prepared with the bridging oxygens labeled (Figures 5 and 7). This information should allow confident assignment of the resonances of the bridging oxygen atoms in ATP.

(2) The resonance associated with the nonbridging oxygens of pyrophosphate undergoes upfield chemical shifts averaging 8.3 ppm when either the tetra- or trianionic species is protonated; the upfield chemical shift per charge neutralized is 50 ppm. We have also observed that the thiophosphoryl oxygens in AMPS and [γ-^{17}O]ATPγS undergo upfield chemical shifts of 22.4 and 24.0 ppm, respectively, when the dianionic species are protonated; assuming that no charge is delocalized on the sulfur, these correspond to upfield chemical shifts of 45 and 48 ppm, respectively, per charge neutralized. We have also noted a dependence of chemical shift on charge neutralized in phosphonates, with upfield chemical shifts averaging 9.8 ppm being found for the protonation of the tetra- and trianionic species of methylene diphosphonate, 18.6 ppm for protonation of the dianionic species of methyl phosphonate, and 18.0 ppm for the terminal oxygens in the β,γ-methylene analogue of ATP (no change is observed in the chemical shift for the β oxygens); these correspond to upfield chemical shifts of 59, 56, and 54 ppm, respectively. These observations provide substantial additional evidence for the hypothesis that ^{17}O NMR chemical shifts may be used to quantitate charge neutralization of phosphoryl oxygens. Some of these experiments were carried out in collaboration with Mark Reynolds and Professor George L. Kenyon, University of California, San Francisco, and with Professor Barry S. Cooperman, University of Pennsylvania.

(3) In collaboration with Virginia W. Miner and Professor James H. Prestegard of this department, we have observed the ^{17}O NMR resonance of multiply labeled AMP bound to ribonuclease A. At 30 °C and pH 5.5, the line width of 3 mM AMP is approximately 300 Hz; in the presence of 4.1 mM ribonuclease A, the line width increases to approximately 2000 Hz. Based upon the literature, these experimental conditions should result in essentially quantitative binding of AMP to the enzyme. The observation of a resonance of moderate line width in the presence of the macromolecule demonstrates that eq 1 in this article cannot be used to predict the line width behavior of ^{17}O NMR nuclei bound to macromolecules; instead, a numerical solution for T_1 and T_2 as a function of $\omega_0\tau_c$ should be used.[30] This analysis reveals that the line widths of the resonances of spin $^5/_2$ nuclei are expected to be maximal when $\omega_0\tau_c = 1$ and to decrease at either shorter or longer values of $\omega_0\tau_c$. Our observation and the numerical solution for T_2 suggest that the common expectation that the ^{17}O NMR resonances of macromolecules will be unobservable due to excessive line widths is incorrect, in agreement with the results recently reported for the ^{43}Ca NMR resonances of enriched Ca^{2+} bound to several proteins.[31]

These and related observations regarding the ^{17}O NMR spectral properties of nucleotides will be described in more detail in forthcoming manuscripts.

Acknowledgment. We thank Professor James H. Prestegard and Dr. Daniel D. Traficante for their interest, encouragement, and advice. Professor W. W. Cleland kindly supplied the computer program used to analyze the titration data for dibasic acids. This research was supported by a grant (GM-22350) from the National Institutes of Health. The high-field NMR spectrometer used in this research is supported in part by a grant from the National Science Foundaton (CHE-791620).

Registry No. [^{17}O]P(O)Cl_3, 66943-75-7; [^{17}O]P(OMe)$_3$, 80777-98-6; [^{17}O]P(OMe)$_2$OH Na, 81246-52-8; [^{17}O]P(OH)$_2$OMe Na, 81246-53-9; [^{17}O]-P(OH)$_3$ NH_3, 81246-54-0; [^{17}O]-AMP 2Et_3N, 81246-56-2; [α-^{17}O]-ADP, 81246-57-3; [B-^{17}O]-ADP, isomer 1, 81246-58-4; [B-^{17}O]-ADP, isomer 2, 81246-59-5; [α-^{17}O]-ATP, 81246-60-8; [B-^{17}O]-ATP, isomer 1, 81246-61-9; [B-^{17}O]-ATP, isomer 2, 81246-62-0; [γ-^{17}O]-ATP, isomer 1, 81246-63-1; [γ-^{17}O]-ATP, isomer 2, 81246-64-2; [^{17}O]P(O)(OH)$_2$SH NH_3, 81246-65-3.

(27) Miner, V. W.; Prestegard, J. H. *J. Am. Chem. Soc.* **1981**, *103*, 5979.
(28) 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.
(29) Peacock, C. J.; Nickless, G. *Z. Naturforsch. A* **1969**, *24A*, 245.
(30) Bull, T. E.; Forsén, S.; Turner, D. L. *J. Chem. Phys.* **1979**, *70*, 3106.
(31) Andersson, T.; Drakenberg, T.; Forsén, S.; Thulin, E.; Swärd, M. *J. Am. Chem. Soc.* **1982**, *104*, 576.