Applicability of the Phosphorus-31 (Oxygen-17) Nuclear Magnetic Resonance Method in the Study of Enzyme Mechanism Involving Phosphorus†

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ABSTRACT: The phosphorus-31 (oxygen-17) [^{31}P (^{17}O)] NMR method [Tsai, M.-D. (1979) *Biochemistry 18*, 1468–1472] is tested for its general applicability by correlation with the line widths of the ^{17}O NMR signals for the following compounds: trimethyl [$^{17}O_4$]phosphate (**1**), [$^{17}O_4$]phosphate (**2**), [α-$^{17}O_2$]adenosine 5′-(thiophosphate) (**3**), [α-^{17}O,$\alpha\beta$-^{17}O]-adenosine 5′-(1-thiotriphosphate) (**4**), [γ-$^{17}O_3$]adenosine 5′-triphosphate (**5**), [$\alpha\beta$,$\beta\gamma$-$^{17}O_2$,β-$^{17}O_2$]adenosine 5′-triphosphate (**6**), magnesium [γ-$^{17}O_3$]adenosine 5′-triphosphate (**7**), and magnesium [$\alpha\beta$,$\beta\gamma$-$^{17}O_2$,β-$^{17}O_2$]adenosine 5′-triphosphate (**8**). It is found that the line broadening effect of ^{17}O on the ^{31}P signals of the directly bonded ^{31}P nuclei is present for all the functional groups in the above compounds which include examples of small ^{17}O line widths (**1** and **2**), of intermediate ^{17}O line widths (**3**, **5**, and the nonbridge ^{17}O of **4** and **6**), and of very large ^{17}O line widths (**7**, **8**, and the bridge ^{17}O of **4** and **6**). On the basis of the established approximate relationship $\Delta X \Delta Q \approx aJ^2$, where ΔX and ΔQ are the ^{31}P and ^{17}O line widths, respectively, of the ^{31}P–^{17}O groups, a is a constant, and J is the ^{31}P–^{17}O coupling constant, the results suggest that for most of the phosphate derivatives of biochemical interest, the line broadening effect of ^{17}O should be present and detectable; i.e., ΔX should be larger than the limiting value (~20 Hz). It is also found that Mg^{2+} causes the ^{17}O signal of **5** and **6** to broaden (ΔQ increases), which in turn causes the ^{31}P signal to sharpen (ΔX decreases). This finding suggests that the ^{31}P (^{17}O) NMR method, in combination with ^{17}O NMR, could become a tool to study diamagnetic metal ion–nucleotide interactions.

We have recently reported that an ^{17}O nucleus directly bonded to a ^{31}P nucleus causes a quantitative decrease in the ^{31}P NMR signal and successfully used this method to elucidate the stereochemical course of acetate activation at the phosphorus center catalyzed by yeast acetyl-coenzyme A synthetase (Tsai, 1979). Whether the ^{31}P (^{17}O) NMR method is generally applicable to other biochemical problems involving phosphoryl transfer has become a question of wide interest. Based on the correlation between the line widths of the ^{17}O and ^{31}P NMR signals, we now present results which suggest that the line broadening effect of ^{17}O on the ^{31}P signals should be generally present in most of the phosphate derivatives of biochemical interest and that the ^{31}P (^{17}O) NMR method could become a useful tool in stereochemical and mechanistic studies of biological phosphoryl transfer reactions, particularly the diamagnetic metal ion–nucleotide interactions.

Materials and Methods

Materials. The 41% $H_2{}^{17}O$ (containing 41% $H_2{}^{17}O$ and 28% $H_2{}^{18}O$) and the 53% $H_2{}^{17}O$ (containing 53% $H_2{}^{17}O$ and 42% $H_2{}^{18}O$) were purchased from Miles Laboratory and Monsanto Co., respectively. Carbamate kinase (*Streptococcus faecalis*; 700–1000 units/mg) was purchased from Sigma Chemical Co. Puratronic $Mg(NO_3)_2$ (99.999% pure) was purchased from Ventron Co. Other biochemicals used were obtained from Boehringer. The chemicals used were of reagent grade of the highest purity available commercially.

Preparation of ^{17}O-Labeled Compounds. [$^{17}O_4$]Phosphate (**2**), [α-$^{17}O_2$]AMPS[1] (**3**), and [α-^{17}O,$\alpha\beta$-^{17}O]ATPαS (**4**) (isomer A) were available from previous work (Tsai, 1979). Trimethyl [$^{17}O_4$]phosphate (**1**) was obtained by the methylation of compound **2** with CH_2N_2. [$\alpha\beta$,$\beta\gamma$-$^{17}O_2$,β-$^{17}O_2$]ATP (**6**) was prepared as previously described (Tsai, 1979), except that 51% ^{17}O-enriched P_i (obtained from PCl_5 and 53% $H_2{}^{17}O$) was used. [γ-$^{17}O_3$]ATP (**5**) was prepared according to the procedure previously used to make [γ-^{32}P]ATP (Mokrascho et al., 1960) by use of 51% ^{17}O-enriched P_i.

NMR Measurements. ^{31}P and ^{17}O NMR spectra were recorded at 32.2 and 10.8 MHz, respectively, at ambient temperature, on a Varian FT-80 NMR spectrometer equipped with a multinuclear probe. The field was locked on deuterium, and all ^{31}P spectra were recorded with broad-band proton decoupling. All samples were dissolved in 1.0–1.5 mL of 99% D_2O in 10-mm tubes, except that trimethyl phosphate was dissolved in $CDCl_3$. The ^{31}P and ^{17}O chemical shifts are expressed relative to 85% H_3PO_4 and $H_2{}^{17}O$, respectively, as external references.

The following conditions were used unless specified: for ^{31}P NMR, spectral width 2000 Hz, acquisition time 2.0 s, pulse width 10 μs, and pulse delay 10 s; for ^{17}O NMR, spectral width 8197 Hz, acquisition time 0.1 s, pulse width 25 μs, pulse delay 0.01 s, and α delay 400 μs. In both cases a large weighting factor was applied in order to obtain a better signal to noise ratio. The small ^{18}O isotope effect on the ^{31}P chemical shift (0.02–0.04 ppm) (Cohn & Hu, 1978) is not detectable under the present conditions. Throughout this manuscript the ^{18}O isotope which is present in the labeled water used is considered as ^{16}O since it has little effect on the apparent intensity of ^{31}P signals.

Rationale

A nucleus with nuclear spin I greater than $^1/_2$ possesses an electric quadrupole moment eQ. The dominant relaxation

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[1] Abbreviations used: AMPS, adenosine 5′-(thiophosphate); ATPαS, adenosine 5′-(1-thiotriphosphate); P_i, inorganic phosphate; AMP, adenosine 5′-phosphate; ATP, adenosine 5′-triphosphate.

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mechanism for quadrupolar nuclei comes from interactions of eQ with an electric field gradient eq at the nucleus and the modulation of these interactions by rotational motion (James, 1975). In the extreme narrowing conditions, i.e., very fast molecular motions with respect to resonance frequency, which is the case for small molecules in solution, the contribution of nuclear quadrupole relaxation to the relaxation rate can be expressed as (Abragam, 1961)

$$\frac{1}{T_q} \approx \frac{1}{T_{1q}} \approx \frac{1}{T_{2q}} \approx \frac{3}{40} \frac{2I+3}{I^2(2I-1)}\left(1+\frac{\eta^2}{3}\right)\left(\frac{e^2qQ}{\hbar}\right)^2 \tau r \quad (1)$$

where $e^2qQ/\hbar$ is the quadrupole coupling constant, η is the asymmetry parameter, and τr is the rotational correlation time.

It is well established (Lehn & Kintzinger, 1973; Abragam, 1961) that when a dipolar nucleus is directly coupled with a quadrupolar nucleus Q, the line shape of the NMR signal of X is governed by the quadrupolar relaxation time (T_q) of Q and the coupling constant (J) between the two nuclei. (A) When T_qJ is large, the signal of X is split by spin–spin coupling. (B) As T_qJ decreases, line broadening of the coupling pattern occurs. (C) When T_qJ is small, the signal of X appears as a broad singlet, the line width of which is determined by

$$1/T_{2x} = a\pi^2 T_q J^2 \quad (2)$$

where $a = (4/3)I(I+1)$ and I is the spin number of Q. Making a reasonable assumption that $\Delta X \approx 1/\pi T_{2x}$ and $\Delta Q \approx 1/\pi T_q$, where ΔX and ΔQ are the line widths of the dipolar nucleus X and the quadrupolar nucleus Q, respectively, one could obtain the following relationship derived from eq 2:

$$\Delta X \Delta Q \approx aJ^2 \quad (3)$$

In the case of ^{31}P–^{17}O, ^{31}P is the dipolar nucleus ($I = 1/2$) and ^{17}O is the quadrupolar nucleus ($I = 5/2$). If T_qJ is in the intermediate range or smaller, the ^{17}O effect can be measured by the decrease in the integration of the ^{31}P NMR signal, as previously described (Tsai, 1979). However, in an extreme case of (C), i.e., when J is very small or ΔQ is very large, ΔX could become so small that the quadrupolar effect is not detectable by the integration of the ^{31}P signal. Such an extreme condition, many examples of which can be found in the compounds containing other quadrupolar nuclei such as Cl, could limit the application of the ^{31}P (^{17}O) method or could lead to an erroneous conclusion if it is not perceived. Whether the ^{31}P (^{17}O) method is generally useful depends on whether ΔX is larger than the limiting value ($\sim$20 Hz) for most of the phosphate derivatives of biological significance.

In principle, ΔX is dependent on both J and ΔQ. The coupling constant J is not readily measurable for most compounds, but J should depend mainly on the functional groups. On the other hand, the line width of the NMR signal of the quadrupolar nucleus, ΔQ, is very sensitive to the rotational correlation time and, to a lesser extent, molecular symmetry (Lehn & Kintzinger, 1973). For compounds with similar functional groups, e.g., various phosphate derivatives, the variation of J with respect to similar P–O bonds is expected to be $\pm$20 Hz, but the variation of ΔQ could be much larger than 10-fold. We tested the general applicability of the ^{31}P (^{17}O) method by correlating ΔX (measured by the apparent decrease in integration) with ΔQ (obtained from ^{17}O NMR) for a series of compounds which cover a large range of ΔQ.

Results

Examples of Small ^{17}O Line Widths. These molecules are usually small and symmetrical, such as trimethyl [$^{17}O_4$]phosphate (**1**) and [$^{17}O_4$]phosphate (**2**). Under certain conditions the ^{31}P–^{17}O coupling can be observed, as shown by the ^{17}O NMR spectrum of **1** (Figure 1). The symmetrical environment of the P=O bond of **1** is reflected in the very narrow ^{17}O line width ($W_{1/2} \approx 50$ Hz) which is smaller than the coupling constant J (154 Hz). Such a coupling has also been observed from the ^{31}P NMR spectrum of ^{17}O=P(OCH$_3$)$_3$ in which the ^{31}P signal is split into six very broad ($W_{1/2} = 90$ Hz) lines (Lowe et al., 1979). These molecules can thus be classified into category B or the borderline between categories B and C. The ^{31}P NMR spectrum of **1** is shown in Figure 2. The coupling pattern is not resolved since the compound contains a mixture of statistically labeled species (e.g., $^{17}O_4$, $^{17}O_3O$, $^{17}O_2O_2$, ...), but it is clear from integration that the ^{31}P signal is spread into a wide range. When the vertical scale is increased and the horizontal scale is decreased, a broad signal can be observed. By taking the integration in the expanded spectrum such that the base line is flattened and the broad signal is not integrated, we can measure the quantitative decrease in the ^{31}P signal. The results are summarized in Table I. The P–O coupling has also been measured for $(CH_3O)_3P$ ($J = 160$ Hz) and $POCl_3$ ($J = 225$ Hz) (Christ et al., 1961).

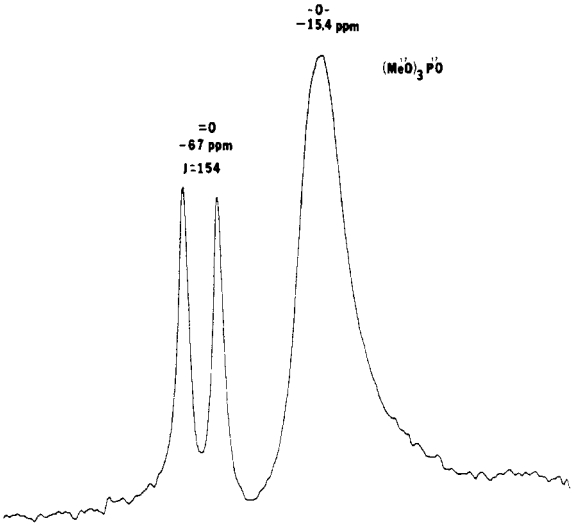


FIGURE 1: ^{17}O NMR spectrum of trimethyl [$^{17}O_4$]phosphate (**1**) (70 μmol in 1.2 mL of $CDCl_3$). Conditions: acquisition time 0.5 s, spectral width 8065 Hz, pulse delay 0.1 s, α delay 800 μs, and 18 567 transients.

Examples of Intermediate ^{17}O Line Widths. In a previous paper (Tsai, 1979) we have shown that a directly bonded ^{17}O nucleus causes the ^{31}P signal to decrease, as measured by integration, in the following nucleotides: [α-$^{17}O_2$]AMPS (**3**), [α-^{17}O,$\alpha\beta$-^{17}O]ATPαS (**4**), and [$\alpha\beta$,$\beta\gamma$-$^{17}O_2$,β-$^{17}O_2$]ATP (**6**). The same effect has been observed for another compound, [γ-$^{17}O_3$]ATP (**5**) (Figure 3A). By comparing the integrations of the whole spectrum of **5** and that of the nonlabeled ATP (Figure 7A), it is evident that there is a very broad signal ($\Delta X > 350$ Hz) underneath the residual Pγ signal of **5**.

As shown in Table I, compounds **3** and **5** have an ^{17}O line width of 450 and 400 Hz, respectively. Compounds **4** and **6** contain both bridge and nonbridge ^{17}O atoms. The ^{17}O NMR spectrum of **4** consists of only a very broad peak ($W_{1/2} \approx$ 1000–1500 Hz) which can hardly be detected. Similarly, only a single ^{17}O signal ($W_{1/2} \approx 500$ Hz) was observed for **6** (Figure 5A). Since the bridge ^{17}O is expected to be more restricted in rotation than the nonbridge ^{17}O, it seems reasonable to attribute the single ^{17}O signal of **4** and **6** to the nonbridge ^{17}O. The signals of the bridge ^{17}O are probably too broad to be observed (i.e., >1500 Hz). Evidence which supports this assignment will be discussed after the Mg^{2+} binding results

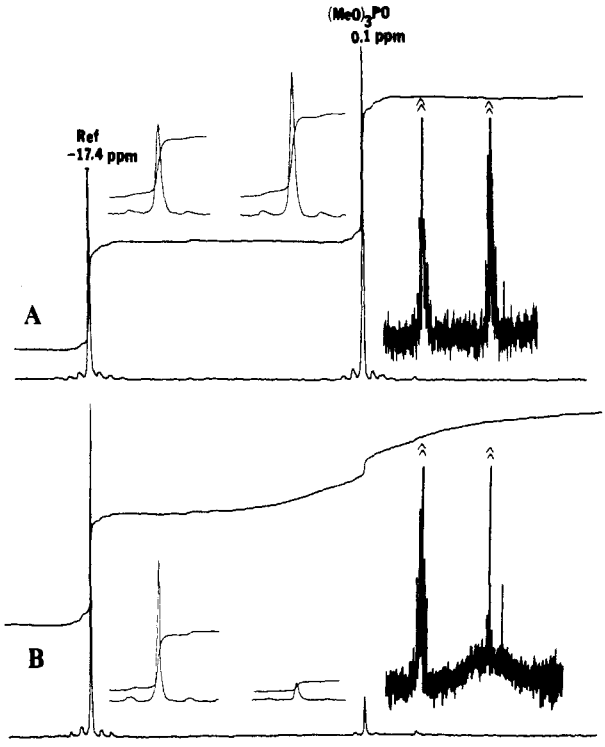


FIGURE 2: ^{31}P NMR spectra of trimethyl phosphate (A) and trimethyl [$^{17}O_4$]phosphate (**1**) (B) (70 μmol in 1.2 mL of $CDCl_3$). The left signal comes from triethyl phosphonoacetate (64 μmol) added as the internal reference. Number of transients = 1000. The inset on the right-hand side represents the spectra with amplified vertical scale (20 times) and reduced horizontal scale (1/4). The other insets represent the expanded integrations of the corresponding signals in the expanded spectra.

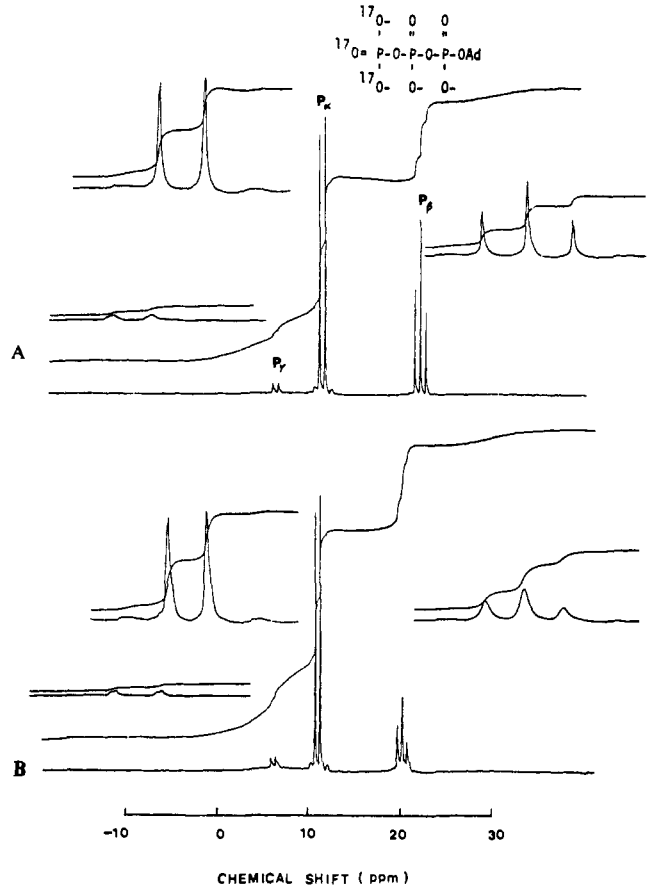


FIGURE 3: ^{31}P NMR spectra. (A) [γ-$^{17}O_3$]ATP (**5**): 50 μmol in 1.0 mL of D_2O containing 0.5 μmol of EDTA, pD 7.6; 2000 transients. (B) is the same as (A) except that 50 μmol of $Mg(NO_3)_2$ was added; 2000 transients. The insets represent the expanded integrations of the corresponding signals.

are described under Examples of Large ^{17}O Line Widths.

Examples of Large ^{17}O Line Widths. It has been well established that Mg^{2+} binds to the β- and γ-phosphate of ATP (Jaffe & Cohn, 1978). As shown in Figure 4, Mg^{2+} causes the ^{17}O NMR signal of [γ-$^{17}O_3$]ATP (**5**) to broaden and disappear. Based on eq 3, ΔX should decrease upon increasing ΔQ, if J(P–O) does not change much. Such an effect can be observed in the ^{31}P NMR spectrum of Mg[γ-$^{17}O_3$]ATP (**7**) (Figure 3B). It is evident by comparing parts A and B of Figure 3 that the broad Pγ signal sharpens up upon binding with Mg^{2+}. However, ΔX of **7** is not so small as to diminish the apparent quadrupolar effect. By integrating the expanded spectrum, the same quantitative decrease of the Pγ signal can still be detected even when $[Mg^{2+}]/[ATP] = 2.0$, as listed in Table I. Although the effect of Mg^{2+} binding is most likely due to the decrease in the rotational freedom, the detailed mechanism remains to be established. Only the relationship between ΔX and ΔQ is used in the present discussion, regardless of the real mechanism of the Mg^{2+} effect.

A similar effect of Mg^{2+} on the ^{17}O NMR of [$\alpha\beta$,$\beta\gamma$-$^{17}O_2$,β-$^{17}O_2$]ATP (**6**) has been observed, as shown in Figure 5. Consistent with this, the Pβ signal of **8** is sharper than that of **6** but not so sharp as to diminish the line broadening effect caused by ^{17}O as shown in Figure 6B. The approximate line widths (ΔX) measured from the enlarged spectra for the compounds **5**–**8** are listed in Table I.

In the previously reported ^{31}P NMR spectrum of **6**, which has only 19% ^{17}O enrichment, the broad signals of Pα and Pγ due to the ^{31}P–^{17}O species are not readily observable, and the ^{17}O effect on Pα and Pγ can only be detected by integrating the expanded spectra. By comparing the ^{31}P spectra of the 51% enriched **6** (Figure 6A) and the nonlabeled ATP (Figure 7A), it is clear that there is a broad signal underneath the sharp peaks. The ΔX of these signals measured from the enlarged spectra are 50 Hz (Pα) and 55 Hz (Pγ), which are relatively small compared to that of Pβ. Under such conditions it is particularly important that the integrations are taken from the expanded spectra such that the base line is flattened over the broad ^{31}P signal. In the present work the horizontal scale was expanded 20 times (spectral width 100 Hz) to obtain the integration of each signal. For a doublet signal, each peaklet was integrated separately.

Binding of Mg^{2+} causes only a slight decrease in the ΔX of Pα and Pγ of **6**, as shown by comparing the ^{31}P NMR spectra of **8** (Figure 6B) and MgATP (Figure 7B). The magnitude of this small change is within the experimental error. Its implication, if significant, requires further investigation.

In the above discussion one should keep in mind that ΔX refers to the line width of the broad ^{31}P signal due to ^{31}P (^{17}O) species, and J refers to ^{31}P–^{17}O coupling constants which are not readily measurable. According to eq 3, ΔX is proportional to J^2. Therefore, a decrease in $J(^{31}P$–$^{17}O)$ upon Mg^{2+} binding would also cause a decrease in ΔX, whereas an increase in $J(^{31}P$–$^{17}O)$ should result in an increase in ΔX. Since an inversely proportional relationship between changes in ΔQ and ΔX was observed experimentally, $J(^{31}P$–$^{17}O)$ does not seem to change greatly upon Mg^{2+} binding. However, it cannot be ruled out that part of the observed decrease in ΔX is caused by a decreasing J in the presence of Mg^{2+}. In addition, J(Pα–Pβ) and J(Pβ–Pγ) are known to decrease by 5 Hz in the presence of Mg^{2+} (Cohn & Hughes, 1962), which can also

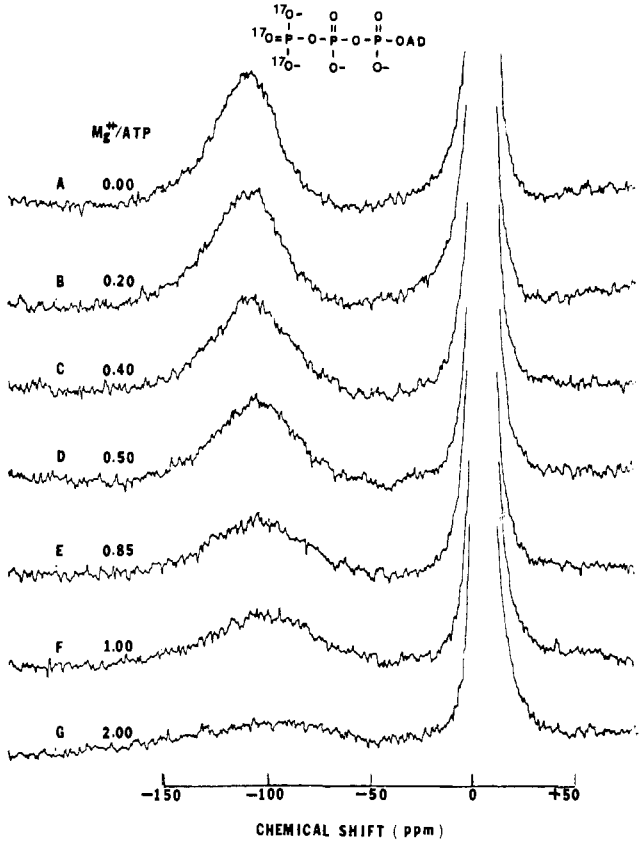


FIGURE 4: ^{17}O NMR spectra of [γ-$^{17}O_3$]ATP (**5**): 25 μmol in 1.0 mL of D_2O containing 0.5 μmol of EDTA, pD 7.6, with varying concentrations of $Mg(NO_3)_2$; 50 000 transients. The sharper signal at 3 ppm is due to D_2O.

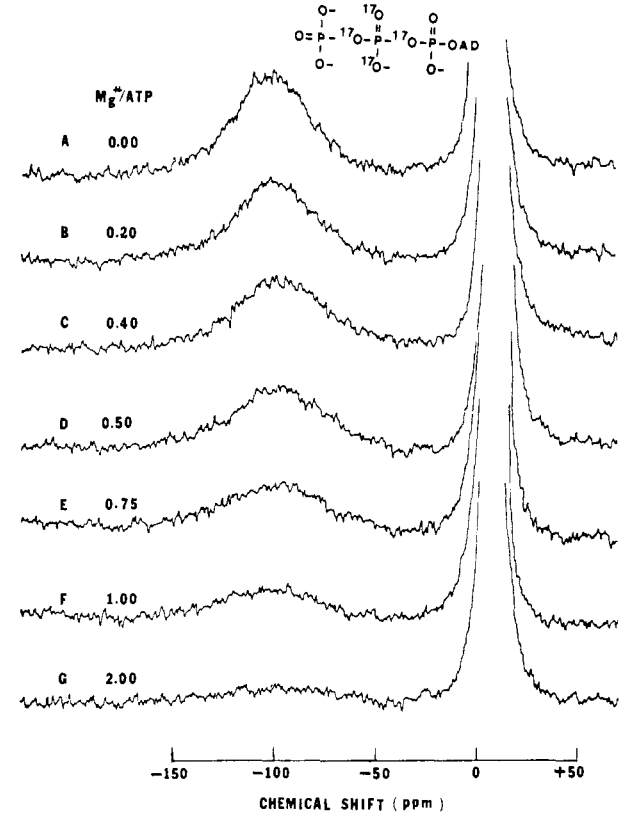


FIGURE 5: ^{17}O NMR spectra of [$\alpha\beta$,$\beta\gamma$-$^{17}O_2$,β-$^{17}O_2$]ATP (**6**): 51% ^{17}O enriched; 25 μmol in 1.0 mL of D_2O (pD 7.6) containing 0.5 μmol of EDTA, with varying concentrations of $Mg(NO_3)_2$; 50 000 transients. The sharper signal at 3 ppm is due to D_2O.

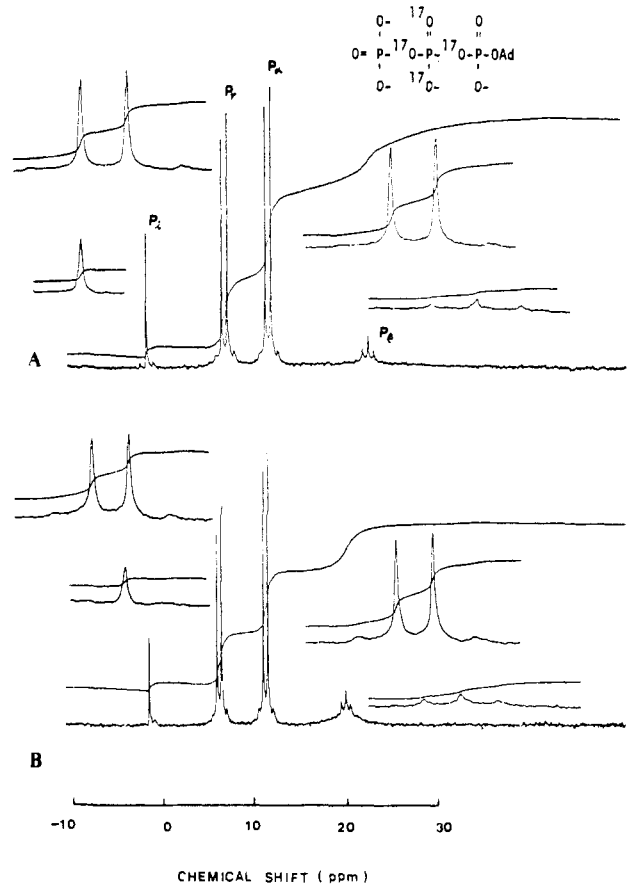


FIGURE 6: ^{31}P NMR spectra. (A) [$\alpha\beta$,$\beta\gamma$-$^{17}O_2$,β-$^{17}O_2$]ATP (**6**): 51% ^{17}O enriched; 93 μmol in 1.0 mL of D_2O containing 0.5 μmol of EDTA, pD 7.6; 1600 transients. (B) is the same as (A) except that 94 μmol of $Mg(NO_3)_2$ was added; 1600 transients. The insets represent the expanded integrations of the corresponding signals. Inorganic phosphate (25 μmol) was added as the internal standard for integration.

be observed in Figures 3, 6, and 7. This small decrease in $J(^{31}P–^{31}P)$ could also cause the ΔX of ^{31}P (^{17}O) species to decrease slightly.

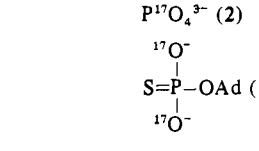
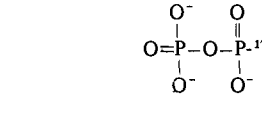
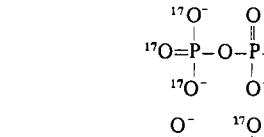
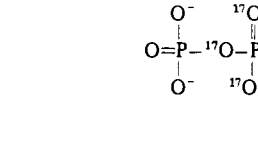
The α coordination of MgATP in solution has remained controversial for many years, although some extent of α coordination has been proposed in the enzyme-bound states (Gupta & Mildvan, 1977). This problem requires further investigation and is beyond the objective of this manuscript.

The assignment of the ^{17}O NMR signals observed for **4** and **6** to nonbridge ^{17}O is supported by the following observations. (1) Mg^{2+} causes dramatic changes in both the ^{17}O NMR signal and the Pβ signal of the ^{31}P NMR of **6** but has only a small effect on the ^{31}P signals of Pα and Pγ. (2) The ΔX values for Pα and Pγ of **6** are smaller than that of Pβ, consistent with the argument that the bridge ^{17}O has a large ΔQ (>1500 Hz) such that its signal is too broad to be detected. (3) No ^{17}O signal of **6** can be detected when $[Mg^{2+}]/[ATP] > 2$.

Discussion

As summarized in Table I, the line broadening effect of ^{17}O on ^{31}P NMR signals has been observed for the $^{31}P–^{17}O$ groups with small ΔQ (**1** and **2**), with intermediate ΔQ (**3**, **5**, and the nonbridge ^{17}O of **4** and **6**), and with very large ΔQ (**7**, **8**, and the bridge ^{17}O of **4** and **6**). Presence of a line broadening effect detectable by integration suggests that ΔX is larger than the limiting value (~20 Hz). Since the compounds (**1**–**8**) investigated are representative of the phosphate derivatives of biological interest, and since their ^{17}O line widths cover a large

Table I: Correlation between ^{17}O Line Widths and Effect of ^{17}O on Integration of ^{31}P NMR Signals

compounds	% ^{17}O enrichment	^{17}O line width,[a] ΔQ (Hz)	decrease of ^{31}P signals (% original) calcd[c]	decrease of ^{31}P signals (% original) obsd[d]	^{31}P line width,[b] ΔX (Hz)
$(CH_3{}^{17}O)_3P={}^{17}O$ (1)	39	50 (P=O) 250 (P-O-)	14	12 ± 2	
$P^{17}O_4{}^{3-}$ (2)	41	320	12	15 ± 2[e]	
 S=P-OAd ([α-$^{17}O_2$]AMPS, 3)	19	450	64	58 ± 4[f]	
 O=P-O-P-^{17}O-P-OAd ([α-^{17}O,αβ-^{17}O]ATPαS, 4)	19	1000-1500 (nonbridge)	66 (Pα)	67 ± 1 (Pα)[f]	
		>1500 (bridge)	81 (Pβ)	83 ± 4 (Pβ)	
 ^{17}O=P-O-P-O-P-OAd ([γ-$^{17}O_3$]ATP, 5)	51	400	12 (Pγ)	11 ± 4 (Pγ)	>350
 O=P-^{17}O-P-^{17}O-P-OAd ([αβ,βγ-$^{17}O_2$,β-$^{17}O_2$]ATP, 6)	51	500 (nonbridge)	49 (Pα)	60 ± 4 (Pα)	50
		>1500 (bridge)	6 (Pβ)	9 ± 2 (Pβ)	>350
			49 (Pγ)	58 ± 5 (Pγ)	55
Mg[γ-$^{17}O_3$]ATP (7)[g]	51	>1500 ([Mg^{2+}]/[ATP] = 2.0)	12 (Pγ)	14 ± 3 (Pγ)	110
Mg[αβ,βγ-$^{17}O_2$,β-$^{17}O_2$]ATP (8)[g]	51	>1500 ([Mg^{2+}]/[ATP] = 2.0)	49 (Pα)	56 ± 5 (Pα)	45
			6 (Pβ)	15 ± 5 (Pβ)	100
			49 (Pγ)	57 ± 6 (Pγ)	50

[a] Obtained from ^{17}O NMR spectra. The limit of detection for a broad signal is ~1500 Hz. Signals too broad to be detected are considered >1500 Hz. [b] Measured from the enlarged ^{31}P NMR spectra. For those ^{31}P nuclei labeled with more than one ^{17}O atom, the ΔX is measured from the apparent signal resulting from a mixture of various species. [c] Calculated percentage of the ^{31}P species free of ^{17}O according to the relationship $[a(^{17}O) + b(^{16}O)]^n$, where a = ^{17}O abundance per labeled oxygen, b = ^{16}O abundance per labeled oxygen, n = number of positions labeled. For example, in the [$^{17}O_4$]P_i the species present are derived from $[0.41(^{17}O) + 0.59(^{16}O)]^4$, where ^{18}O is considered as ^{16}O since ^{18}O has no effect on the peak intensity. The $P^{16}O_4$ species is therefore equal to 0.59^4 of the total species, i.e., 12%. [d] Observed percentage of the ^{31}P signal of the labeled compound relative to that of the nonlabeled compound. Integrations were taken from the expanded spectra such that the broad signal was not integrated, and the signal of a nonlabeled ^{31}P nucleus in the same compound or an added compound was used as an internal reference. [e] The higher value obtained previously (Tsai, 1979) was caused by slow hydrolysis of the internal reference thiophosphate to phosphate. [f] Obtained from previous report (Tsai, 1979). [g] The data listed in Table I for 7 and 8 are for [Mg^{2+}]/[ATP] = 2.0, whereas the ^{31}P NMR spectra shown (Figures 3b and 6b) are for [Mg^{2+}]/[ATP] = 1.0.

range, it is expected that the line broadening effect of ^{17}O on the ^{31}P signal of the directly bonded ^{31}P nucleus should be present for most of the phosphate derivatives of biochemical significance (not including macromolecules such as phosphorylated enzymes). The ^{31}P (^{17}O) NMR method should therefore be generally useful in the mechanistic studies involving phosphoryl transfer.

Binding of Mg^{2+} with ATP causes the β- and γ-^{17}O NMR signal to broaden and disappear, which in turn causes the ^{31}P NMR signal to sharpen. These results are consistent with the results of the previous ^{31}P NMR study (Jaffe & Cohn, 1978) which show that Mg^{2+} binds to the β- and γ-phosphate of ATP. If it can be unequivocally established by further investigation that the Mg^{2+} effect on ^{17}O and ^{31}P NMR is a direct measure of the extent of binding, the combined use of the ^{31}P (^{17}O) and ^{17}O NMR methods could be very useful in solving the problems such as binding of Mg^{2+} at the α-phosphate of ATP, which are difficult to resolve based on the previously used methods, e.g., the ^{31}P NMR of nonlabeled nucleotides (Jaffe & Cohn, 1978). The methods may also be used in the enzyme-bound states. Although the ^{17}O signal may be too broad to be observed in the enzyme–substrate complexes, the ^{31}P signal could be further sharpened and readily measurable for its line width. It should be noted that the coupling constant J could also change upon Mg^{2+} binding, but this change does not seem to be large compared to the change in ΔQ.

A particularly significant aspect of the generalization of the ^{17}O effect on ^{31}P NMR signals is that it would, in combination with the ^{31}P (^{18}O) isotope-shift method (Cohn & Hu, 1978), allow the analysis of the configuration of chiral [^{16}O,^{17}O,^{18}O]phosphate monoester (Abbott et al., 1979) or of chiral [^{16}O,^{17}O,^{18}O]thiophosphate[2] by ^{31}P NMR. Displacement of one of the three oxygen isotopes by a different group gives a mixture of three species, two of which contain one ^{17}O atom. Only the third species, which contains one ^{16}O atom and one ^{18}O atom, will give an unquenched ^{31}P NMR signal. Stereochemical information can then be obtained by determining whether the ^{18}O isotope is located at the *pro-R* or *pro-S* position. This can be achieved by a stereospecific derivatization

[2] Chiral thio[^{16}O,^{17}O,^{18}O]phosphates have been prepared independently by D. R. Trentham and co-workers (personal communication) and by us.

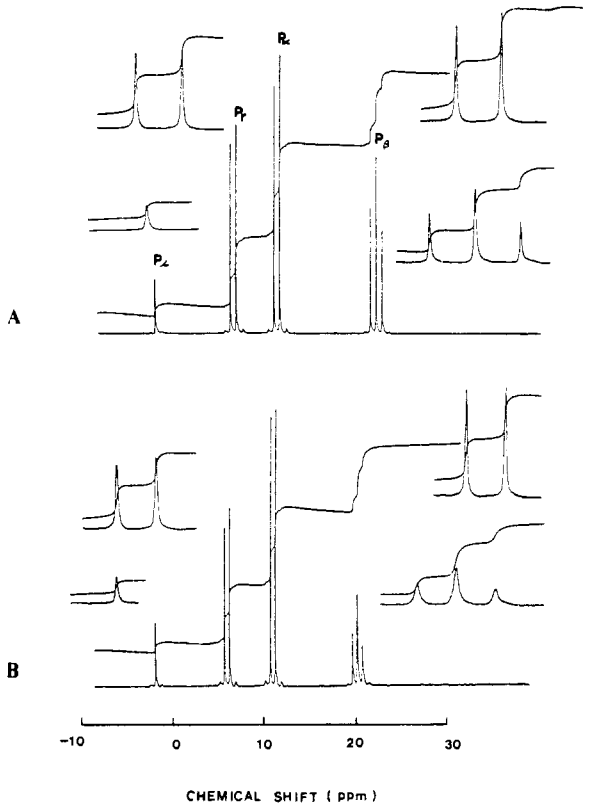


FIGURE 7: ^{31}P NMR spectra. (A) ATP: 93 μmol in 1.0 mL of D_2O containing 0.5 μmol of EDTA, pD 7.6; 1400 transients. (B) is the same as (A) except that 94 μmol of $Mg(NO_3)_2$ was added; 1400 transients. The insets represent the expanded integrations of the corresponding signals. Inorganic phosphate (25 μmol) was added as the internal standard for integration.

and by distinguishing the ^{18}O isotope shifts between the bridge and nonbridge positions.

The qualitative nature of this work should be realized. The relationship $\Delta X \Delta Q \approx aJ^2$ is approximate by itself and is derived for a group with a single quadrupolar nucleus. Most of the compounds investigated are multilabeled and contain a mixture of different isotopic species. An extreme case in which ΔX is very small is not impossible, namely, when J is small, as in the case of ^{13}C bonded to ^{17}O in some organic compounds (C. C. Chang and M.-D. Tsai, unpublished experiments).

Acknowledgments

The authors are indebted to Professor H. G. Floss of this department, Professor J. B. Grutzner of Purdue University, and Professors M. Cohn and G. Reed of the University of Pennsylvania for useful discussions.

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