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Effects of ^{17}O and ^{18}O on ^{31}P NMR: Further Investigation and Applications

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Abstract: An approximately linear relationship between the magnitude of the ^{18}O isotope effect in ^{31}P chemical shifts (S) and the spin–spin coupling constant between ^{17}O and ^{31}P (J) has been observed. Such a correlation is useful in systems where only one of the two parameters can be measured. In addition, we have discussed ^{31}P–^{17}O interactions in $^{31}P(^{17}O)$ NMR using some model compounds and addressed the relationship $\Delta P \Delta O \simeq (35/3)J^2$, where ΔP and ΔO are line widths of the $^{31}P(^{17}O)$ NMR signal and the ^{17}O NMR signal, respectively. By use of such correlations and chirally labeled [α-^{17}O]adenosine 5′-diphosphate (ADP), the interactions of Mg^{2+} and Co^{3+} with ADP have been investigated in detail. The results unambiguously established that binding of Co^{3+} with [α-^{17}O]ADP results in an upfield signal (−82 ppm) in ^{17}O NMR due to O═P—$^{17}O^{-}$···Co^{3+} and a downfield signal (98 ppm) due to Co^{3+}···$^{-}$O—P═^{17}O and that binding of Mg^{2+} with [α-^{17}O]ADP results in an averaged signal due to rapid exchange of the two species. Finally, we have shown that ^{17}O can be used as a "label" of oxygen and phosphate in macromolecular systems, which can be detected by ^{31}P NMR due to quadrupolar or dipolar broadening.

Three NMR[2] techniques involving oxygen isotopes have recently been introduced in studies of various physical and biochemical problems involving biochemical phosphates.[3] The ^{18}O isotope effect in ^{31}P chemical shifts,[4] which will be referred to as the $^{31}P(^{18}O)$ method in this paper, has been widely used to locate a labeled oxygen and to follow the exchange of an oxygen or a phosphoryl group.[5,6] The ^{17}O quadrupolar effect in ^{31}P NMR,[7] referred to as the $^{31}P(^{17}O)$ method,[8] has become an indispensable tool in some stereochemical analyses[9] and has been used to quantitate ^{17}O.[8,10] Recently, ^{17}O NMR has been useful for studying diamagnetic metal ion–nucleotide interactions,[8,11] protonation of adenine nucleotides,[11,12] and differentiation of diastereotopic oxygens.[13]

There are limitations in the applications of all three methods. The $^{31}P(^{18}O)$ method requires a high-resolution spectrometer and is limited to small molecules that give very sharp ^{31}P NMR signals. The ^{18}O "label" cannot be detected by ^{31}P NMR in macromolecules or even in small molecules such as phospholipids in solution. The $^{31}P(^{17}O)$ method is mainly used in stereochemical analysis of small molecules. In ^{17}O NMR analysis of phosphates, the ^{31}P–^{17}O spin–spin coupling constant (designated as J) is obtained only for some relatively small and symmetrical molecules and only at elevated temperatures.[12,14] Some ^{17}O NMR signals may be too broad to be detected even in small molecules unless a high-power, high-recovery probe can be used.[8,11]

Table I. Correlation between the ^{18}O Isotope Shift ($S^{31}P-^{18}O$) and the ^{31}P–^{17}O Coupling Constant ($J^{31}P-^{17}O$)[a,c]

compound	condition	$S^{31}P-^{18}O$, ppm[b]	$J^{31}P-^{17}O$, Hz	temp, °C
$H_4P^{17}O_4^+ClO_4^-$		0.0188 ± 0.0007	83.0 ± 2.4	95
$KH_2P^{17}O_4$	pH 2.1	0.0201 ± 0.0007	87.9 ± 2.4	80
	pH 2.6	0.0200 ± 0.0011	88.7 ± 2.4	95
$K_2HP^{17}O_4$	pH 8.6	0.0218 ± 0.0007	95.0 ± 2.4	95
$(CH_3O)_3P^{17}O$	$CDCl_3$	0.0392 ± 0.0029	153.8 ± 2.4	30
$Ph_3P^{17}O$	$CDCl_3$	0.0399 ± 0.0007	160 ± 2.4	30
$(PhO)_3P^{17}O$	$CDCl_3$	0.0391 ± 0.0029	158.7 ± 2.4	30
$(PhO)_2P^{17}OO$	pD 5.4	0.0293 ± 0.0007	121 ± 2.4	95
[α-$^{17}O_2$]ADP		0.0286 ± 0.0015	123 ± 2.4	95
[α-$^{17}O_2$]AMPS		0.0331 ± 0.0007	131 ± 2.4	95
[α-^{17}O]-β-CNEt-ADPαS	pD 6.4, R_p	0.0363 ± 0.0045	146 ± 2.4	97
	pD 6.4, S_p	0.0363 ± 0.0045	148 ± 2.4	97

[a] The same sample was used for both ^{31}P NMR (determining $S^{31}P-^{18}O$) and ^{17}O NMR (determining $J^{31}P-^{17}O$). [b] Measured at 81 or 121 MHz, at ambient temperatures. Gaussian multiplication was applied to obtain a near base-line separation of peaks. Although it is desirable to measure S values at the same temperature as in ^{17}O NMR experiments, it is hard to obtain a good resolution (to resolve ^{18}O shifts) at near-boiling temperatures, particularly during a long accumulation. [c] The correlation should be applied to only phosphates and derivatives of phosphates.

These limitations prompted us to investigate further the three NMR methods and their applicability. In this paper we present results of recent work on three aspects of these phenomena. Part A deals with a newly unmasked empirical correlation between the magnitudes of ^{18}O isotope shifts in ^{31}P NMR (designated as S) and the magnitudes of the ^{31}P–^{17}O spin–spin coupling constant (designated as J), as well as the interaction between ^{17}O and ^{31}P in small molecules. In part B, we have used the above correlations and chirally labeled [α-^{17}O]ADP to perform a detailed investigation of the interaction of Mg^{2+} and Co^{3+} with ADP. Part C further evaluates the use of ^{17}O as a label of oxygen and phosphate in macromolecular systems.

Results and Discussion

(A) Further Investigation in the NMR Methods. (1) Correlation between J and S. Determination of both J and S for a given phosphate is limited to certain conditions, so it would be useful

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(2) Abbreviations: P_i, inorganic orthophosphate; AMP, adenosine 5′-phosphate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; AMPS, adenosine 5′-thiophosphate; ADPαS, adenosine 5′-(1-thiodiphosphate); EDTA, ethylenediaminetetraacetate; DE, preacquisition delay; HPLC, high-pressure liquid chromatography; J, ^{31}P–^{17}O spin–spin coupling constant; S, ^{18}O isotope shift in ^{31}P NMR; THF, tetrahydrofuran.

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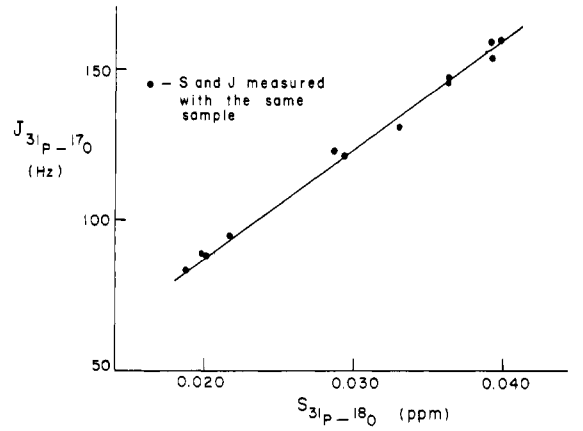

Figure 1. Correlation between $S_{^{31}P-^{18}O}$ and $J_{^{31}P-^{17}O}$ (from Table I), for the data that were obtained from our laboratory, using identical samples for the measurements of both S and J.

if the value of one could be obtained from the measured value of the other. Since both J and S were expected to be related to the P–O bond order, we have sought a correlation between the two parameters. The large amounts of data on both J and S available in the literature have been measured under various conditions, with variable resolution, and could be accurate to within only ±20%. We therefore measured the J and S values given in Table I for a number of compounds, using the same sample to determine J (by ^{17}O NMR) and S (by ^{31}P NMR; the shift is due to the ^{18}O isotope always associated with ^{17}O). In cases where peaks overlapped, the J and S values were determined by spectral simulation. When J was plotted vs. S, as shown in Figure 1, an approximately linear relationship, J (Hz) $\simeq (3.65 \times 10^3)S$ (ppm) + 14, was obtained, confirming the existence of a relationship between these parameters for biochemical phosphates.

(2) $^{31}P-^{17}O$ Interaction in Small Molecules. For small biochemical phosphates in solution, the line widths of ^{17}O NMR signals (ΔO) can be related to the quadrupolar relaxation time T_q by eq 1:[11]

$$\Delta O \simeq \frac{1}{\pi T_q} \simeq \frac{12\pi}{125}\left(1 + \frac{\eta^2}{3}\right)\left(\frac{e^2qQ}{h}\right)^2 \tau_r \qquad (1)$$

where e^2qQ/h is the quadrupolar coupling constant, η is the asymmetry parameter, and τ_r is the rotational correlation time. When ^{31}P is bonded directly to ^{17}O, the ^{31}P nucleus will also be relaxed by virtue of its spin–spin coupling with ^{17}O. This is termed "scalar relaxation of the second kind" by Abragam.[15] Such a scalar relaxation is dependent upon the magnitudes of the longitudinal relaxation time T_1 of the quadrupolar nucleus (which is approximately equal to T_q under present conditions) and the spin–spin coupling constant J. When the product T_qJ is sufficiently small, the scalar relaxation dominates the relaxation of ^{31}P and results in the collapse of the multiplet. Suzuki and Kubo[16] have calculated the line shape of a dipolar nucleus coupled to a quadrupolar nucleus with $I = 5/2$ at various values of T_qJ.

Figure 2 shows the ^{17}O and $^{31}P(^{17}O)$ NMR spectra of $P^{17}OCl_3$ (Figure 2A), $(CH_3O)_3P^{17}O$ (Figure 2B), $(PhO)_3P^{17}O$ (Figure 2C), and $Ph_3P^{17}O$ (Figure 2D). These compounds are all symmetrical small molecules with a P=O bond that have relatively long T_q and large J, thus showing fully or partially resolved ^{17}O and $^{31}P(^{17}O)$ NMR spectra. It can be seen in Figure 2 that as the ^{17}O NMR coupling pattern collapses (decreasing T_qJ), the ^{31}P NMR coupling pattern also collapses.

For biochemical phosphate molecules T_q is generally shorter, due to a larger molecular size and a smaller degree of symmetry, and J is generally smaller, due to a P–O bond with a smaller π-character, than for the molecules in Figure 2. Therefore, the

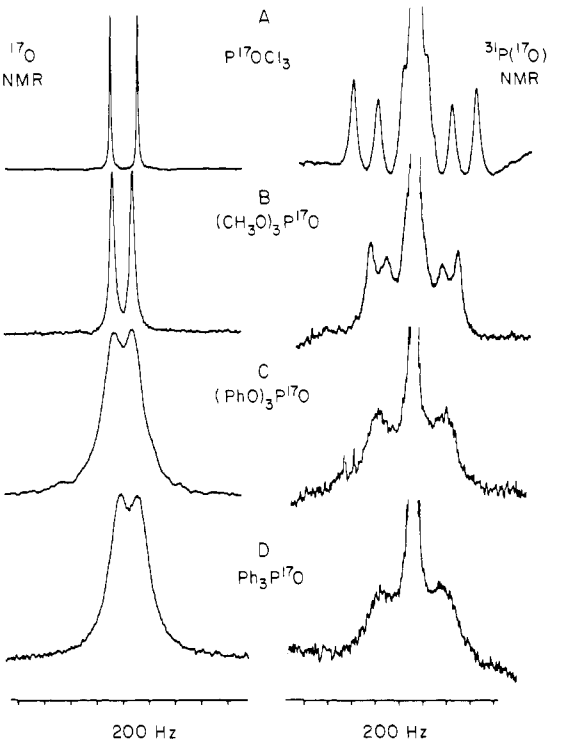

Figure 2. Line shapes of ^{17}O NMR (left, at 27.1 MHz) and $^{31}P(^{17}O)$ NMR (right, at 81.0 MHz). (A) $P^{17}OCl_3$ in tetrahydrofuran, using acetone-d_6 for the external lock, $\delta = 210$ for ^{17}O and +2.5 for ^{31}P; (B) $(CH_3O)_3P^{17}O$ in $CDCl_3$, $\delta = 73.6$ for ^{17}O and 2.6 for ^{31}P; (C) $(PhO)_3P^{17}O$ in $CDCl_3$, $\delta = 91.2$ for ^{17}O and −17.9 for ^{31}P; (D) $(Ph)_3P^{17}O$ in $CDCl_3$, $\delta = 43.3$ for ^{17}O and 28.8 for ^{31}P. ^{17}O NMR parameters: spectral width 10 kHz; acquisition time 0.4 s; pulse width 70 μs (90° ≃ 100 μs); ^1H decoupled; 8K data points; DE = 25 μs. ^{31}P NMR parameters: spectral width 2000 Hz; acquisition time 2 s; acquisition delay 3 s; 75° pulse; ^1H decoupling. All spectra were run at 31 °C and processed with a 5-Hz line broadening. The strong central peaks in ^{31}P spectra are due to non-^{17}O species.

^{17}O NMR signals of biophosphates are broader and less well resolved, and the $^{31}P(^{17}O)$ NMR signals of biochemical phosphates appear as a "broad singlet".[8] Under this condition ($T_qJ < 1$) the scalar relaxation contributes to the relaxation of the dipolar nucleus according to[15,17]

$$\frac{1}{T_{1sc}} = \frac{8\pi^2J^2I(I+1)}{3}\frac{T_q}{1 + (\omega_p - \omega_o)^2T_q^2} \qquad (2)$$

$$\frac{1}{T_{2sc}} = \frac{4\pi^2J^2I(I+1)}{3}\left[T_q + \frac{T_q}{1 + (\omega_p - \omega_o)^2T_q^2}\right] \qquad (3)$$

where $I = 5/2$, $J = J_{^{31}P-^{17}O}$, $1/T_{1sc}$ and $1/T_{2sc}$ are the contribution of scalar relaxation to the longitudinal and the transverse relaxations, respectively, of ^{31}P, T_q is the quadrupolar T_1 relaxation time of ^{17}O, and ω_p and ω_o are the angular precession frequencies of ^{31}P and ^{17}O, respectively.

For small biochemical phosphate molecules at the extreme narrowing limit ($\omega^2\tau_c^2 \ll 1$), T_q is in the order of 10^{-2}–10^{-4} s. Since $\omega_p - \omega_o \simeq 10^7$–$10^8$ Hz, $(\omega_p - \omega_o)^2T_q^2 \gg 1$, and eq 4 and 5 can be reduced to

$$\frac{1}{T_{1sc}} \simeq 0 \qquad (4)$$

$$\frac{1}{T_{2sc}} \simeq \frac{35}{3}\pi^2J^2T_q \qquad (5)$$

Under this condition, $1/T_2 \simeq 1/T_{2sc}$ for ^{31}P, and $T_1 \simeq T_2 \simeq T_q$

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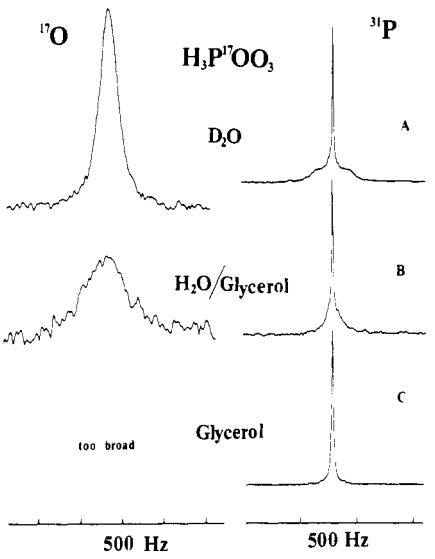


Figure 3. ^{17}O NMR spectra (at 27.1 MHz) and $^{31}P(^{17}O)$ NMR spectra (at 81.0 MHz) of $H_3P^{17}OO_3$ (50 atom % ^{17}O) in D_2O (A), H_2O/glycerol (1/1 volume ratio) (B), and glycerol (C). ^{17}O NMR parameters: spectral width 10 kHz; acquisition time 0.05 s; pulse width 100 μs; ^1H decoupled; 1K data points; DE = 12 μs; line broadening 20 Hz, ^{31}P NMR parameters: spectral width 3012 Hz; acquisition time 2.7 s; acquisition delay 1 s; 75° pulse; ^1H decoupling; line broadening 4 Hz. All spectra were obtained at 30 °C.

for ^{17}O, which justifies the approximations of $\Delta O \simeq 1/(\pi T_q)$ and $\Delta P \simeq 1/(\pi T_{2sc})$. The following approximate relationship can be obtained from eq 5

$$\Delta P \Delta O \simeq (35/3)J^2 \quad (6)$$

where ΔP and ΔO represent the line widths of $^{31}P(^{17}O)$ and ^{17}O NMR signals, respectively.

While the quantitative nature of eq 6 remains to be established by detailed experimental measurements, the relationship between ΔP and ΔO is approximately true in many systems. As one example, Figure 3 shows the ^{17}O NMR and the $^{31}P(^{17}O)$ NMR signals of $H_3P^{17}OO_3$ in D_2O (Figure 3A), H_2O/glycerol (Figure 3B), and glycerol (Figure 3C). In Figure 3A, the ΔO is 160 Hz (after correcting for a 20-Hz line broadening and $J_{^{31}P-^{17}O} = 88$ Hz) while the ΔP is 390 Hz. The product $\Delta P \Delta O \simeq 62400$ Hz^2, which is ca. 30% smaller than $(35/3)J^2$ ($\simeq$90350 Hz^2). However, as ΔO increases due to an increased viscosity, which is not expected to change J, the ΔP decreases correspondingly, showing the inversely proportional relationship between ΔP and ΔO. The significance of Figure 3C will be discussed further in part C.

(B) Interactions of Mg^{2+} and Co^{3+} with ADP: Complete Study by Three Techniques and Chiral [α-^{17}O]ADP. Recently we have introduced the use of ^{17}O NMR to study the binding of Mg^{2+} with adenine nucleotides,[11] which is based on the observation that binding of Co^{3+} with [α-$^{17}O_2$]ADP (and other ^{17}O-labeled nucleotides) resulted in two signals: one slightly shifted downfield (1–9 ppm) and slightly broadened; the other greatly shifted upfield (180–200 ppm) and significantly broadened. In Mg^{2+} complexes only a single signal with a small upfield shift (<6 ppm) has been observed. Although it has been concluded, on an empirical basis, that Mg^{2+} interacts with both the α-phosphate and β-phosphate of ADP, and with all the α-, β-, and γ-phosphates of ATP (with a smaller extent of interaction with the α-phosphate of ATP), several important problems on the methodology remain to be established.

In the following sections we described detailed study of Mg^{2+} and Co^{3+} binding with ADP by use of all three NMR techniques and chirally labeled ADP.

(1) Effects of Metal Ions on *S* and *J* in Metal–Nucleotide Complexes. The effect of Co^{3+} binding on the *S* values of nucleotides has been reported[18,19] but not the effect of Mg^{2+} binding.

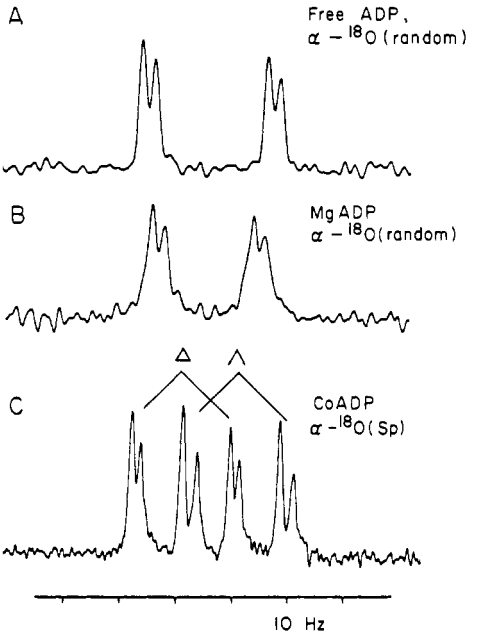
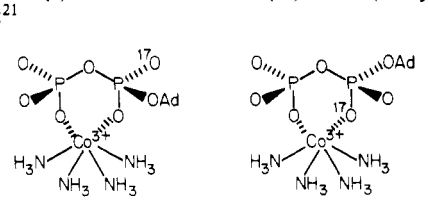


Figure 4. ^{31}P NMR spectra (81.0 MHz) showing the effect of metal ion binding on the ^{18}O isotope shift (at the P_α signal) of [α-^{17}O]ADP. (A) Free [α-^{17}O]ADP, randomly labeled, 25 mM in D_2O, pD 7.8; (B) Mg-[α-^{17}O]ADP, randomly labeled, 25 mM in D_2O, pD 7.8; (C) Co-$(NH_3)_4$-(S_p)-[α-^{17}O]ADP, Λ plus Δ isomers, in 50% D_2O, pH 5.5. Spectral parameters for (A) and (B): spectral width 2500 Hz; acquisition time 3.3 s; 75° pulse; 16K data points; resolution 0.305 Hz/point; temperature 30 °C; ^1H decoupled; Gaussian multiplication (LB −0.8, GB 0.04). Spectrum C was obtained as previously described.[18]

A possible reason is that the ^{31}P NMR signals of Mg^{2+} complexes are slightly broadened at high magnetic fields.[20] At a medium magnetic field, we have observed the ^{18}O isotope effect on the P_α signal of free ADP (Figure 4A), MgADP (Figure 4B), and CoADP (Figure 4C) as the α,β-bidentate mixture of Λ and Δ isomers obtained from (S_p)-[α-^{18}O]ADP. The reported *S* values for O=P—$^{18}O^-$···Co^{3+} are 0.018 and 0.020 ppm, and those for ^{18}O=P—O^-···Co^{3+} are 0.032 and 0.033 ppm,[18] which give an average value of 0.026 ppm. The *S* values measured from Figure 6 for free [α-^{17}O]ADP and Mg[α-^{17}O]ADP are 0.0276 and 0.0259 ppm, respectively. Thus, Mg^{2+} and Co^{3+} binding does not seem to change the *S* value (as an average) appreciably (<10% decrease, which is within the limit of detection).

The *J* values of CoADP and MgADP are not readily measurable due to the relatively broad ^{17}O NMR signals. However, on the basis of the correlation in Figure 1 between *S* and *J*, the *J* values of MgADP (J_b) and CoADP (as an average of O=P—$^{17}O^-$···Co^{3+} and ^{17}O=P—O^-···Co^{3+}) should be within 10% of that of free ADP (J_f).

(2) Unequivocal Assignments of ^{17}O NMR Signals. As indicated in an earlier paper,[11] the unequivocal assignment of the two ^{17}O NMR signals of $Co(NH_3)_4$[α-$^{17}O_2$]ADP awaited the preparation of stereospecifically labeled compounds. Following the procedure previously developed for the synthesis of chiral [α-^{18}O]ADP,[18] we have synthesized (R_p)- and (S_p)-[α-^{17}O]ADP. Interaction of (S_p)-[α-^{17}O]ADP with $[Co(NH_3)_4CO_3]NO_3$ gave a mixture of the Λ isomer (I) and the Δ isomer (II) of $Co(NH_3)_4$-(S_p)-[α-^{17}O]ADP:[21]



I, Λ isomer II, Δ isomer

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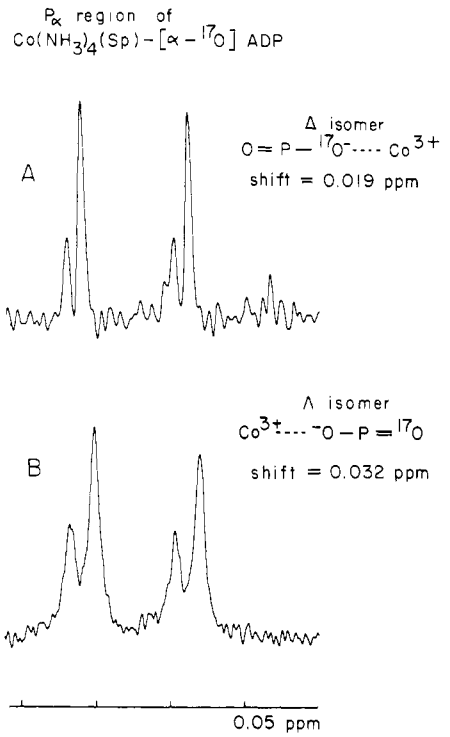


Figure 5. ^{31}P NMR spectra (121 MHz) showing the ^{18}O isotope shift in the P_α signal of $Co(NH_3)_4$-(S_p)-[α-^{17}O]ADP. (A) Δ isomer (II), with bridging ^{18}O isotope; (B) Λ isomer (I), with nonbridging ^{18}O isotope. Sample conditions: 12 mM, 10% D_2O, pH 5.5. Spectral parameters: spectral width 600 Hz; acquisition time 7 s; 90° pulse; line broadening −0.5 Hz; Gaussian broadening 0.05 Hz; ^1H decoupled; resolution 0.082 Hz/point; temperature 28 °C.

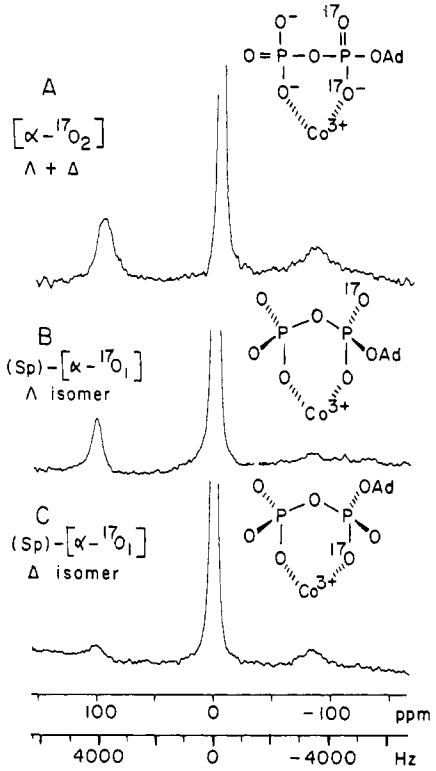


Figure 6. ^{17}O NMR spectra (40.65 MHz) of ^{17}O-labeled $Co(NH_3)_4ADP$ (α,β-bidentate) showing the unequivocal assignments of the downfield peak to P=^{17}O and the upfield peak to P-$^{17}O^-\cdots Co^{3+}$. (A) From [α-$^{17}O_2$]ADP, Λ isomer plus Δ isomer; (B) from (S_p)-[α-^{17}O]ADP, Λ isomer; (C) from (S_p)-[α-^{17}O]ADP, Δ isomer. Sample conditions: (A) 12 mM, D_2O, pD 4.0; (B and C) 7 mM, 10% D_2O, pH 5.5. Spectral parameters: spectral width 20 000 Hz; acquisition time 0.102 s; 4K data points; DE = 12 μs; line broadening 50 Hz; ^1H decoupled; temperature 50 °C. The small amount of the Δ isomer present in the spectrum of the Λ isomer (and vice versa) is due to epimerization between the two isomers during accumulation.

We separated the Λ and Δ isomers of $Co(NH_3)_4ADP$ by high-pressure liquid chromatography (HPLC) as described under Experimental Section and identified them as Λ and Δ isomers based on the ^{31}P NMR spectra. Shown in Figure 5 are the P_α signals of the resolved Λ and Δ isomers of $Co(NH_3)_4$-(S_p)-[α-^{17}O]ADP, which exhibit ^{18}O isotope shifted lines due to the ^{18}O species present in the starting ^{17}O-enriched water. Both the stereochemical purity of (S_p)-[α-^{17}O]ADP and the diastereomeric purity of I and II must be >95% on the basis of Figure 5.

Figure 6 shows the ^{17}O NMR spectra (at 40.65 MHz) of $Co(NH_3)_4$[α-$^{17}O_2$]ADP (Figure 6A), in which the α-phosphate of ADP is randomly labeled with ^{17}O at nonbridging positions. Also shown are the Λ isomer, I, (Figure 6B), in which ^{17}O is specifically located at the uncoordinated position, and the Δ isomer, II, (Figure 6C), in which ^{17}O is directly coordinated to Co^{3+}. These results unambiguously establish that the upfield signal (−82 ppm) is due to O=P—$^{17}O^-\cdots Co^{3+}$, whereas the downfield signal (98 ppm) is due to ^{17}O=P—$O^-\cdots Co^{3+}$. We attribute the presence of ca. 20% downfield signal in Figure 2C to epimerization between the Λ isomer and the Δ isomer during 2 h of data accumulation at 50 °C. We confirmed this by redetermining the ^{31}P NMR spectrum subsequent to the ^{17}O experiments and verifying the presence of ^{31}P NMR signals corresponding to the two isomers. No appreciable dissociation to free ADP or monodentate CoADP was detected by ^{31}P NMR.

(3) $^{31}P(^{17}O)$ NMR Studies of Mg^{2+} and Co^{3+} Binding to ADP. In the Mg^{2+} complexes of ^{17}O-labeled ADP and ATP only one signal at the low field (broadened by 2–4 times) was observed. It was not clear whether this signal was due to the average of ^{17}O=P—$O^-\cdots Mg^{2+}$ and O=P—$^{17}O^-\cdots Mg^{2+}$, or whether it represented essentially only the signal of ^{17}O=P—$O^-\cdots Mg^{2+}$, the upfield signal being too broad to be detected. This question has now been resolved by the $^{31}P(^{17}O)$ NMR method, as described below.

Figure 7 shows the ^{31}P NMR spectra of free ADP (Figure 7A) and free [α-^{17}O]ADP (Figure 7B), the difference spectrum B − A (Figure 7C), the ^{31}P NMR spectra of MgADP (Figure 7D) and Mg[α-^{17}O]ADP (Figure 7E), and the difference spectrum E − D (Figure 7F). By comparing the broad P_α signals in parts C and F of Figure 7, it is obvious that the apparent ΔP of MgADP has decreased by ca. 50%. Such a "line sharpening effect" in $^{31}P(^{17}O)$ NMR is predictable based on eq 6. The line widths of the broad P_α signals, measured at the half-height and corrected for the spin–spin coupling constant between P_α and P_β, are 470 Hz for free ADP (ΔP_f) and 250 Hz for MgADP (ΔP_b).

Figure 8 shows the ^{31}P NMR spectra of $Co(NH_3)_4ADP$, the Λ isomer (Figure 8A), and the corresponding ^{17}O-labeled compound I (Figure 8B), the difference spectrum B − A (Figure 8C), the ^{31}P NMR spectra of $Co(NH_3)_4ADP$, the Δ isomer (Figure 8D), and the corresponding ^{17}O-labeled compound II (Figure 8E), and the difference spectrum E − D (Figure 8F). The ΔP of the broad P_α signals of I and II, as measured from parts C and F of Figure 8, respectively, and corrected for J, are 290 and 170 Hz, respectively. If the Λ and Δ isomers were in rapid exchange, as in MgADP, the average ΔP_b would be 230 Hz, which is the same as the ΔP_b of MgADP within experimental error. The ratio of $\Delta P_f/\Delta P_b$ is ca. 1.9 for MgADP and 2.0 for CoADP.

Therefore, Mg^{2+} and Co^{3+} have approximately the same effect on both J (as described in section 1) and ΔP upon binding with [α-^{17}O]ADP. On the basis of eq 6, they should also have the same effect on ΔO. According to the previous report[11] for $Co(NH_3)_4$[α-^{17}O]ADP, $\Delta O_b/\Delta O_f \simeq 3.0$–5.2 for the upfield signal and $\simeq 1$ for the downfield signal, which give an average value of 2.0–3.1. For Mg[α-$^{17}O_2$]ADP, $\Delta O_b/\Delta O_f \simeq 2.2$–2.8 for the single

(21) (a) Cornelius, R. D.; Hart, P. A.; Cleland, W. W. *Inorg. Chem.* **1977**, *16*, 2799–2805. (b) Cornelius, R. D.; Cleland, W. W. *Biochemistry* **1978**, *17*, 3279–3286.

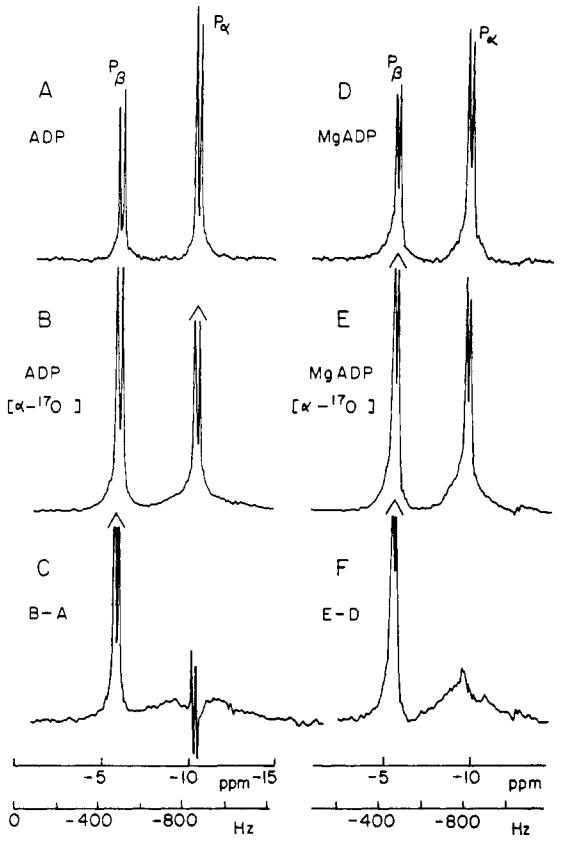


Figure 7. "Line sharpening effect" of Mg^{2+} binding in $^{31}P(^{17}O)$ NMR (81.0 MHz). (A) Free ADP; (B) free [α-^{17}O]ADP; (C) 100% ^{17}O-labeled ADP obtained by subtracting (A) from (B); (D) MgADP; (E) Mg[α-^{17}O]ADP; (F) 100% ^{17}O-labeled MgADP obtained by subtracting D from E. Sample conditions: 50 mM (A, D) and 25 mM (B, E) in D_2O, pD 7.9. NMR parameters: spectral width 5000 Hz; acquisition time 0.82 s; acquisition delay 3 s; line broadening 6 Hz; number of scans 9000 (B, E), 1800 (A), and 600 (D); temperature 30 °C.

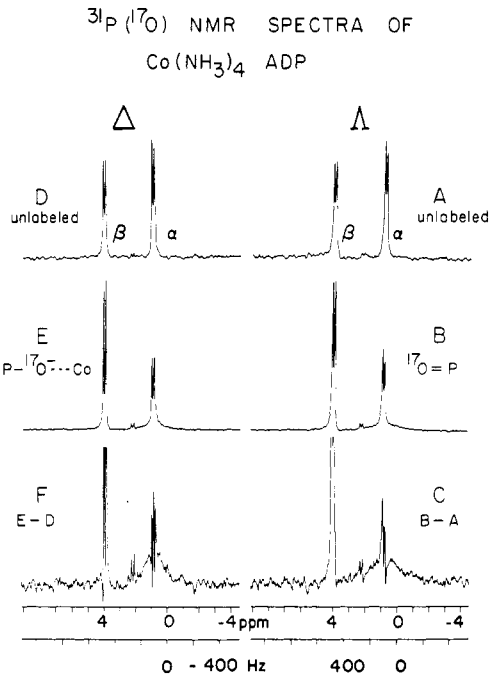


Figure 8. $^{31}P(^{17}O)$ NMR spectra (121 MHz) showing the ^{31}P–^{17}O interaction in $Co(NH_3)_4$-(S_p)-[α-^{17}O]ADP. (A) $Co(NH_3)_4$ADP, Λ isomer, unlabeled; (B) $Co(NH_3)_4$-(S_p)-[α-^{17}O]ADP, Λ isomer (compound I), in which ^{17}O is not coordinated (^{17}O=P—O^-···Co^{3+}); (C) subtraction of A from B; (D) $Co(NH_3)_4$ADP, Δ isomer, unlabeled; (E) $Co(NH_3)_4$-(S_p)-[α-^{17}O]ADP, Δ isomer (compound II), in which ^{17}O is coordinated (Co^{3+}···$^{17}O^-$—P=O); (F) subtraction of D from E. Sample conditions: 7 mM; 10% D_2O; pH 5.5. Spectra B and E were taken before the ^{17}O NMR experiments and were diastereomerically pure. The small doublet (<5%) at 2.1 ppm is due to contaminating β-monodentate, which had been removed by passing through a column of DEAE-Sephadex A-25 prior to ^{17}O NMR experiments. Spectral parameters: spectral width 2994 Hz; acquisition time 2.736 s; 90° pulse; ^1H decoupled; 16K data points; line broadening 9 Hz (A, D) and 5 Hz (B, E); temperature 27 °C.

observed signal, which is approximately the same as the $\Delta O_b/\Delta O_f$ of CoADP (as the average of two signals). Thus, it seems unlikely to have a broad, undetected signal for Mg[α-$^{17}O_2$]ADP.[22]

(C) ^{17}O as a Label in Macromolecular Systems. Figure 3C shows that the "quadrupolar broadening" diminishes in the ^{31}P NMR of $H_3P^{17}OO_3$/glycerol, which suggests that the "line broadening effect" of ^{17}O on ^{31}P NMR may not be assumed to be present in all circumstances. It should be noted, however, that the case of $H_3P^{17}OO_3$/glycerol is unique in that the τ_r (ca. 10^{-9} s) is slow enough to diminish the quadrupolar effect, but fast enough to average out ^{31}P–^{17}O dipolar coupling. In macromolecular systems, the line broadening effect of ^{17}O on ^{31}P NMR may persist due to the dipolar effect rather than the quadrupolar effect. It is beyond the scope of this paper to treat the ^{31}P–^{17}O dipolar interaction quantitatively. However, we present two examples, one in enzyme–substrate complexes ($\tau_r \simeq 10^{-7}$–10^{-9} s) and the other in phospholipid bilayers ($\tau_r > 10^{-7}$ s), which show the dipolar broadening of ^{31}P NMR by ^{17}O.

Figure 9 shows the ^{31}P NMR spectra of ADP bound to arginine kinase (represented by E, M_r 40000) (Figure 9A), E·ADP·Mg^{2+} (Figure 9B), free [β-$^{17}O_3$,$\alpha\beta$-^{17}O]ADP (Figure 9C), E·[β-$^{17}O_3$,$\alpha\beta$-^{17}O]ADP (Figure 9D), and E·[β-$^{17}O_3$,$\alpha\beta$-^{17}O]ADP·Mg^{2+} (Figure 9E). The P_β signal is broadened by ^{17}O in free ADP due to scalar relaxation and in enzyme complexes due most likely to dipolar coupling.[23] Although the upfield peak has been assigned to the P_α of ADP in both E·ADP and E·ADP·Mg^{2+} on the basis of the chemical shifts of free ADP and titration of ADP with the enzyme,[24] the "^{17}O label" provides an alternative, unequivocal assignment.

The dipolar broadening is also present in phospholipid bilayers. Figure 10 shows the ^{31}P NMR spectra of dipalmitoylphosphatidylcholine (DPPC) dispersed in H_2O (Figure 10A), the corresponding spectrum of [^{17}O]DPPC (50 atom % ^{17}O) (Figure 10B), and the difference spectrum (Figure 10C). The spectrum of DPPC (above transition temperature) is characteristic of lipid bilayers, but that of [^{17}O]DPPC is broadened.

Our results suggest that ^{17}O is a useful label of oxygen or phosphate in both small molecules and macromolecular systems, except in some very unique cases (τ_r ca. 10^{-9} s) such as $H_3P^{17}OO_3$/glycerol. By use of $^{31}P(^{17}O)$ NMR, the position of ^{17}O can be located and quantitated. In systems where there is more than one phospho group, the ^{31}P chemical shifts can be unequivocally assigned by specific ^{17}O labeling followed by ^{31}P-(^{17}O) NMR analysis.

Experimental Section

Materials. The following compounds were prepared as previously described or were available from previous work:[8,11] [$^{17}O_4$]P_i, [α-$^{17}O_2$]-ADP, [β-$^{17}O_3$,$\alpha\beta$-^{17}O]ADP, [α-$^{17}O_2$]AMPS, and $Co(NH_3)_4$[α-$^{17}O_2$]-ADP. The [α-^{17}O]ADP (randomly labeled at P_α) used in Figures 4 and 7 is indeed a sample of [α-$^{17}O_2$]ADP, with lower atom percent enrich-

(22) The conclusion that Mg[α-$^{17}O_2$]ADP is in the "fast exchange limit" on the time scale of ^{17}O NMR may not seem reasonable considering the fact that the two signals of Co[α-$^{17}O_2$]ADP are separated by ca. 200 ppm (8×10^3 Hz at 40 MHz). However, it can easily be explained by the "epimerization" process mentioned in section 2 of part B. The epimerization is intramolecular and should be much faster than the chemical exchange (MgADP $\rightleftarrows$ Mg^{2+} + ADP). In the case of Co[α-^{17}O]ADP, no dissociation to free ADP or the monodentate complex was detectable when ca. 30% of epimerization had occurred.

(23) It is not impossible that the "quadrupolar relaxation" is partially or fully responsible for the observed broadening, if the bound ADP has a large internal rotational freedom and therefore a very small τ_r.

(24) Rao, B. D. N.; Cohn, M. *J. Biol. Chem.* **1977**, *252*, 3344–3350.

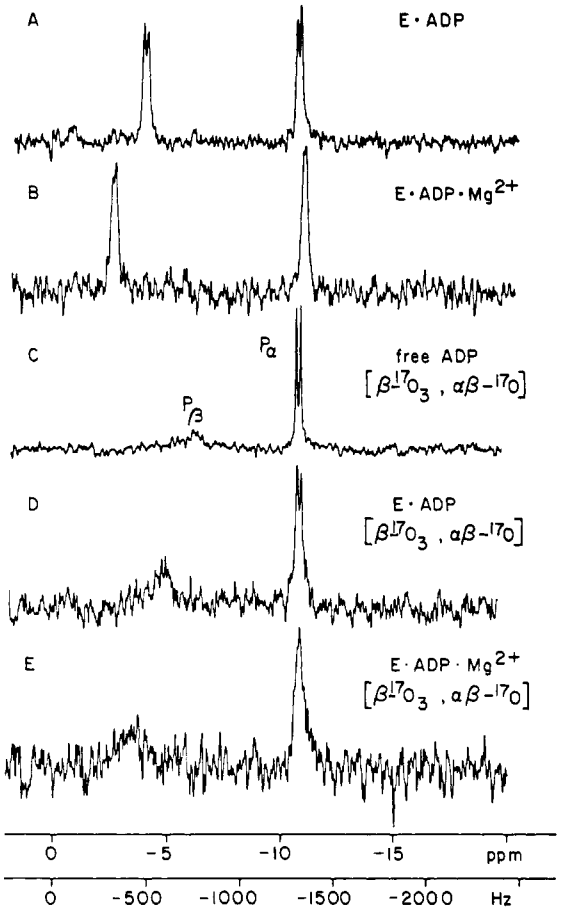
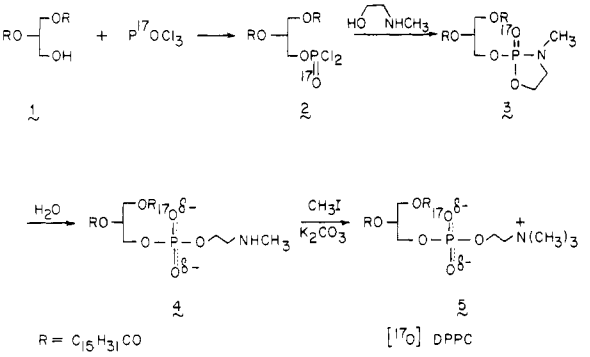


Figure 9. ^{31}P NMR spectra (121.5 MHz) of ADP·arginine kinase (AK) complexes in 50 mM Hepes buffer (10% D_2O), pH 8.0. (A) 2.6 mM AK, 2.0 mM ADP, 0.67 mM EDTA, 4260 scans; (B) same as A, 4.65 mM $MgCl_2$, 1530 scans; (C) free [β-$^{17}O_3$,$\alpha\beta$-^{17}O]ADP, 6.7 mM in D_2O, 458 scans; (D) 2.0 mM AK, 1.4 mM [^{17}O]ADP, 0.53 mM EDTA, 5000 scans; (E) same as D, 4.74 mM $MgCl_2$, 8000 scans. Sample volumes: 1.5–2.0 mL. Line broadening 5 Hz; acquisition time 1.36 s; temperature 27 °C; ^1H decoupling.

Scheme I



ments ($^{16}O/^{17}O/^{18}O \simeq 0.52/0.29/0.19$). Due to this pattern of enrichment, the major labeled species are the singly labeled ones, as is evident from Figure 4. The $H_2{}^{17}O$ (52.4% ^{17}O, 35.1% ^{18}O) was obtained from Monsanto. The puratronic-grade (99.999%) $Mg(NO_3)_2$ was purchased from Ventron Co. Arginine kinase was purified and assayed as previously described.[25] Other biochemicals were obtained from Sigma. Other chemicals used were of reagent grade or highest purity available commercially.

Synthesis of [^{17}O]DPPC. Scheme I outlines the synthesis of [^{17}O]DPPC. To a solution of 5.25 mol of $P^{17}OCl_3$ (52 atom % ^{17}O) in dry THF was added ca. 6 mmol of triethylamine, followed with 2.0 g of (*S*)-(−)-1,2-dipalmitin (**1**) in THF. After being stirred for 3 h at room temperature, the solvent and excess $P^{17}OCl_3$ and triethylamine were removed under vacuum, and the resulting phosphorodichloridate **2** was dissolved in THF at 0 °C and then added to a mixture of 2-(methylamino)ethanol (0.32 g) and triethylamine (2.2 mL) in THF. The reaction was allowed to proceed for 1 h at room temperature. After filtration and evaporation, 1.6 g of the product **3** was isolated by column chromatography on silica gel. The structure of **3** was characterized by ^1H and ^{13}C NMR. ^{31}P NMR analysis in $CDCl_3$ showed two peaks due to P–^{16}O and P–^{18}O (0.039 ppm upfield), which is characteristic of a P=O double bond. Calculation on the basis of the known $^{17}O/^{18}O$ ratio and the observed $^{18}O/^{16}O$ ratio indicated that the atom percent ^{17}O enrichment is 50%. ^{17}O NMR analysis (60 °C, in $CDCl_3$) showed $\delta = 67$ and $J_{^{31}P-^{17}O} = 150$ Hz. Hydrolysis of **3** in H_2O gave [^{17}O]-*N*-methyldipalmitoylphosphatidylethanolamine (**4**). Methylation of **4** in $CHCl_3$ with CH_3I, using a heterogeneous catalyst (2 M aqueous K_2CO_3 containing benzyltriethylammonium chloride), gave [^{17}O]DPPC (**5**), which was characterized by ^1H and ^{13}C NMR.

Synthesis of the Λ and Δ Isomers of Co(NH$_3$)$_4$-(S_p)-[α-^{17}O]ADP. (R_p)- and (S_p)-[α-^{17}O]ADP were synthesized according to the procedure used for the synthesis of (R_p)- and (S_p)-[α-^{18}O]ADP,[18] except that $H_2{}^{17}O$ was introduced in the first step (synthesis of [α-$^{17}O_2$]AMPS) and desulfurization was carried out in unlabeled H_2O. The procedure of Cornelius et al.[21a] was followed to prepare Co(NH$_3$)$_4$[α-^{17}O]ADP from (S_p)-[α-^{17}O]ADP, which was then purified as previously described.[18] The ^{17}O enrichment was calculated as 52% on the basis of the ^{18}O enrichment (measured from ^{31}P NMR) and the known $^{17}O/^{18}O$ ratio in the starting $H_2{}^{17}O$.

The Λ and Δ isomers of Co(NH$_3$)$_4$ADP had been separated previously on a cycloheptaamylose column,[21b] but we have separated the two isomers on a Waters μBondapak C_{18} reverse-phase HPLC column using 50 mM acetate at pH 6.3 as the eluting buffer. The Λ and Δ isomers were eluted at 33 and 39 min, respectively. The assignment of peaks was based on the known ^{31}P chemical shifts of the two diastereomers.[21b] The first band gave the more upfield P_α resonance (corresponding to the Λ isomer) and the second band gave the more downfield resonance (corresponding to the Δ isomer). Remixing of half of the two isomers in a 2/1 ratio gave the expected pattern of the P_α signal.

Synthesis of Model Compounds. $P^{17}OCl_3$ was prepared by hydrolyzing 10.4 g of PCl_5 with 1 mL of $H_2{}^{17}O$ at −78 °C followed by distillation under vacuum (88% yield). Treatment of $P^{17}OCl_3$ with a severalfold excess of a MeOH/trimethylamine mixture at room temperature gave $(CH_3O)_3P^{17}O$. The atom percent ^{17}O enrichment in $(CH_3O)_3P^{17}O$ was 52% on the basis of the percent ^{18}O enrichment (determined by ^{31}P NMR) and the known ratio of $^{17}O/^{18}O$. $(PhO)_3P^{17}O$ was prepared

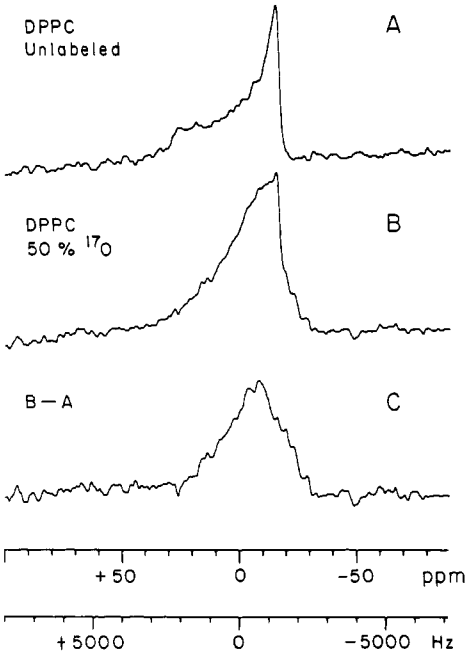


Figure 10. ^{31}P NMR spectra (at 81.0 MHz) of unsonicated lipid bilayers. (A) Dipalmitoylphosphatidylcholine (DPPC), unlabeled; (B) [$^{17}O_1$]DPPC, 50 atom % ^{17}O at phosphorus; (C) subtraction of A from B. Sample conditions: 100 mg of DPPC mixed with 1.5 mL of D_2O by vortexing at 50 °C. Spectral parameters: spectral width 25 000 Hz; ^1H decoupling (decoupler power 2.5 W); acquisition time 0.082 s; 40,000 scans; line broadening 100 Hz; 45 °C.

(25) (a) Buttlaire, D. H.; Cohn, M. *J. Biol. Chem.* **1974**, *249*, 5733–5740. (b) Blethen, S. L.; Kaplan, N. O. *Biochemistry* **1967**, *6*, 1413–1421.

analogously to $(CH_3O)_3P^{17}O$ except that phenol was used instead of methanol. $Ph_3P^{17}O$ (49 atom % ^{17}O) was prepared by oxidizing triphenylphosphine with the mixture $Et_3N/CCl_4/H_2{}^{17}O$ (5 equiv) in dry dimethoxyethane[26] followed by silica gel chromatography. $(PhO)_2P^{17}OO$ was a byproduct of the coupling reaction of [α-$^{17}O_2$]AMPS to cyanoethyl phosphate, the second step in the synthesis of chiral [α-^{17}O]ADP. $H_4P^{17}O_4{}^+ClO_4{}^-$ was obtained by dissolving $H_3P^{17}O_4$ (1 mmol) in 5 mL of D_2O followed by addition of 631 μL of 70% $HClO_4$. The final solution contained 1.4 M $HClO_4$ and 0.2 M $H_3P^{17}O_4$.

Spectral Methods. ^{17}O NMR spectra were obtained from a Bruker WM-300 spectrometer and ^{31}P NMR spectra from both WP-200 and WM-300 spectrometers. A deuterium lock was used in all cases. The ^{17}O chemical shifts reported are relative to external $H_2{}^{17}O$ (at 25 °C), and the ^{31}P chemical shifts are referenced to external 1 M H_3PO_4. The positive sign represents a downfield shift in both ^{17}O and ^{31}P NMR. Spectral simulations were performed with a program written by Drs. C. Cottrell and A. G. Marshall.

Most of the NMR work described in this paper dealt with ^{17}O-labeled compounds that were also enriched with ^{18}O. There are two different types of ^{31}P NMR work: in the so-called $^{31}P(^{17}O)$ NMR[7,8] a large spectral width and a large line broadening were used such that the broad signal due to ^{31}P–^{17}O species can be observed; in the determination of ^{18}O isotope shift,[4] a small spectral width and a small line broadening (or Gaussian multiplication) were used to obtain high resolution. In the latter case, the broad ^{31}P–^{17}O signal was not detectable.

MgADP was prepared from free ADP and puratronic-grade $Mg(NO_3)_2$ as previously described.[11] Sample sizes were 1.5 mL in most NMR experiments. The preparation of arginine kinase·ADP complexes for ^{31}P NMR studies followed essentially the procedure of Rao and Cohn.[24]

The estimated error in the measurements of "broad $^{31}P(^{17}O)$ NMR signals" is ±10%.

Acknowledgment. This work was supported by National Institutes of Health Grants GM 29041 (M.-D.T.) and GM 30480 (P.A.F.). The NMR spectrometers used (at The Ohio State University) were supported by National Institutes of Health Grant GM 27431 and National Science Foundation Grant CHE 7910019. We thank Drs. A. G. Marshall and C. Cottrell at The Ohio State University for providing computer programs for curve fitting, Ru-Tai Jiang and Yeun-Jung Shyy (also at The Ohio State University) for assistance in the synthesis of stereoisomers of $Co(NH_3)_4$-(S_p)-[α-^{17}O]ADP, and Judy Hart for isolating arginine kinase.

Registry No. Λ-$Co(NH_3)_4{}^-$(S_p)-[α-^{17}O]ADP, 86119-73-5; Δ-$Co(NH_3)_4$-(S_p)-[α-^{17}O]ADP, 86119-74-6; $Co(NH_3)_4$ADP, 63937-09-7; $Co(NH_3)_4$-[α-$^{17}O_2$]ADP, 80539-98-6; Mg[α-^{17}O]ADP, 86119-85-9; MgADP, 7384-99-8; [^{17}O]DPPC, 86119-75-7; DPPC, 2644-64-6; [α-^{17}O]ADP, 81246-59-5; (R_p)-[α-^{17}O]ADP, 83541-22-4; (S_+)-[α-^{17}O]-ADP, 85550-14-7; [α-$^{17}O_2$]ADP, 80547-13-3; [β-$^{17}O_3$,$\alpha\beta$-^{17}O]ADP, 80547-17-7; [α-$^{17}O_2$]AMPS, 80547-08-6; [α-^{17}O]-β-CNEt-ADPαS, 86119-83-7; $H_4P^{17}O_4{}^+ClO_4{}^-$, 86119-77-9; $KH_2P^{17}O_4$, 86119-78-0; $K_2HP^{17}O_4$, 86119-79-1; $(CH_3O)_3P^{17}O$, 80777-98-6; $Ph_3P^{17}O$, 86119-80-4; $(PhO)_3P^{17}O$, 86119-81-5; $(PhO)_2P^{17}OO$, 86119-82-6; $P^{17}OCl_3$, 66943-75-7; $H_3P^{17}OO_3$, 86119-84-8; P, 7723-14-0; ^{17}O, 13968-48-4; ^{18}O, 14797-71-8.

(26) Appel, R. *Angew. Chem., Int. Ed. Engl.* **1975**, *14*, 801–811.

Stereochemistry of Lysine 2,3-Aminomutase Isolated from *Clostridium subterminale* Strain SB4

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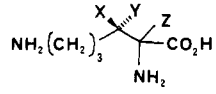
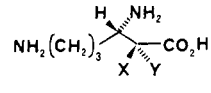
Received December 2, 1982

Abstract: The stereochemistry of lysine 2,3-aminomutase in *Clostridium subterminale* strain SB4 has been elucidated. Deuterium NMR has been used to show that the transformation of (2*S*)-α-lysine to (3*S*)-β-lysine proceeds with transfer of the 3-*pro-R* hydrogen of α-lysine to the 2-*pro-R* position of β-lysine. The 3-*pro-S* hydrogen of α-lysine is retained at C-3 of β-lysine. Also the C-2 hydrogen of α-lysine is retained at the 2-*pro-S* position of β-lysine. Thus, the reaction proceeds with inversion of configuration at C-2 and C-3. Experiments with [2-^{15}N,3-^{13}C]-α-lysine have shown that the amino group transfer takes place completely intramolecularly. However, conversion of α-lysine-3,3-d_2 led to the formation of mainly β-lysine-d_1 indicating substantially or completely intermolecular hydrogen transfer in the reaction.

The transformation of α-L-lysine, **1a**, into β-L-lysine, **2a**, by the



1 a X = Y = Z = H (2S)
b X = Y = D, Z = H
c X = Y = H, Z = D
d X = D, Y = Z = H
e X = Z = H, Y = D



2 a X, Y = H_2
b X = H, Y = D
c X = D, Y = H

enzyme lysine 2,3-aminomutase constitutes the first step of a major metabolic pathway of lysine in *Clostridia* and other bacteria.[2] The transformation also takes place in several species of *Nocardia* or *Streptomyces*, in which the metabolic product, β-L-lysine, occurs as a constituent of several antibiotics, including myomycin[3] and related compounds,[4] viomycin,[5] roseothricin,[6] geomycin,[7] tuberactinomycin (containing γ-hydroxy-β-lysine),[8] and the strepto-

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