nucleases as well as for the nonspecific phosphohydrolase from *Enterobacter aerogenes*.[40]

The stereochemical courses of phosphohydrolases that produce phosphate can be determined by the use of chirally substituted [^{18}O]phosphorothioates or [^{17}O,^{18}O]×phosphorothioates synthesized by the procedures described in the first section. Hydrolysis of such substrates in $H_2{}^{17}O$ (or H_2O) produces [^{16}O,^{17}O,^{18}O]phosphorothioate, whose configurational analysis is described by Webb this volume, Article [17].

The stereochemical course of snake venom phosphodiesterase and cAMP phosphodiesterase have been determined in $H_2{}^{18}O$ using (R_p) ATPαS[24] or (R_p) *p*-nitrophenyl AMPS[33] as substrates for venom phosphodiesterase and (S_p) cAMPS as the substrate for cAMP phosphodiesterase.[41] The hydrolysis product was (S_p) AMPS,^{18}O in both cases, whose configuration was determined by the methods presented in the second section. It was therefore concluded that the snake venom phosphodiesterase reaction proceeded with overall retention and the cAMP phosphodiesterase reaction with inversion of stereochemical configuration at phosphorus.

Chiral Phosphorothioates as Precursors of Chiral Phosphates

The availability of chirally substituted phosphorothioates makes them logical precursor molecules for syntheses of chirally substituted phosphates, if stereochemically defined methods can be devised for displacing sulfur from the chiral centers with ^{18}O or ^{17}O. A reasonable approach to the synthesis of (R_p) or (S_p)[α-^{18}O]ADP would be to displace sulphur from ADPαS with an ^{18}O atom by a stereochemically defined process. ADPαS is easily synthesized, and $H_2{}^{18}O$ is available. Similarly, the (R_p) and (S_p) epimers of ADPβS,β^{18}O and AMPS,^{18}O can be synthesized in good yields by the methods described in the first section, and they may potentially serve as precursors for [β-^{16}O,^{17}O,^{18}O] ADP and [α-^{16}O,^{17}O,^{18}O]AMP upon displacement of sulfur by $H_2{}^{17}O$.

One such synthesis has recently been completed by Sammons in the author's laboratory.[42] β-Cyanoethyl-ADPαS was synthesized from AMPS and cyanoethylphosphate by the Michelson phosphoanhydride synthesis procedure,[7] and the (S_p) and (R_p) epimers were separated by reverse phase HPLC. (S_p) and (R_p) β-cyanoethyl-ADPαS were then converted to (R_p) and (S_p) β-cyanoethyl-[α-^{18}O]ADP in excellent yields by reaction

[40] J. A. Gerlt and W. H. Y. Wan, *Biochemistry* **18**, 4630 (1979).

[41] P. M. J. Burgers, F. Eckstein, D. H. Hunneman, J. Baraniak, R. W. Lesiak, and W. J. Stec, *J. Biol. Chem.* **254**, 9959 (1979).

[42] R. D. Sammons and P. A. Frey, *J. Biol. Chem.* **257**, 1138 (1982).

with cyanogen bromide in $H_2{}^{18}O$. The products were converted to (R_p) and (S_p)[α-^{18}O]ADP by treatment with base to remove the cyanoethyl protecting groups.

In this reaction the electrophilic cyanogen bromide cyanylates the sulfur in β-cyanoethyl-ADPαS in the first step. The strongly electron-withdrawing cyanide group, now covalently bonded to sulfur, converts it into an excellent leaving group as thiocyanate for displacement by $H_2{}^{18}O$ in the second step. The displacement proceeds with inversion of configuration. Similar results have been privately communicated to the author by F. Eckstein, who used *N*-bromosuccinimide to desulfurize ADPαS in dioxane/$H_2{}^{18}O$.

By coupling highly stereoselective or stereospecific enzymatic phosphorylations of ADPβS,β^{18}O or AMPS,^{18}O with the above described desulfurization in H_2O or $H_2{}^{17}O$, it should be possible to synthesize ATP or ADP with ^{18}O or/and ^{17}O enrichment at any position of the polyphosphate system at which its presence might be desired or required, including either bridging oxygen as well as any of the four diastereotopic oxygens at P_α or P_β of ATP.

Acknowledgments

The author is grateful to the National Institute of General Medical Sciences and the National Science Foundation for financial support.

[15] Use of ^{31}P(^{18}O), ^{31}P(^{17}O), and ^{17}O NMR Methods to Study Enzyme Mechanisms Involving Phosphorus[1]

By MING-DAW TSAI

Introduction

Oxygen is perhaps the most important atom in biological functions. However, biochemical studies based on the physical and chemical properties of the oxygen atom have been few, presumably due to the "magnetic inertness" of ^{16}O ($I = 0$, 99.759% natural abundance) and ^{18}O ($I = 0$, 0.204% natural abundance) and the large quadrupole moment as-

[1] Abbreviations used: P_i, inorganic phosphate; P_{si}, inorganic thiophosphate; PEP, phosphoenolpyruvate; EDTA, ethylenediaminetetraacetic acid; PGK, phosphoglycerate kinase; PP_i, inorganic pyrophosphate; ○, oxygen-16; ⊖, oxygen-17; ●, oxygen-18; AMP, adenosine 5′-monophosphate; AMPS, adenosine 5′-thiophosphate; ADPαS, adenosine 5′-

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sociated with ^{17}O (0.037% natural abundance, $I = 5/2$, $Q = -2.6 \times 10^{-2}$ b).

Physicists have long been aware of the magnetic resonance properties of quadrupolar nuclei[2] and the presence of isotope effects on NMR signals[3] has been known since the 1950s. However, until 1978 most biochemical research using oxygen isotopes had involved only the magnetic resonance of $H_2{}^{17}O$ and the mass spectral analysis of ^{18}O-labeled compounds. Stimulated by the need for methodology to study the mechanism of enzyme reactions involving phosphorus, biochemists finally "demonstrated" (not "discovered") the ^{18}O isotope shift effect[4] and the ^{17}O quadrupolar effect[5] on ^{31}P NMR. This review intends to cover the recent developments and applications of these techniques, as well as some related new developments in magnetic resonance studies involving oxygen isotopes. This chapter emphasizes the methodology and the rationale of application, rather than the detailed insights of various mechanistic problems.

Principles of NMR Methods

$^{31}P(^{18}O)$ NMR. Ramsey and Purcell[3] first predicted the effect of isotope substitution on the magnetic shielding of nuclei. This effect has been observed in various systems: see Ref. 6 for a recent summary. With few exceptions,[7] substitution by a heavier isotope causes the NMR signal of a neighboring nucleus to shift upfield. The magnitude of the shift is related to the fractional change in mass, the chemical shift range of the nucleus being observed, and the structure of the compounds.[8] In the case of multiple substitution, the magnitude of shift is generally additive.[9,10]

(1-thiodiphosphate); ATPαS, adenosine 5′-(1-thiotriphosphate); ADPβS, adenosine 5′-(2-thiodiphosphate); ATPβS, adenosine 5′-(2-thiotriphosphate); ATPγS, adenosine 5′-(3-thiotriphosphate); NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; Glc-1-P, glucose 1-phosphate; MS, mass spectroscopy; diastereomers A and B, designated on the basis of their enzymic activity[50]; NMR, nuclear magnetic resonance; DE, preacquisition delay; T_1, spin–lattice relaxation time; T_2, spin–spin relaxation time.

[2] A. Abragam, "The Principles of Nuclear Magnetism." Oxford Univ. Press, London and New York, 1961.

[3] N. F. Ramsey and E. M. Purcell, *Phys. Rev.* **85**, 143 (1952).

[4] M. Cohn and A. Hu, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 200 (1978).

[5] M.-D. Tsai, *Biochemistry* **18**, 1468 (1979).

[6] J. M. Risley and R. L. Van Etten, *J. Am. Chem. Soc.* **102**, 4609 (1980).

[7] Y. Kanazawa, J. D. Baldeschwieler, and N. C. Craig, *J. Mol. Spectrosc.* **16**, 325 (1965); G. Fraenkel, Y. Asahi, H. Batiz-Hernandez, and R. A. Bernheim, *J. Chem. Phys.* **44**, 4647 (1966).

[8] H. Batiz-Hernandez and R. A. Bernheim, *Prog. Nucl. Magn. Reson. Spectrosc.* **3**, 63 (1967).

[9] J. M. Risley and R. L. Van Etten, *J. Am. Chem Soc.* **102**, 6699 (1980).

[10] M. Cohn and A. Hu, *J. Am. Chem. Soc.* **102**, 913 (1980).

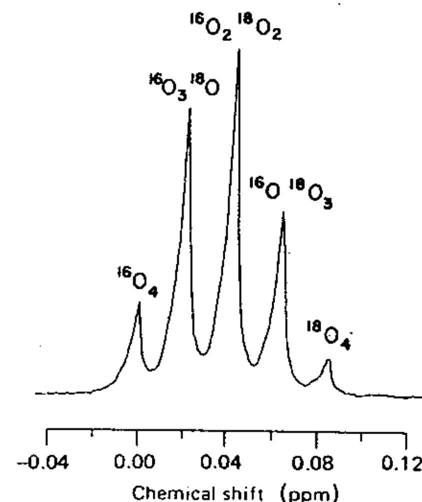


FIG. 1. ^{31}P NMR spectrum of $[^{18}O_4]P_i$ (44 atom% ^{18}O) at 145.7 MHz. (In all figures, unless otherwise specified, the ^{31}P chemical shifts are referenced to external H_3PO_4 with the + sign indicating upfield, whereas the ^{17}O chemical shifts are referenced to external $H_2{}^{17}O$, with the + sign indicating downfield.) [From Ref. 4 with permission.]

The ^{18}O isotope effect in ^{31}P NMR was first reported by Cohn and Hu[4] and others.[11] Figure 1 shows the ^{31}P NMR spectrum of $[^{18}O_4]PO_4^{3-}$ (44 atom% ^{18}O). The five observed peaks correspond to the five isotopic isomers: $^{16}O_4$, $^{16}O_3{}^{18}O$, $^{16}O_2{}^{18}O_2$, $^{16}O^{18}O_3$, and $^{18}O_4$.

The magnitude of the ^{18}O shift on the ^{31}P resonance of phosphate derivatives ranges from 0.01 to 0.04 ppm, and correlates well with the double-bond character of the P–O bond.[10] The resonance frequency of the ^{31}P nucleus at a magnetic field of 2.35 T (corresponding to 100 MHz proton resonance) is 40.5 MHz. To resolve a shift of <0.03 ppm, a resolution of <1 Hz is necessary, and such a high resolution at low magnetic field has been achieved.[11–13] In general, a medium-field spectrometer (4.7–8.5 T) is required for adequate resolution and sensitivity. Since the ^{31}P nucleus is of 100% natural abundance, it is possible to obtain a satisfactory spectrum from an overnight run of a small amount of sample (e.g. <5 μmol) on a medium-field instrument. Figure 2 shows the high-field (14.1 T, 235 MHz ^{31}P resonance) spectrum of the β-phosphate of $[\beta\text{-}^{18}O_2, \beta\gamma\text{-}^{18}O, \gamma\text{-}^{18}O_3]$ATP (63 atom% ^{18}O), in which all possible peaks for the P_β signal are well resolved.

[11] G. Lowe and B. S. Sproat, *J. Chem. Soc., Chem. Commun.* p. 565 (1978); O. Lutz, A. Nolle, and D. Z. Staschewski, *Z. Naturforsch.* **33A**, 380 (1978).

[12] R. L. Van Etten and J. Risely, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4784 (1978).

[13] D. G. Gorenstein and R. Rowell, *J. Am. Chem. Soc.* **102**, 6165 (1980).

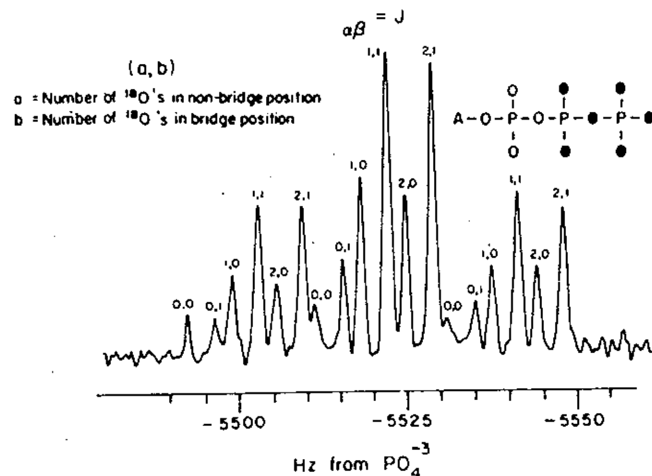


FIG. 2. ^{31}P NMR spectrum of the β-P of [β-$^{18}O_2$, βγ-^{18}O, γ-$^{18}O_3$]ATP (63 atom% ^{18}O) at 235 MHz, by correlation spectroscopy. [From Ref. 10 with permission.]

^{17}O NMR. The observed relaxation times of a nuclear spin system can be expressed as

$$\frac{1}{T_1} = \frac{1}{T_{1d}} + \frac{1}{T_{1c}} + \frac{1}{T_{1s}} + \frac{1}{T_{1r}} + \frac{1}{T_{1q}} + \frac{1}{T_{1p}} \quad (1)$$

$$\frac{1}{T_2} = \frac{1}{T_{2d}} + \frac{1}{T_{2c}} + \frac{1}{T_{2s}} + \frac{1}{T_{2r}} + \frac{1}{T_{2q}} + \frac{1}{T_{2p}} \quad (2)$$

where d is the magnetic dipole-dipole relaxation, c the chemical shift anisotropy relaxation, s the scalar coupling relaxation, r the spin rotation relaxation, q the electric quadrupole relaxation, and p the paramagnetic relaxation.

The most difficult part of the NMR relaxation study of phosphates[14] is to ascribe the experimentally measured T_1 and T_2 to the various specific relaxation mechanism. A commonly used method to solve this problem is to use a "paramagnetic probe," so that the paramagnetic relaxation becomes the dominant relaxation process.[15] However, such a paramagnetic probe may not be feasible or desirable in some problems. Therefore we can adopt ^{17}O as a "diamagnetic probe."[5,16-18] As will be explained later,

[14] D. C. McCain and J. L. Markley, *J. Am. Chem. Soc.* **102,** 5559 (1980).
[15] A. S. Mildvan and M. Cohn, *Adv. Enzymol.* **33,** 1 (1970).
[16] M.-D. Tsai, S. L. Huang, J. F. Kozlowski, and C. C. Chang, *Biochemistry* **19,** 3531 (1980).
[17] S. L. Huang and M.-D. Tsai, *Biochemistry* **21,** 951 (1982).
[18] G. Lowe, B. V. L. Potter, B. S. Sproat, and W. E. Hull, *J. Chem. Soc., Chem. Commun.* p. 733 (1979).

the quadrupolar relaxation is the dominant relaxation process for both T_1 and T_2 in ^{17}O NMR, and the scalar coupling relaxation is the dominant relaxation process for T_2 in ^{31}P (^{17}O) NMR. Without perturbing the system these techniques provide ways to directly observe the behavior of ^{17}O nuclei, which are often directly involved in inter- or intramolecular interactions.

The theoretical basis of the following derivations is derived from several monographs or review articles.[2,19-21] The predominant relaxation mechanism for a quadrupolar nucleus results from the interaction of the electric quadrupole moment eQ with the electric field gradient eq at the nucleus and the modulation of this interaction by rotational motions. In the extreme narrowing conditions—i.e., very fast molecular motions with respect to resonance frequencies, which is the case for most small molecules in solution—the contribution of quadrupolar relaxation to the relaxation rates of the quadrupolar nucleus can be expressed by Eq. (3), where e^2qQ/h is the nuclear quadrupolar coupling

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

constant (NQCC), η is the asymmetry parameter, and τ_r is the effective local correlation time of the quadrupolar nucleus. Under this condition, Eq. (1) and (2) can be approximated by

$$\frac{1}{T_1} \approx \frac{1}{T_{1q}} \approx \frac{1}{T_q} \quad (4)$$

$$\frac{1}{T_2} \approx \frac{1}{T_{2q}} \approx \frac{1}{T_q} \quad (5)$$

Since $1/T_q$ is very large, it is reasonable to assume that the linewidth of the ^{17}O NMR signal $\Delta O \approx 1/\pi T_q$.

Thus, the linewidth of ^{17}O NMR signals is directly related to the rotational correlation time τ_r and the electric field gradient. Obviously a useful variable of ^{17}O NMR is the linewidth. However, most previous work on ^{17}O NMR dealt with chemical shifts.[22-25] The majority of ^{17}O NMR work

[19] T. L. James, "Nuclear Magnetic Resonance in Biochemistry," Academic Press, New York, 1975.
[20] J. M. Lehn and J. P. Kintzinger, *in* "Nitrogen NMR" (M. Witanowski and G. A. Webb, eds.), p. 80. Plenum, New York, 1973.
[21] K. Wuthrich, "NMR in Biological Reactions." Academic Press, New York, 1976.
[22] C. Rodger, N. Sheppard, C. McFarlane, and W. McFarlane, *in* "NMR and the Periodic Table" (R. K. Harris and B. E. Mann, eds.), Chapter 12A. Academic Press, New York, 1978.
[23] H. A. Christ, P. Diehl, H. R. Schneider, and H. Dahn, *Helv. Chim. Acta* **44,** 865 (1961); W. G. Klemperer, *Angew. Chem., Int. Ed. Engl.* **17,** 246 (1978).

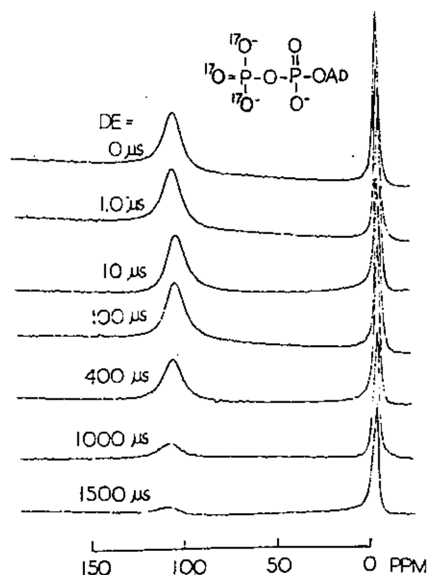


FIG. 3. ^{17}O NMR spectra at 33.9 MHz (5.87 T) showing the effect of the preacquisition delay (DE) on the ^{17}O NMR signals of [β-$^{17}O_3$]ADP.

in biochemical systems dealt with only $H_2\,^{17}O$.[26] Only recently have a few papers concerning biomolecules and linewidth problems[27-31] appeared.

The technical problems in ^{17}O NMR can best be explained by Fig. 3, which shows the ^{17}O NMR signal of [β-$^{17}O_3$]ADP with different preacquisition delays (DE) at 33.9 MHz in a magnetic field of 5.87 T. The ^{17}O NMR signal has a sensitivity of 2.91×10^{-2} relative to that of ^1H, which reduces to only 1.08×10^{-5} for naturally abundant samples. However, both T_1 and T_2 of ^{17}O nuclei are generally very short (<5 msec), so that a very short recycle time (e.g., 50 msec) can be used. Thus the unenriched water gives a reasonably good signal in 1 min. For the biophosphate

[24] J. K. Crandall and M. A. Centeno, *J. Org. Chem.* **44,** 1183. (1979); J. K. Crandall, M. A. Centeno, and S. Borresen, *ibid.* p. 1184.

[25] C. Delseth, T. T.-T. Nguyen, and J.-P. Kintzinger, *Helv. Chim. Acta* **63,** 498 (1980).

[26] I. D. Kuntz, Jr. and W. Kauzmann, *Adv. Protein Chem.* **28,** 239 (1974); M. S. Zetter, G. Y.-S. Lo, H. W. Dodgen, and J. P. Hunt, *J. Am. Chem. Soc.* **100,** 4430 (1978).

[27] H. Pearson, D. Gust, I. M. Armitage, H. Huber, J. D. Roberts, R. E. Stark, R. R. Vold, and R. L. Vold, *Proc. Natl. Acad. Sci. U.S.A.* **72,** 1599 (1975).

[28] K. D. Rose and R. G. Bryant, *J. Am. Chem. Soc.* **102,** 21 (1980).

[29] B. Valentine, T. St. Amour, R. Walter, and D. Fiat, *J. Magn. Reson.* **38,** 413 (1980).

[30] H. M. Schwartz, M. MacCoss, and S. S. Danyluk, *Tetrahedron Lett.* **21,** 3837 (1980).

[31] A. I. Popov, A. J. Smetana, J.-P. Kintzinger, and T. T.-T. Nguyen, *Helv. Chim. Acta.* **63,** 668 (1980); B. Tiffon, B. Ancian, and J.-E. Dubois, *Chem. Phys. Lett.* **73,** 89 (1980).

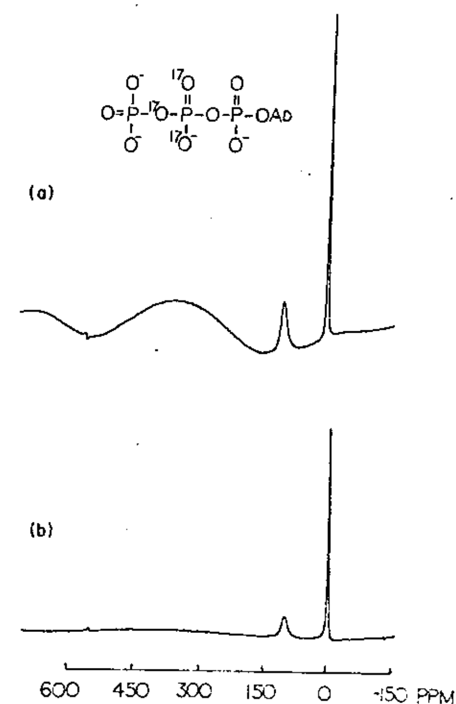


FIG. 4. ^{17}O NMR spectra of [β-$^{17}O_2$,$\beta\gamma$-^{17}O]ATP at 33.9 MHz, showing the effect of preacquisition delay on the base line. (a) DE = 1.0 μsec, which results in a rolling base line. (b) DE = 400 μsec, which gives an undistorted base line.

work, however, an enriched sample is required for several reasons. First, the sample is generally in a dilute (25–50 m*M*) aqueous solution. The solvent signal will be overwhelmingly strong if an unenriched sample is used. Second, the phosphate signals are more than 5–10 times broader than the solvent signal. The S/N ratio of the phosphate signal thus decreases. Third, as the signal is broad (short T_1 and T_2), the loss of data during the preacquisition delay, which is the delay time between the end of a pulse and the beginning of data acquisition, becomes appreciable. As shown in Fig. 3, the S/N ratio of the sharp solvent signal is less affected by the increasing DE values. However, the S/N ratio of the free ADP signal decreases substantially when DE changes from 400 μsec to 1000 μsec. Thus the broader the signal is, the shorter the DE should be in order to detect the signal. Unfortunately, use of a short DE inevitably causes a pulse breakthrough, which results in a rolling baseline,[32] as shown in the ^{17}O NMR spectrum of [β-$^{17}O_2$,$\beta\gamma$-^{17}O] ATP in Fig. 4. The sharp and broad sig-

[32] D. Canet, C. Goulon-Ginet, and J. P. Marchal, *J. Magn. Reson.* **22,** 537 (1976).

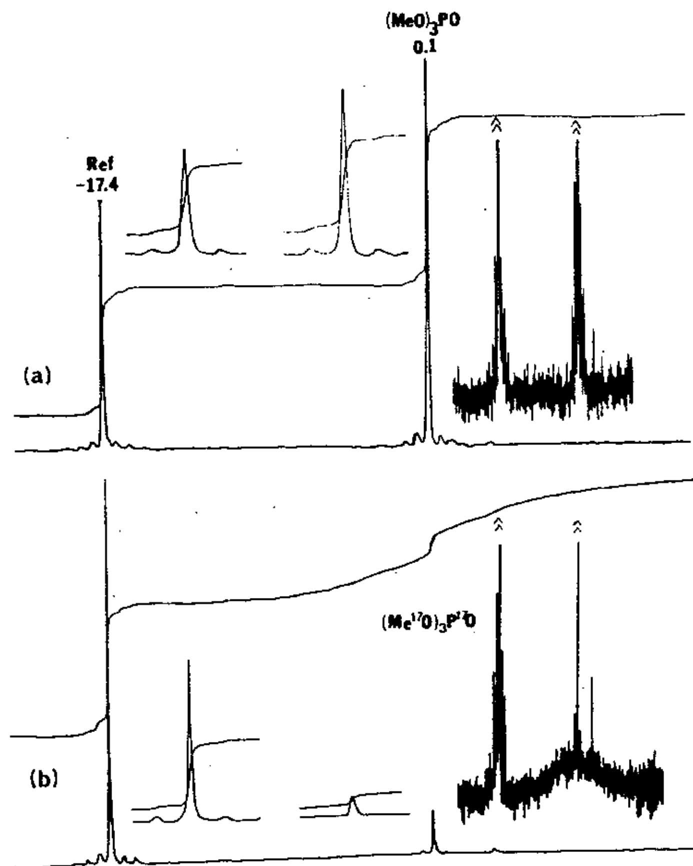


FIG. 5. ^{31}P NMR spectra (at 32.2 MHz) of (a) trimethyl phosphate and (b) trimethyl [$^{17}O_4$]phosphate (70 μmol in 1.2 ml of $CDCl_3$). The left-hand signal comes from triethyl phosphonoacetate (64 μmol) added as the internal reference. The inset on the right-hand side represents the spectra with amplified vertical scale (20 times) and reduced horizontal scale (⇟). The other insets represent the expanded integrations of the corresponding signals in the expanded spectra. [From Ref. 16 with permission.]

nals are due to D_2O and the nonbridging ^{17}O. The bridging ^{17}O signal is either too broad to be observed, or buried under the rolling baseline.[33] This problem is the key factor in determining whether a spectrometer is suitable for a specific ^{17}O NMR experiment. We have so far tried seven different spectrometers: Varian FT-80, JEOL FX-90Q, Bruker WP-200, CXP-180, CXP-300, and the home-made EO-360 and NSF-250 at the NSF

[33] In our high-field (8.456 T) spectrum of [$\alpha\beta,\beta\gamma$-$^{17}O_2$,β-$^{17}O_2$]ATP, there is an evidence for the bridging ^{17}O signal, which is located at 120–125 ppm downfield from $H_2{}^{17}O$ with $\Delta O \approx 1600$ Hz. Since the signal is partially overlapped with a nonbridging ^{17}O signal, it is to be further confirmed.

NMR Center at Illinois. The high-power probe of the CXP-300 (at Procter and Gamble's laboratory) gives the best baseline and S/N ratio for broad signals.

$^{31}P(^{17}O)$ NMR. Figure 5 shows the ^{31}P NMR spectrum of trimethyl [$^{17}O_4$] phosphate (TMP) (39 atom% ^{17}O) in comparison with the corresponding nonlabeled TMP. It is clear that the sharp signal of the ^{17}O-TMP decreases substantially. The integration curve indicates the presence of a very broad signal, which becomes observable when the vertical scale is enlarged, as shown by the insets. In fact the residual sharp signal is due to non-^{17}O species, while the broad signal is due to ^{17}O-labeled species ($^{17}O_4$, $^{17}O_3O$, $^{17}O_2O_2$, $^{17}OO_3$, and O_4). The theoretical basis and experimental problems of the ^{31}P(^{17}O) method are described as follows.[2,19-21] In all discussions, ΔP designates the linewidth of the "broad" instead of the "sharp" ^{31}P NMR signal.

When a dipolar nucleus X (^{31}P in the present case) is bonded directly to a quadrupolar nucleus Q (^{17}O in the present case), X will also be relaxed by virtue of its spin–spin coupling with Q. This was termed "scalar relaxation of the second kind" by Abragam.[2] The effect of such a scalar relaxation on the line shape of ^{31}P signals is dependent on the quadrupolar relaxation time T_q of ^{17}O and the spin–spin coupling constant J between ^{31}P and ^{17}O. When T_qJ is large, the signal of ^{31}P is split by spin–spin coupling, as in the case of $P^{17}OCl_3$. As T_qJ decreases, line broadening of the coupling pattern occurs.[34] When T_qJ is small ($T_q < 1/2\pi J$), the multiplet splitting of ^{31}P collapses and the signal appears as a broad singlet. Under this condition, the scalar relaxation contributes to the relaxation of ^{31}P:

$$\frac{1}{T_{1s}} = \frac{8\pi^2J^2I(I+1)}{3}\frac{T_q}{1+(\omega_p-\omega_o)^2T_q^2} \tag{6}$$

$$\frac{1}{T_{2s}} = \frac{4\pi^2J^2I(I+1)}{3}\left[T_q + \frac{T_q}{1+(\omega_p-\omega_o)^2T_q^2}\right] \tag{7}$$

where I is the spin quantum number of ^{17}O, J is the spin–spin coupling constant between ^{31}P and ^{17}O, and ω_P and ω_O are the angular precession frequencies of ^{31}P and ^{17}O, respectively. When $(\omega_P - \omega_O)^2T_q^2 >> 1$, which is the case for ^{31}P–^{17}O in most compounds, Eqs. (6) and (7) can be simplified to

$$\frac{1}{T_{1s}} \approx 0 \tag{8}$$

$$\frac{1}{T_{2s}} \approx \frac{4\pi^2J^2I(I+1)}{3}T_q \tag{9}$$

[34] M. Suzuki and R. Kubo, *Mol. Phys.* 7, 201 (1964).

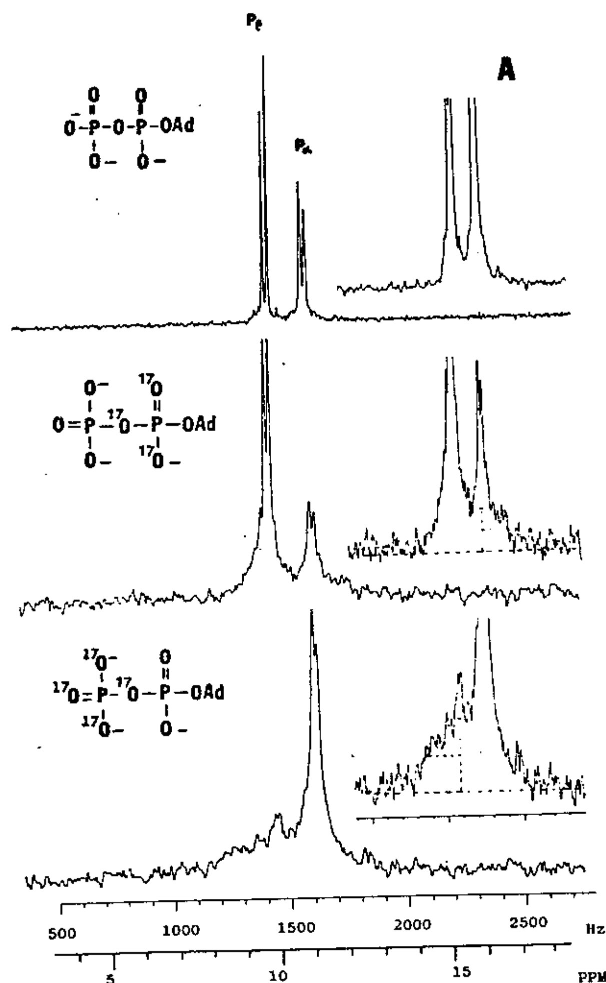


FIG. 6. ^{31}P NMR spectra (at 145.7 MHz) of nonlabeled ADP, [α-$^{17}O_2$,$\alpha\beta$-^{17}O]ADP (39 at. % ^{17}O) and [$\alpha\beta$-^{17}O, β-$^{17}O_3$]ADP (51 at. % ^{17}O), showing that the ΔP dramatically decreases, but not to the limit of extremely small ΔP, when ADP binds with excess ribonuclease A. (A) Free ADP, δP_α = 10.9 ppm, δP_β = 9.8 ppm. (B) Bound ADP, δP_α = 10.1 ppm, δP_β = 6.0 ppm. Sample conditions: 0.12-*M* NaOAc buffer, pH 6.2, containing 25% D_2O, 10 mg EDTA. A 7.0 Hz line broadening has been applied to the spectra in (A) and 4.0 Hz to that in (B). Temperature: 25–26°.

Thus, the scalar relaxation due to ^{17}O contributes negligibly to T_1 but significantly to T_2 of ^{31}P. Since scalar relaxation is the predominant spin–spin relaxation mechanism in this case, Eq. (2) for ^{31}P can be reduced to

$$\frac{1}{T_2} \approx \frac{1}{T_{2s}} \approx \frac{4\pi^2 I(I+1)}{3} T_q J^2 \tag{10}$$

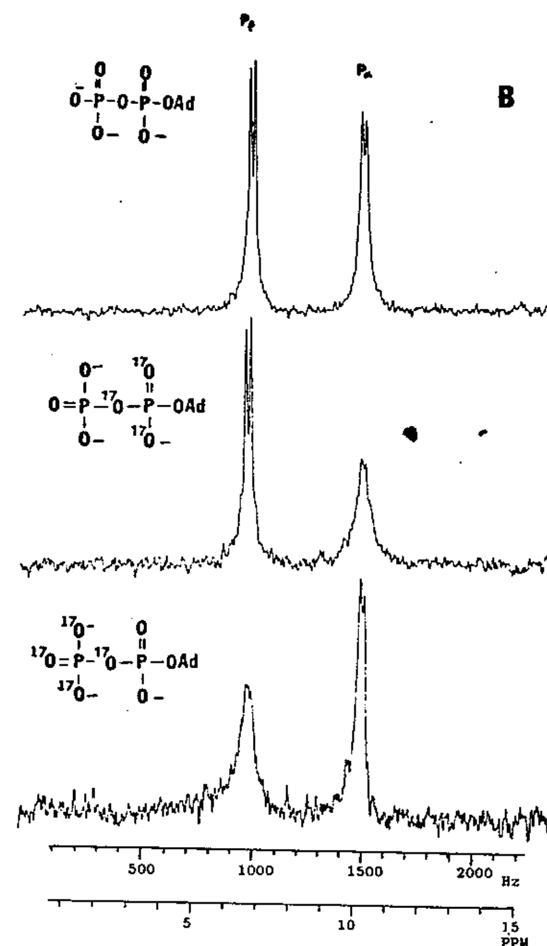


FIG. 6. (*continued*)

Making a reasonable assumption that $\Delta P \approx 1/\pi T_2$ and $\Delta O \approx 1/\pi T_q$, one can derive the following relationship from Eq. (10):

$$(\Delta P)(\Delta O) \approx \frac{4I(I+1)}{3} J^2 = \frac{35}{3} J^2 \tag{11}$$

Equation (11) is approximately true at least under extremely narrowing conditions. It suggests that when ΔP is not extremely small (<5 Hz), the ^{31}P(^{17}O) NMR method can be useful in three ways: (1) to determine the isotope enrichment of ^{17}O[5], which has been used in a few examples[17,35]; (2) the result of a large ΔP is that the ^{31}P signal is "quenched" by ^{17}O, such an

[35] G. H. Reed and T. S. Leyh, *Biochemistry* **19**, 3472 (1980).

effect is useful in stereochemical studies to be discussed later; (3) since ΔP is related to ΔO, the $^{31}P(^{17}O)$ NMR may be used to deduce ΔO when the ^{17}O signal is too broad to be detected. However, since the observed ΔP represents a mixture of different isotopic species (100% enriched $H_2{}^{17}O$ is not available), the third aspect of application is only qualitatively useful at this stage. An important question to ask is under what condition will the ΔP be too small to be useful. We have shown that an extremely small ΔP will not occur with most biophosphate compounds (including both bridging and nonbridging ^{17}O)[16] or even when α- and β-^{17}O-ADP are bound to ribonuclease A, as shown in Fig. 6. However, an extremely small ΔP has been observed for $H_3P^{17}O_4$/glycerol, presumably due to the high viscosity of glycerol (785 times more viscous than water at 30°).

Application of NMR Methods

Numerous problems can be or have been solved by the $^{31}P(^{18}O)$ method. Almost everything that mass spectroscopy can do can be done by the NMR method in a nondestructive, simpler, and more informative way. The only advantages that MS can still claim are higher accuracy and smaller sample size. The latter is, however, counterbalanced by the need to degrade and derivatize biophosphates for MS analysis.

It is therefore impractical to describe all cases of application of the $^{31}P(^{18}O)$ NMR method in this chapter. Readers interested in such an extensive coverage should refer to the review by Cohn[36] and other chapters in this volume that discuss specific types of problems. The earlier work has been covered by two reviews.[37,38]

Instead, the application of the $^{31}P(^{18}O)$ method is discussed in this chapter on the basis of its merit and rationale. The four main types of application are (1) oxygen isotope exchange, (2) phosphate exchange, (3) positional isotope exchange, and (4) stereochemistry. At least one example for each type of application is given and compared with examples of the application of the $^{31}P(^{17}O)$ NMR method. Although fewer examples are available for the $^{31}P(^{17}O)$ method, they require a more elaborate explanation. The stereochemical problems are emphasized since this has been a most important topic relating to enzyme mechanisms at phosphorus in the past, and is also one of the main themes of this volume.

[36] M. Cohn, *Annu. Rev. Biophys. Bioeng.* (in press).
[37] J. R. Knowles, *Annu. Rev. Biochem.* **49,** 877 (1980).
[38] J. J. Villafranca and F. M. Raushel, *Annu. Rev. Biophys. Bioeng.* **9,** 363 (1980).

$^{31}P(^{18}O)$ NMR: A Substitute for Mass Spectrometry and ^{32}P-Radioisotope Labeling.

Oxygen Isotope Exchange. The enzymes that catalyze hydrolysis of phosphate monoesters very often also catalyze $P_i \rightleftharpoons H_2O$ oxygen exchange,[39] presumably through the reverse pathway of hydrolysis. This subject has been investigated extensively by Boyer and Hackney by MS, as reviewed recently.[40] The $^{31}P(^{18}O)$ NMR method offers an alternative tool for such studies, but has not completely replaced the MS method. For this specific type of work, MS can still be advantageous for detailed kinetic study, which requires precise analysis of a large number of small samples. Figure 7 shows the ^{31}P NMR experiment on the $P_i \rightleftharpoons H_2O$ oxygen exchange catalyzed by native alkaline phosphatase from *E. coli* and by cobalt alkaline phosphatase, clearly showing different kinetics for the two enzyme forms.[41] The starting $KH_2P^{18}O_4$ (P_i), as shown in Fig. 7a, is "statistically labeled" with 90.3% ^{18}O for its oxygen. The zinc alkaline phosphatase (native enzyme) catalyzes the exchange of only one oxygen per encounter, so that the partially exchanged P_i is still statistically labeled, as shown in Fig. 7b. However, Fig. 7c shows that the experimentally observed distribution of isotopic species for the cobalt enzyme-exchanged P_i is not a binomial distribution, which suggests that more than one oxygen of P_i is exchanged per encounter. It is interesting to note that while there are a number of reports on the $P_i \rightleftharpoons H_2O$ oxygen exchange studied by $^{31}P(^{18}O)$ NMR,[4,12,41–43] the ^{31}P NMR study of inorganic thiophosphate (P_{si}) is far less popular. The $P_{si} \rightleftharpoons H_2O$ oxygen exchange has been observed (by MS analysis),[44] but the detailed kinetics have not been established.

Oxygen exchange study by use of $^{31}P(^{18}O)$ NMR has also been done for enzymes other than phosphomonoesterases, and for substrates other than P_i. For example, glutamine synthetase catalyzes oxygen exchange of Pi,[43] and carbamoyl-phosphate synthetase catalyzes oxygen exchange of the γ-phosphate of ATP,[45] both via reversible reaction processes.

[39] D. D. Hackney and P. D. Boyer, *Proc. Natl. Acad. Sci. U.S.A.* **75,** 3133 (1978).
[40] D. D. Hackney, K. E. Stempel, and P. D. Boyer, in "Methods in Enzymology" (D. L. Purich, ed.), Vol. 64, p. 60. Academic Press, New York, 1980; D. D. Hackney, *J. Biol. Chem.* **255,** 5320 (1980).
[41] J. L. Bock and M. Cohn, *J. Biol. Chem.* **253,** 4082 (1978).
[42] M. R. Webb, G. G. McDonald, and D. R. Trentham, *J. Biol. Chem.* **253,** 2908 (1978).
[43] M. S. Balakrishnan, T. R. Sharp, and J. J. Villafranca, *Biochem. Biophys. Res. Commum.* **85,** 991 (1978).
[44] M.-D. Tsai, *Biochemistry* **19,** 5310 (1980); M.-D. Tsai and T. T. Chang, *J. Am. Chem. Soc.* **102,** 5416 (1980).
[45] F. M. Raushel and J. J. Villafranca, *Biochemistry* **18,** 3424 (1979).

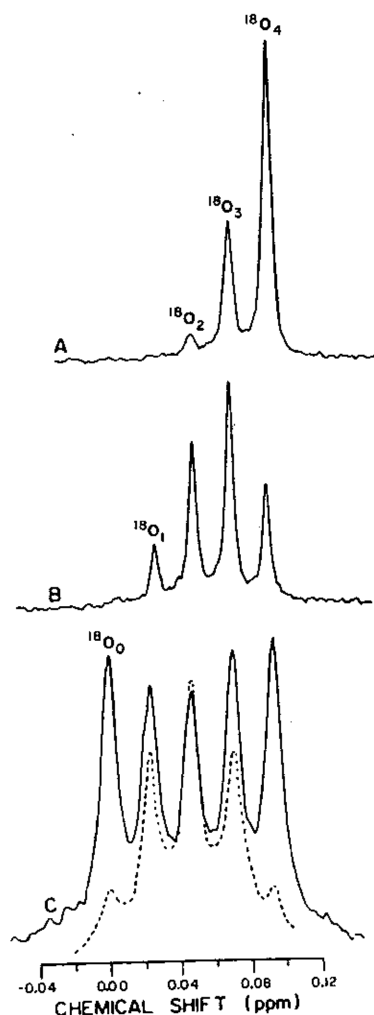
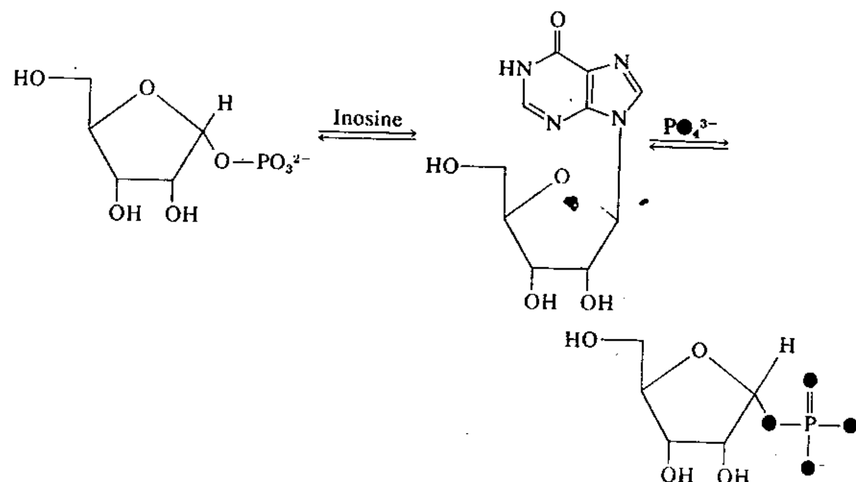


FIG. 7. ^{31}P NMR spectra at 145.7 MHz showing the enzyme-catalyzed Pi ⇌ H_2O oxygen exchange. (A) Initial $KH_2P^{18}O_4$, 90.3 at. % ^{18}O, 10mM. (B) After reaction with 7μM native alkaline phosphatase for 3 hr at 22° (66.5 at. % ^{18}O). (C) After reaction with 13 μM cobalt alkaline phosphatase for 55 min at 22°. The dashed line in (C) is the calculated spectrum for binomial distribution of 51.3 at. % [$^{18}O_4$]Pi. [From Ref. 41 with permission.]

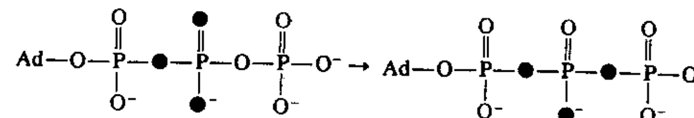
Phosphate Exchange. It has been established by ^{32}P radioisotope experiments that the polynucleotide phosphorylase catalyzes the P_i ⇌ ADP exchange reaction. Cohn and Hu[4] have demonstrated that by using [$^{18}O_4$]P_i, the $^{31}P(^{18}O)$ NMR method can not only observe the exchange process, but also establish that the P_i molecule is exchanged with all four oxygens remaining intact.

Jordan *et al.*[46] have followed the exchange between ribose 1-phosphate and [$^{18}O_4$]P_i, catalyzed by the purine-nucleoside phosphorylase from calf spleen using ^{31}P NMR. The results show that the ^{18}O distribution is the same in the starting P_i and the phosphate of ribose 1-phosphate after exchange, suggesting that the C–O bond rather than the P–O bond of ribose 1-phosphate is cleaved:



In both examples, the [$^{18}O_4$]P_i functions as a ^{32}P label. In addition, it also establishes the position of bond cleavage (or bond formation).

Positional Isotope Exchange. Positional isotope exchange is an extremely important way of investigating enzyme mechanism. Using the MS method, Rose has developed and reviewed this subject.[47] Owing to the development of the $^{31}P(^{18}O)$ NMR method, the bridging and nonbridging ^{18}O can now be easily distinguished with the intact nucleotide. Article [12] in this volume specifically deals with the use of $^{31}P(^{18}O)$ NMR for positional isotope exchange studies. Only an example is presented here. Lowe and Sproat[48] have shown that pyruvate kinase catalyzes the following positional isotope exchange in the absence of any acceptor:



[46] F. Jordan, J. A. Patrick, and S. Salamone, Jr., *J. Biol. Chem.* **254**, 2384 (1979).
[47] I. A. Rose, *Adv. Enzymol.* **50**, 361 (1979).
[48] G. Lowe and B. S. Sproat, *J. Chem. Soc. Perkin Trans. 1* p. 1622 (1978).

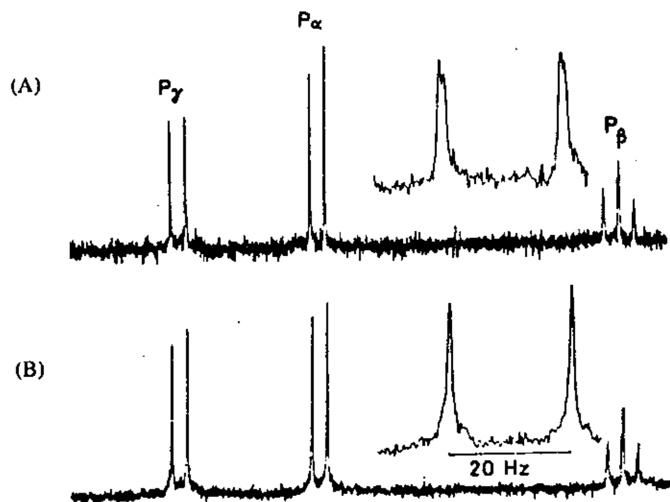


FIG. 8. ^{31}P NMR spectra at 36.43 MHz showing the positional isotope exchange of [$\alpha\beta$-^{18}O, β-$^{18}O_2$]ATP. The inset is the expanded P_γ resonance. The starting ATP clearly shows no ^{18}O isotope shift at P_γ. (A) After incubation with pyruvate kinase in the absence of pyruvate, the P_γ signal shows presence of ^{18}O isotope. (B) A control experiment which shows that such a positional isotope exchange does not occur when ATP binding is prevented by a saturated concentration of PEP. [From Ref. 48 with permission.]

The ^{31}P NMR spectra in Fig. 8 clearly show such a conversion. The authors used such evidence to argue that the phosphoryl transfer catalyzed by pyruvate kinase proceeds by the "metaphosphate" mechanism (dissociative). However, alternative explanations such as transfer of the γ-phosphoryl group to an unidentified nucleophile have not been ruled out.[37]

^{31}P(^{18}O) or ^{31}P(^{17}O) NMR: Stereochemistry at a Prochiral or Proprochiral Phosphorus Center

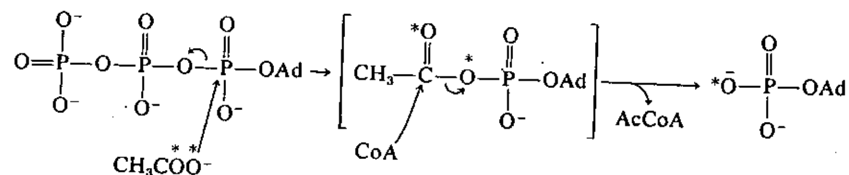
The enzyme-catalyzed reactions involving a P–O bond cleavage can be categorized into the following types based on the stereochemistry involved:

(a) $ROPO_2OR'$ (prochiral) $\rightleftharpoons$ $ROPO_2OR''$ (prochiral)
(b) $ROPO_2OR'$ (prochiral) $\rightleftharpoons$ $ROPO_3$ (pro-prochiral)
(c) $ROPO_3$ (pro-prochiral) $\rightleftharpoons$ $R'OPO_3$ (pro-prochiral)
(d) $ROPO_3$ (pro-prochiral) $\rightleftharpoons$ PO_4 (pro-pro-prochiral)
(e) PO_4 (pro-pro-prochiral) $\rightleftharpoons$ PO_4 (pro-pro-prochiral)

Reaction types (a) and (b) have been studied by the ^{31}P(^{18}O) or the ^{31}P(^{17}O) NMR method, covered in this section. Reaction types (c) and (d) have been studied by combined use of the ^{31}P(^{18}O) *and* the ^{31}P(^{17}O) NMR methods, discussed in the next section. In all cases, the MS method can

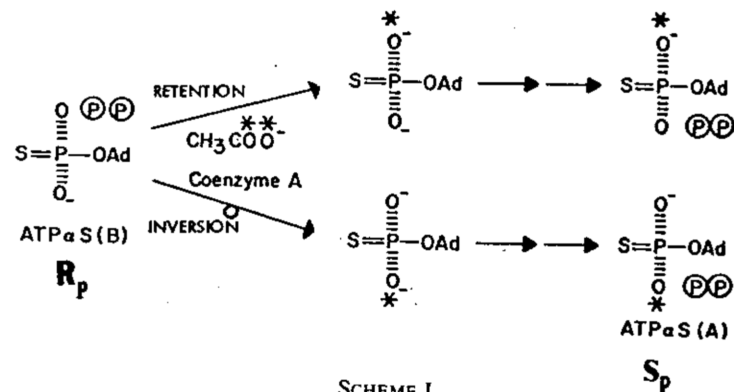
also be used in a less direct way. Earlier work on these problems has been reviewed.[37,38,49,50] In addition, some articles in this volume review some specific types of reactions.

Acetyl-CoA synthetase[51] catalyzes the following reaction, which is an example of reaction type (b):



To elucidate the stereochemical course of the displacement at phosphorus, we made use of the thiophosphate analogs of ATP developed by Eckstein.[49,50] It was found that acetyl-CoA synthetase is specific to (R_p) ATPαS but not to (S_p) ATPαS. As shown in Scheme I, when (R_p)-ATPαS and ^{17}O-acetate are used as substrates, the ^{17}O from acetate will be incorporated into the pro-*S* position of AMPS if the reaction proceeds with retention of configuration, or the pro-*R* position if inversion occurs. By converting the AMPS to (S_p) ATPαS with known procedures,[52,53] the pro-*S* oxygen of AMPS should be located at the nonbridging position of (S_p) ATPαS while the pro-*R* oxygen should be at the bridging position.

Our original idea was to use ^{17}O NMR to distinguish the bridging and nonbridging ^{17}O. However, the synthesized (S_p) ATPαS labeled with ^{17}O at both the α-nonbridging and α,β-bridging positions gave only one ^{17}O



SCHEME I

[49] F. Eckstein, *Angew. Chem. Int. Ed. Engl.* **14**, 160 (1975).
[50] F. Eckstein, *Acc. Chem. Res.* **12**, 204 (1978).
[51] P. D. Boyer, O. J. Koeppe, and W. W. Luchsinger, *J. Am. Chem. Soc.* **78**, 356 (1956).
[52] K. F. R. Sheu and P. A. Frey, *J. Biol. Chem.* **252**, 4445 (1977).
[53] E. K. Jaffe and M. Cohn, *Biochemistry* **17**, 652 (1978).

NMR signal (133 ppm). Later, after more extensive model studies, we realize that the signal of a bridging ^{17}O is too broad to be detected. Although there is a difference between the bridging and nonbridging ^{17}O, since the absence of a signal cannot be used to conclude the presence of a bridging ^{17}O, the ^{17}O NMR does not seem to be a satisfactory method, unless better instrumentation can be used to observe the signal of the bridging ^{17}O.[33]

This was, however, the starting point of all our later work using ^{17}O. Even with the low ^{17}O enrichment (20%) in the earlier work and the fair ^{31}P NMR spectra at a low magnetic field (1.88 T), we noticed that the intensity of the ^{31}P NMR signal decreases when the ^{31}P nucleus is directly bonded to ^{17}O. The theoretical basis of this ^{17}O quadrupolar effect has been discussed earlier in this article. Figure 9 shows the ^{31}P NMR spectra of (A) nonlabeled (S_p) ATPαS, (B) the synthesized (S_p) [α-^{17}O, $\alpha\beta$-^{17}O] ATPαS, and (C) the (S_p) ATPαS obtained from the enzyme reactions shown in Scheme I. The ^{17}O isotope used was 20% enriched, and the enrichment of ^{17}O-acetate was determined as 19%. In Fig. 9(B), the P_α signal decreases to 67 ± 1% and the P_β signal to 83 ± 4%. In Fig. 9(C), the P_α signal decreases to 80 ± 4% and P_β signal to 82 ± 5%. Since both P_α and P_β have decreased in Fig. 9(C), the results indicate that ^{17}O must be located at the bridging position, and the reaction catalyzed by acetyl-CoA synthetase must proceed with inversion of configuration.[5]

In our experience, integration is more accurate than peak height to measure the peak "intensity." The height of a ^{31}P NMR signal can have a large error if the signal-to-noise ratio is not very good. The signal to be integrated should be fully expanded such that the "broad signal" due to ^{31}P–^{17}O species is not integrated. The insets in Fig. 9 show the integration of each expanded signal. It should also be kept in mind that although the line-broadening effect of ^{17}O in ^{31}P NMR has been shown to be generally present for small biophosphate molecules,[16] it is not unlikely that under certain conditions an extreme case of very large ΔO and very small ΔP [Eq. (11)] could occur.

The same problem could have been solved by use of the ^{31}P(^{18}O) NMR method to distinguish the bridging ^{18}O and the nonbridging ^{18}O. This has not been done for this specific enzyme, but Midelfort and Sarton-Miller[54] have studied the same problem by the same approach, using the ^{18}O isotope. Instead of analyzing the ^{18}O-labeled (S_p) ATPαS by ^{31}P NMR [their work was done prior to publication of the ^{31}P(^{18}O) NMR], they determined the ^{18}O position by MS analysis of the degraded compound.

Indeed, most of other work on reaction types (a) and (b) have been performed by use of thiophosphate analogs and the ^{18}O isotope. The

[54] C. F. Midelfort and I. Sarton-Miller, *J. Biol. Chem.* **253**, 7127 (1978).

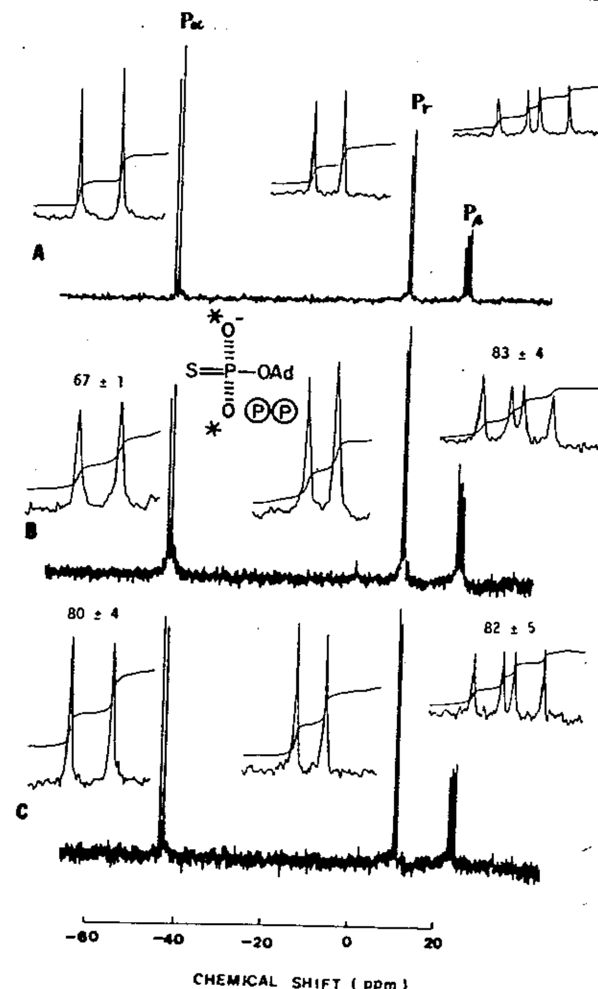
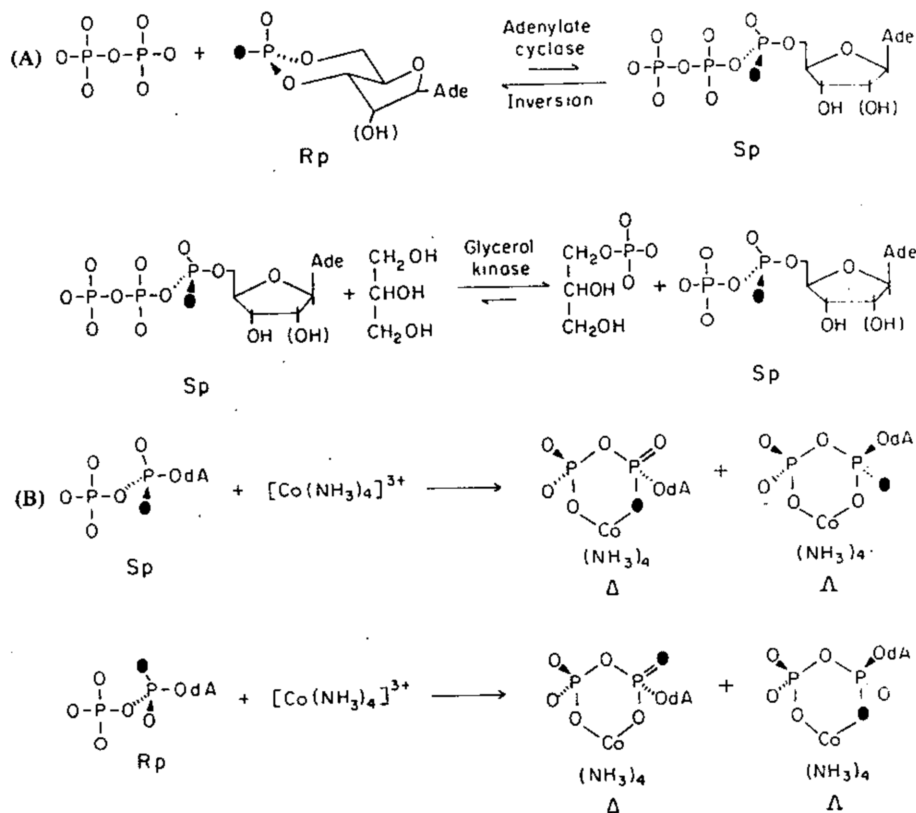


FIG. 9. ^{31}P NMR spectra (at 32.2 MHz) showing the results of acetyl-CoA synthetase. (A) Nonlabeled (Sp)-ATPαS. (B) Synthesized (Sp)-[α-^{17}O,$\alpha\beta$-^{17}O]ATPαS. (C) The (Sp)-ATPαS from [$^{17}O_2$] acetate. The insets represent the integrations of the corresponding signals. [From Ref. 5 with permission.]

method of analysis used was in most cases either MS or ^{31}P(^{18}O) NMR. The work up to early 1980 has been reviewed.[37,38,50]

Recently, Gerlt's group has investigated reaction types (a) and (b) by use of substrates labeled with only oxygen isotopes (without a sulfur substitution). In general, such an approach requires more sophisticated synthetic procedures and configurational analysis. However, the problem of low enzyme activity toward sulfur analogs can be avoided, and the results



SCHEME II

are certainly more authentic. Two recent pieces of work involve the stereochemistry of cyclic AMP hydrolysis catalyzed by the cyclic AMP phosphodiesterase[55] (type b), which will be covered in the next section, and the stereochemistry of adenylate cyclase[56] (type a), which deserves an elaborate presentation.

The experimental approach to elucidate the stereochemistry of adenylate cyclase is shown in Scheme II. They first synthesized (R_p) and (S_p) cyclic [^{18}O]AMP with known configuration, which reacted with pyrophosphate and glycerol in the presence of the adenylate cyclase from *B. liquefaciens* and yeast glycerol kinase to yield two diastereomers of [α-^{18}O]dADP (Scheme IIA). To determine the configuration of P_α, they converted the two isomers to the α,β-bidentate $Co(NH_3)_4$dADP complexes

[55] J. A. Coderre, S. Mehdi, and J. A. Gerlt, *J. Am. Chem. Soc.* **103**, 1872 (1981).

[56] J. A. Coderre and J. A. Gerlt, *J. Am. Chem. Soc.* **102**, 6594 (1980).

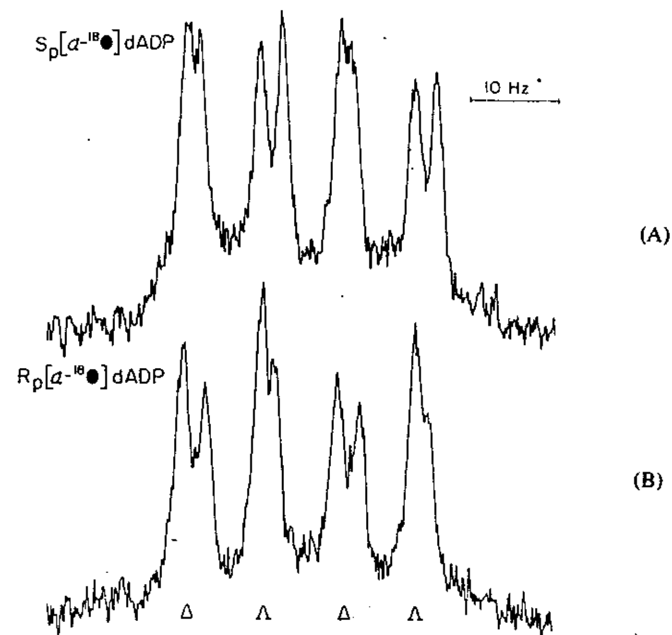


FIG. 10. The α-phosphorus region of the 81.0-MHz ^{31}P NMR spectra of $Co(NH_3)_4$dADP prepared from the enzymatic products, which had been isotopically diluted with an equal amount of unlabeled dADP. The top spectrum is that of the sample prepared from the [α-^{18}O]dADP predicted to have the S_p configuration, and the bottom spectrum is that of the sample prepared from the [α-^{18}O]dADP predicted to have the R_p configuration. The approximate chemical shift of the center of the multiplets is +0.80 ppm. The first and third sets of resonances are associated with the Δ screw sense diastereomer, and the second and fourth are associated with the Λ screw sense diasteromer (two sets of resonances are present for each diastereomer because of spin–spin coupling to the β-phosphorus atoms). [From Ref. 56 with permission.]

by the procedure of Cornelius *et al.*,[57] which gave a mixture of the Δ and Λ isomers[58] (Scheme IIB). The ^{31}P NMR spectra in Fig. 10 clearly show that for the sample synthesized from the R_p diastereomer of cyclic [^{18}O]dAMP [Fig. 10(A)], the ^{18}O shift for the Δ isomer is 1.3 Hz and that for the Λ isomer is 2.4 Hz, which suggests that in the Δ isomer ^{18}O is at the P—O···Co bridging position, while in the Λ isomer, ^{18}O is not directly chelated with Co^{3+}. The results from the opposite isomer [Fig. 10(B)] show just the reverse. On the basis of all of the stereochemistry involved, the configuration of the [α-^{18}O]dADP can be assigned and the stereochemistry of adenylate cyclase can be determined. The result is inversion of

[57] R. D. Cornelius, P. A. Hart, and W. W. Cleland, *Inorg. Chem.* **16**, 2799 (1977).

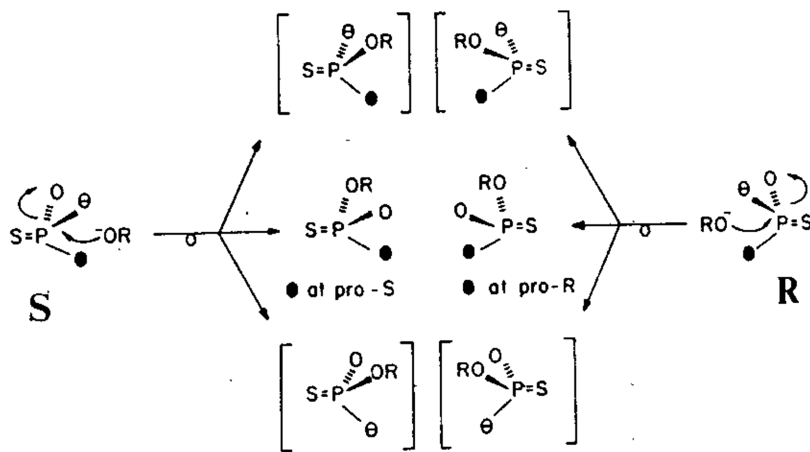
[58] D. Dunaway-Mariano and W. W. Cleland, *Biochemistry* **19**, 1496 (1980).

configuration, consistent with the results of using thiophosphate analogs.[59]

$^{31}P(^{18}O)$ and $^{31}P(^{17}O)$ NMR: Stereochemistry at a Pro-prochiral or Pro-pro-prochiral Phosphorus Center

In a paper discussing the general applicability of the $^{31}P(^{17}O)$ NMR method,[16] we have pointed out that in addition to the examples described above, two other important application aspects are: (1) in combination with the $^{31}P(^{18}O)$ NMR method, to analyze the configuration of chiral [^{16}O,^{17}O,^{18}O]thiophosphates (P_{si}) or chiral [^{16}O,^{17}O,^{18}O]phosphate monoesters; and (2) in combination with ^{17}O NMR, to study binding of nucleotides in diamagnetic systems. These two subjects are covered in this and the following sections, respectively.

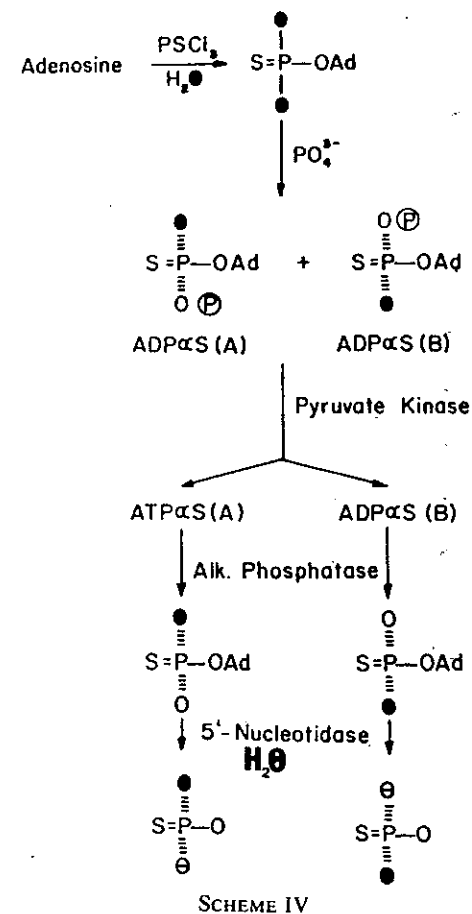
Scheme III illustrates the rationale of configurational analysis for chiral P_{si}.[44] The same principle applies to chiral phosphate monoesters, where the P–S bond is replaced by P–OR. Displacement of one of the three oxygen isotopes of (*S*)-[^{16}O,^{17}O,^{18}O]P_{si} by a nucleophile (RO^-) gives a mixture of three inseparable, isotopically different species. Among them, two (those in brackets) contain an ^{17}O isotope, which should quench the corresponding ^{31}P NMR signals. Only the species that contains only ^{16}O and ^{18}O (^{18}O at the pro-*S* position) should give a sharp, unquenched ^{31}P NMR signal. Analogously, the (*R*)-[^{16}O,^{17}O,^{18}O]P_{si} should give a corresponding non-^{17}O-containing species with ^{18}O at the pro-*R* position.



SCHEME III

[59] J. A. Gerlt, J. A. Coderre, and M. S. Wolin, *J. Biol. Chem.* **255**, 331 (1980).

Thus, determination of whether ^{18}O is at the pro-*R* or pro-*S* position would tell the configuration of chiral P_{si} or chiral phosphate monoesters. A general way to achieve this is to stereospecifically derivatize the pro-*R* or pro-*S* oxygen. The $^{31}P(^{18}O)$ NMR method can then be used to distinguish the bridging and nonbridging ^{18}O on the basis of the different magnitude of isotope shifts.[10,18,60] Therefore, two main chemical steps need to be done to convert the chiral phosphoryl group to an analyzable form: a displacement with known stereochemistry, and stereospecific derivatization of the prochiral oxygens. These two chemical steps vary from compound to compound, but the underlying principles remain the same.

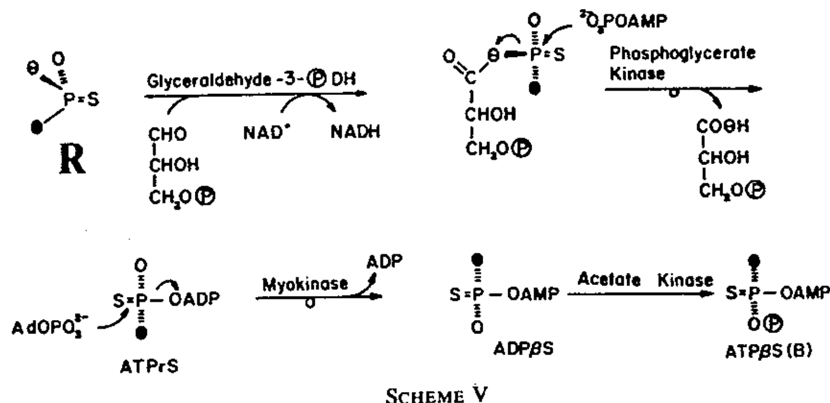


SCHEME IV

[60] D. G. Gorenstein and R. Rowell, *J. Am. Chem. Soc.* **102**, 6166 (1980).

To date, the ^{31}P NMR method has been used to solve at least one problem for each of the reaction types (b), (c), and (d). However, the first application was for reaction type (d), since at that time the type (b) had been solved by use of thiophosphate analogs and a solution for type (c), the phosphoryl-transfer reaction of phosphate monoesters, had been well demonstrated by the elegant MS analysis developed by Knowles.[37] On the other hand, reaction type (d) remained a challenging problem since it involved a pro-pro-prochiral phosphorus center. To make the inorganic phosphate (P_i) chiral, it is necessary to use the sulfur analog (P_{si}), since only three oxygen isotopes are available (other isotopes have a lifetime of less than 1 min).

Venom 5′-nucleotidase furnishes a good example of reaction type (d), since it catalyzes hydrolysis of AMP to adenosine and P_i but does not catalyze transphosphorylation or $P_i \rightleftharpoons H_2O$ oxygen exchange.[61] Our approach to this problem is shown in Scheme IV, which shows the synthesis of (R_p) [^{18}O]AMPS and (S_p) [^{18}O] AMPS and hydrolysis of these two isomers in $H_2{}^{17}O$ to give two chiral [^{16}O,^{17}O,^{18}O] P_{si} enantiomers with unknown configuration. The "two main steps" required were indeed available in the literature,[62–64] as shown in Scheme V. The stereochemical course of each step in Scheme V had been elucidated separately,[63,64] except that of phosphoglycerate kinase, which was elucidated by Webb and Trentham using synthesized chiral P_{si} of known configuration[65] on the basis of the same NMR analysis discussed below.



SCHEME V

[61] D. E. Koshland, Jr. and S. S. Springhorn, *J. Biol. Chem.* **221**, 469 (1956).
[62] F. Eckstein, *Biochim. Biophys. Acta* **483**, 1 (1977).
[63] J. P. Richard and P. A. Frey, *J. Am. Chem. Soc.* **100**, 7757 (1978).
[64] J. P. Richard, H.-T. Ho, and P. A. Frey, *J. Am. Chem. Soc.* **100**, 7756 (1978).
[65] M. R. Webb and D. R. Trentham, *J. Biol. Chem.* **255**, 1775 (1980).

TABLE I
^{31}P NMR ANALYSIS OF THE ATPβS(B) DERIVED FROM CHIRAL THIOPHOSPHATES

P_{si} samples	Intensity (%)[a] a	b	c	d	F Value	Configuration
$PS^{18}O_3{}^{3-}$	41.3± 1.2	24.6± 0.1	22.1± 0.0	11.8± 1.2	1.11	
(S_p) AMPS	8.8± 0.5	42.8± 0.6	28.1± 0.5	20.3± 0.5	1.52	S
(R_p) AMPS	12.2± 0.5	26.5± 1.6	38.8± 0.1	22.4± 2.0	0.68	R
Calc[b]	7.8	47.3	25.9	19.0	1.82	S
	7.8	25.9	47.3	19.0	0.55	R

[a] Obtained from peak height measurements for the P_β signal of ATPβS. The errors represent deviations between the two nonoverlapping halves of the two doublets.
[b] Calculated for chiral P_{si} of 47.5% purity and 90% chirality expected based on isotopic enrichments.

According to Scheme V, the (R_p)-chiral P_{si} should give ATPαS(B) with ^{18}O located specifically at the β-nonbridging position. The (S_p) enantiomer should give ATPβS(B) with ^{18}O at the $\beta\gamma$-bridging position. It is known that a bridging ^{18}O should cause a smaller isotope shift in ^{31}P NMR than a nonbridging ^{18}O does. On this basis, the configuration can be determined. However, Scheme V only shows the species that will give an unquenched ^{31}P NMR signal. In reality, each chiral P_{si} species should give a mixture of three ATPβS(B) species (Ia,Ib, and Ic in Scheme VI). In addition, it is impossible to have a chiral P_{si} of 100% purity. A chiral P_{si} sample actually contains up to six isotopic species, as shown in the left column of Scheme VI; two of them are identical species, and each gives three ATPβS(B) species. Fortunately, a careful examination of Scheme VI reveals that there are only four different non-^{17}O-containing species **a**, **b**, **c**, and **d**, and that all the nonchirally labeled P_{si} species contribute *equally* to species **b** and **c**. Only the [^{16}O,^{17}O,^{18}O]P_{si} species gives specifically **b** or **c**, depending on whether the configuration is S or R, respectively. The amounts of species **a** and **d** have to do with isotopic enrichments, not configuration.

Figure 11 shows the P_β signal of the ATPβS(B) obtained from $PS^{18}O_3{}^{3-}$ and the two chiral P_{si} enantiomers. The signal contains two overlapping doublets due to ^{31}P–^{31}P coupling. Each half of a doublet contains four lines due to the four species **a**, **b**, **c**, and **d**. The results are summarized in Table I, where F value is defined as the ratio **b**/**c**, purity refers to the percentage of chirally labeled P_{si} species, and chirality refers to the optical purity of chirally labeled P_{si} species. The results indicate that 5′-nucleotidase catalyzes the hydrolysis of AMPS with inversion of configuration, as

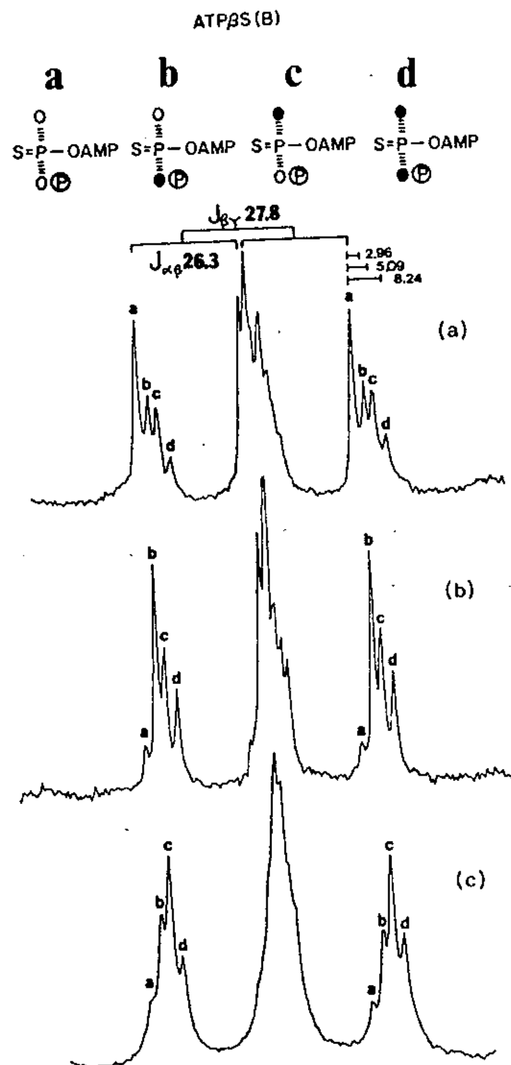
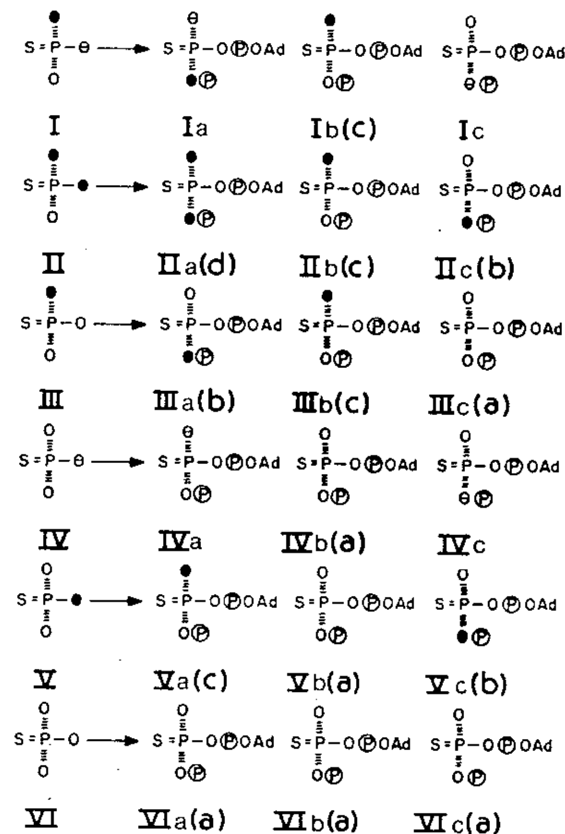


FIG. 11. The P_β signals of the ^{31}P NMR spectra of the ATPβS(B) obtained from $[^{18}O_3]P_{si}$(A) and from the two chiral P_{si} from Scheme IV. The sample (30 μmol) was dissolved in 2.5 ml of D_2O containing 10 m*M* EDTA, and the spectra were recorded at 145.7 MHz at ambient temperature. The coupling constants and isotope shifts are expressed in hertz. The chemical shift of the P_β signal is −29.8 ppm from H_3PO_4. [From Ref. 44 with permission.]



SCHEME VI

shown in Scheme VII. The causes for deviation of observed *F* values from theoretical values have been discussed.[44]

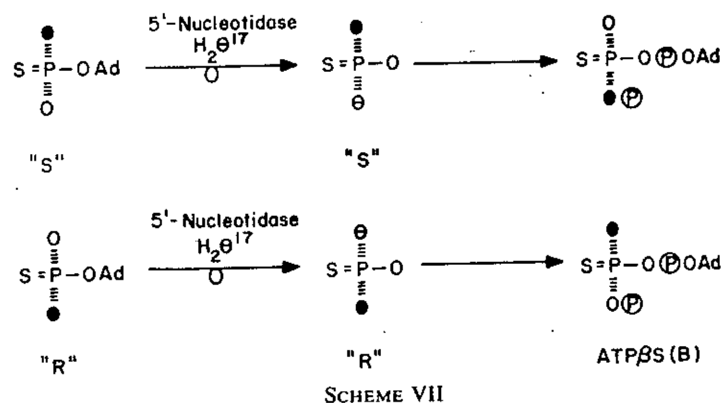
The significant work of Webb and Trentham on the stereochemistry of nucleoside triphosphatases is reviewed by Webb in this volume, Article [17]. In summary, they found that the stereochemical course of ATPγS hydrolysis (to ADP and P_{si}) is inversion for myosin ATPase[66] and mitochondrial ATPase,[67] and retention for sarcoplasmic reticulum ATPase.[68]

The stereochemical study of type (d) reactions is limited by several factors. First, the $P_{si} \rightleftharpoons H_2O$ oxygen exchange should not occur exten-

[66] M. R. Webb and D. R. Trentham, *J. Biol. Chem.* **255,** 8629 (1980).

[67] M. R. Webb, C. Grubmeyer, H. S. Penefsky, and D. R. Trentham, *J. Biol. Chem.* **255,** 11637 (1980).

[68] M. R. Webb and D. R. Trentham, *J. Biol. Chem.* **256,** 4884 (1981).



SCHEME VII

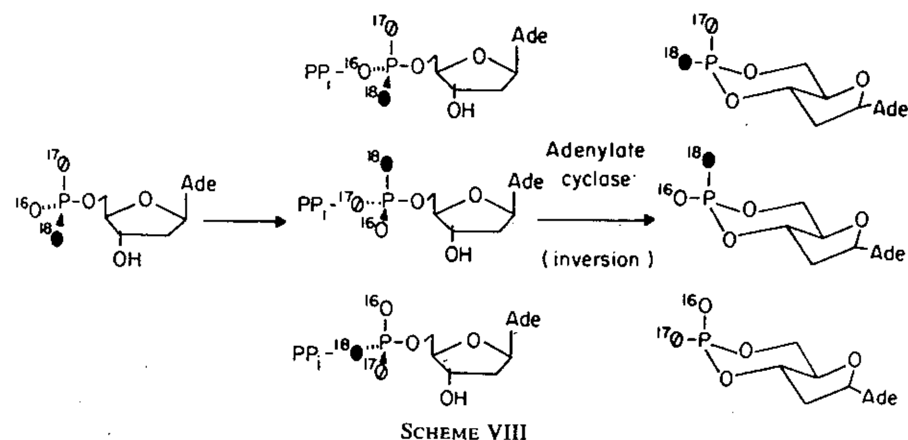
sively. We have found at least one case in which the isolated P_{si} has such a low oxygen enrichment that stereochemical study can no longer be done. Second, P_{si} is chemically unstable.[69] The enzyme reaction has to be faster than the decomposition of P_{si} (to P_i). This is difficult if a sulfur-chelating metal such as Zn^{2+} or Cd^{2+} is required for enzyme catalysis. These metal ions catalyze hydrolysis of P_{si} to P_i drastically.[70] Third, the enzyme reaction also has to be faster than the decomposition of an unstable substrate such as ATPγS. For the metal ions that may be present in enzyme catalysis, we have found[70] that Mg^{2+} and Ca^{2+} catalyze hydrolysis of ATPγS to ADP, Zn^{2+} slows down this process, and Cd^{2+} catalyzes hydrolysis of ATPγS to ATP![71]

Reaction type (c), the phosphoryl transfer between phosphate monoesters, need not be explained in detail since it has been reviewed by Knowles previously,[37] and in this volume Article [16]. The configuration of chiral [$^{16}O,^{17}O,^{18}O$]phosphate monoesters was first analyzed by linked-scan metastable ion mass spectrometry, in its theoretical base superficially similar to the ^{31}P NMR analysis. Indeed, the two chemical conversion steps required for NMR analysis had already been achieved in the MS analysis. In the paper reporting the ^{31}P NMR analysis of phosphate monoesters,[72] the authors have pleased those who prefer NMR by saying that the NMR method is "simpler both conceptually and practically." By use of the ^{31}P NMR method, they have determined that the phosphoryl-transfer reaction catalyzed by creatine kinase proceeds with inversion,[73]

[69] D. C. Dittmer and O. B. Ramsay, *J. Org. Chem.* **28,** 1268 (1963).
[70] M.-D. Tsai and L. J. Chern, unpublished results.
[71] The detailed mechanism for such observations remains to be established.
[72] S. L. Buchwald and J. R. Knowles, *J. Am. Chem. Soc.* **102,** 6601 (1980).
[73] D. E. Hansen and J. R. Knowles, *J. Biol. Chem.* **256,** 5967 (1981).

whereas that catalyzed by the acid phosphatase from bovine liver proceeds with retention.[74]

An example of reaction type (b), hydrolysis of cyclic AMP and cyclic dAMP catalyzed by the cyclic nucleotide phosphodiesterase, has been investigated independently by Lowe[75] and Gerlt,[55] respectively, by use of the ^{31}P NMR analysis. Since the report of Lowe has been found to be ambiguous in the configurational assignment of the synthesized chirally labeled cAMP,[55] only the work of Gerlt will be described. The (R_p) cyclic [$^{17}O,^{18}O$]dAMP was hydrolyzed in $H_2{}^{16}O$ by bovine heart cyclic nucleotide phosphodiesterase to give chiral [$^{16}O,^{17}O,^{18}O$]-5'-dAMP with unknown configuration. The "displacement step" was achieved by first converting the dAMP to dATP, followed by ring closure catalyzed by adenylate cyclase, as shown by Scheme VIII. The "stereospecific derivatization step" was achieved by nonstereospecifically methylating one of the two prochiral oxygens. Since the resulting two diastereomers (with methyl group at the pro-R or pro-S oxygen) have different ^{31}P chemical shifts, the ^{31}P NMR can be directly observed without separation of diastereomers. Figure 12 shows the predicted ^{31}P NMR spectra for an inversion and a retention steric course. The observed spectra indicate that the reaction catalyzed by the cyclic nucleotide phosphodiesterase proceeds with inversion of configuration, which again accords with results using sulfur analogs of cAMP.[76]



SCHEME VIII

[74] M. S. Saini, S. L. Buchwald, R. L. Van Etten, and J. R. Knowles, *J. Biol. Chem.* **256,** 10453 (1981).
[75] R. L. Jarvest, G. Lowe, and B. V. L. Potter, *J. Chem. Soc., Chem. Commun.* p. 1142 (1980); R. L. Jarvest and G. Lowe, *ibid.* p. 1145.
[76] P. M. J. Burgers, F. Eckstein, D. H. Hunneman, J. Baraniak, R. W. Kinas, K. Lesiak, and W. J. Stec, *J. Biol. Chem.* **254,** 9959 (1979).

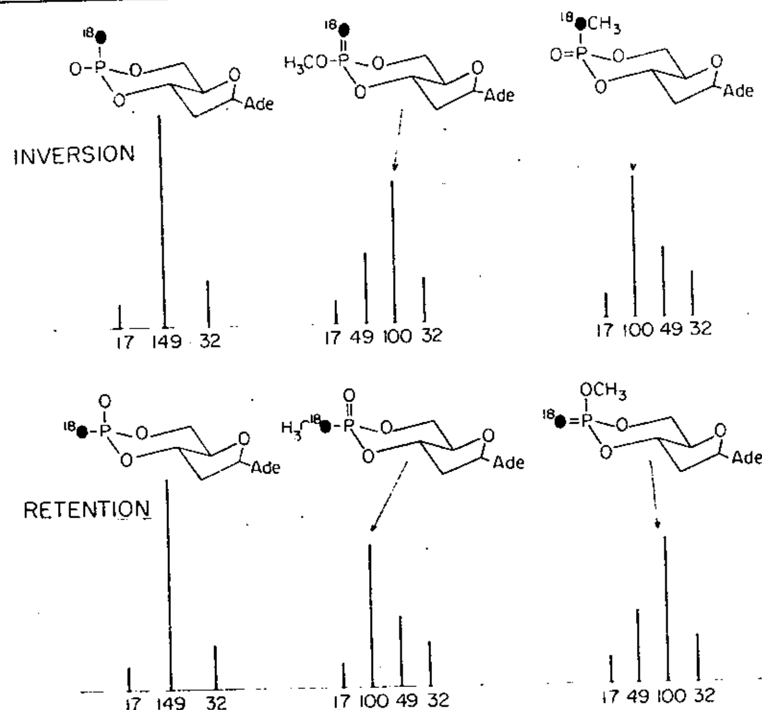


FIG. 12. The predicted ^{31}P NMR spectra for an inversion and a retention steric course of the hydrolysis of cyclic dAMP catalyzed by cyclic nucleotide phosphodiesterase. [From Ref. 55 with permission.] The observed spectra at 81 MHz are almost identical with the spectra predicted for inversion.

Table II summarizes the examples in which the combination of the ^{31}P(^{17}O) and the ^{31}P(^{18}O) NMR methods has been used to analyze the configuration of chiral [^{16}O,^{17}O,^{18}O]P_{si} or chiral [^{16}O,^{17}O,^{18}O]phosphate monoesters obtained from enzyme-catalyzed reactions. Almost 10 examples have been reported in less than 2 years.

The only other method that has been used for configurational analysis of this type is the circular dichroic method. However, only one enantiomer of the methyl [^{16}O,^{17}O,^{18}O]phosphate has been reported.[77] For chiral P_{si}, the Raman CD[78] remains a potential alternative. To elucidate the stereochemical course of the type (e) reaction—i.e., the $P_{si} \rightleftharpoons H_2O$ oxygen exchange—a more direct way of measuring chirality is desirable.

[77] P. M. Cullis and G. Lowe, *J. Chem. Soc., Chem. Commun.* p. 512 (1978).
[78] L. D. Barron, *Acc. Chem. Res.* **13**, 90 (1980).

TABLE II
STEREOCHEMICAL STUDY BY USE OF THE COMBINATION OF ^{31}P(^{17}O) AND ^{31}P(^{18}O) NMR FOR CONFIGURATIONAL ANALYSIS

Enzyme	Reaction type	Result	Compound analyzed	Reference
Phosphoglycerate kinase	c	Inversion	[^{16}O,^{17}O,^{18}O]P_{si}	65
5′-Nucleotidase	d	Inversion	[^{16}O,^{17}O,^{18}O]P_{si}	44
Myosin ATPase	d	Inversion	[^{16}O,^{17}O,^{18}O]P_{si}	66
Mitochondrial ATPase	d	Inversion	[^{16}O,^{17}O,^{18}O]P_{si}	67
Sarcoplasmic reticulum ATPase	d	Retention[a]	[^{16}O,^{17}O,^{18}O]P_{si}	68
Creatine kinase	c	Inversion	[^{16}O,^{17}O,^{18}O] Phosphopropanediol	73
Acid phosphatase	c	Retention[a]	[^{16}O,^{17}O,^{18}O] Phosphopropanediol	74
Cyclic nucleotide phosphodiesterase	b	Inversion	5′-[^{16}O,^{17}O,^{18}O]dAMP	55
Cyclic nucleotide phosphodiesterase	b	?[b]	5′-[^{16}O,^{17}O,^{18}O]AMP	75
—	—	—	[^{16}O,^{17}O,^{18}O]Glucose 6-phosphate	75

[a] For these enzymes there is evidence for a phosphoryl-enzyme intermediate.
[b] A retention mechanism was first reported, but later found to be incorrect.[55]

^{17}O NMR and ^{31}P(^{17}O) NMR: Binding Study of Nucleotides in Diamagnetic Systems

In many cases, oxygen is directly involved in inter- or intramolecular interactions. However, most magnetic resonance studies deal with atoms next to oxygen (e.g., ^{1}H,^{13}C,^{31}P). On the other hand, the NMR of a number of quadrupolar ions (e.g., Cl^-, Na^+, Mg^{2+}, Ca^{2+}) has been widely used in binding studies.[38,79] For example, it has been shown that the NMR signal of $^{25}Mg^{2+}$ broadens upon chelating with ATP.[80] However, the binding study by use of ^{17}O NMR has involved only H_2 ^{17}O.[26] The paucity of ^{17}O NMR studies of biological problems is perhaps due to the difficulty in quantitation and in experimental techniques.

As pointed out earlier in this article, the broad lines in ^{17}O NMR, though undesirable in terms of quantitation and instrumentation, is indeed advantageous since the quadrupolar relaxation process becomes the dominant relaxation mechanism. The contribution by other relaxation mecha-

[79] R. G. Bryant, *Annu. Rev. Phys. Chem.* **29**, 167 (1978).
[80] R. G. Bryant, *J. Magn. Reson.* **6**, 159 (1972); J. A. Magnusson and A. A. Bothner-By, *in* "Magnetic Resonance in Biological Research" (C. Franconi, ed.), p. 365. Gordon & Breach, New York, 1971.

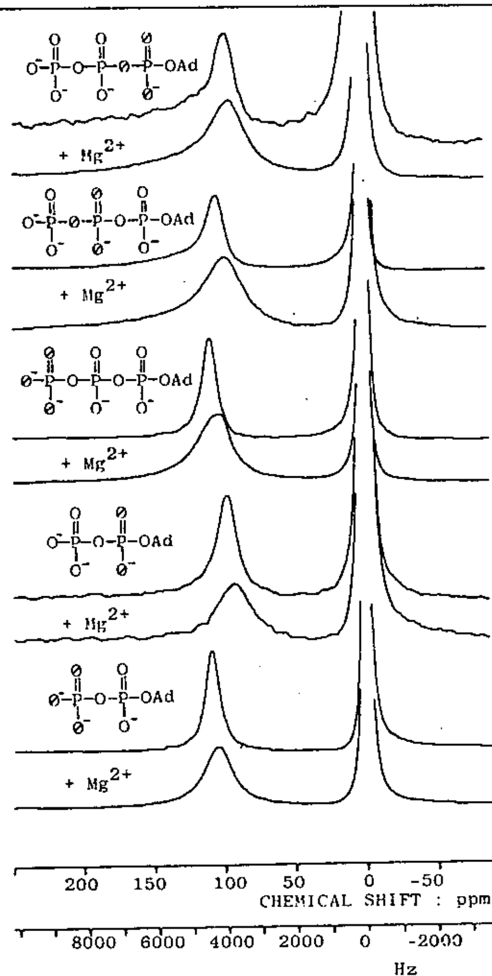


FIG. 13. ^{17}O NMR at 40.67 MHz, showing the effect of Mg^{2+} binding with [α-^{17}O]ATP, [β-^{17}O]ATP, [γ-^{17}O]ATP, [α-^{17}O]ADP, and [β-^{17}O]ADP. Sample conditions: 25 m*M* in D_2O, pD 7.8. Spectrometer (Bruker CXP-300) conditions: DE = 10 μsec, 4K data points, spectral width 50 KHz, unlocked, nonspinning, line broadening 100 Hz. Chemical shifts are referenced to external $H_2{}^{17}O$. The sharper signal is due to D_2O. [From Ref. 17 with permission.]

nisms or nonspecific factors to the line width becomes relatively insignificant. As shown by Eq. (3), the quadrupolar relaxation time T_q (and thus the linewidth ΔO) is directly related to $(NQCC)^2$ and τ_r (since $0 \leq \eta \leq 1$, the effect of η is relatively small). In the past 2 years we have tried, using limited instrumentation, to develop the ^{17}O NMR method for binding study of nucleotides in two aspects: In systems where τ_r is relatively constant, the change in ΔO can be related to the change in NQCC. In systems where the change in τ_r is large compared to the change in NQCC, the change in ΔO can be related to the change in τ_r. On the basis of Eq. (11), the $^{31}P(^{17}O)$ NMR method can be used complementarily to confirm the changes in ΔO, or to deduce the changes in ΔO when ^{17}O NMR signals are too broad to be detected.

By use of a low-field NMR spectrometer (1.879 T, ^{17}O at 10.85 MHz, ^{31}P at 32.4 MHz), we have first shown that Mg^{2+} causes the ^{17}O NMR signals of [γ-^{17}O]ATP and [β-^{17}O]ATP to "broaden," which in turn causes the corresponding ^{31}P NMR signals to "sharpen."[16] Since then, we have taken three steps[17]: (1) Investigate the Mg^{2+} effect of [α- and β-^{17}O]ADP and [α-,β-, *and* γ-^{17}O]ATP at different magnetic fields and different spectrometers. (2) Show that the metal-ion effect on ^{17}O NMR is *specific to sites of chelation* by use of substitution-inert Co^{3+} complexes of ADP (α,β-bidentate) and ATP (β,γ-bidentate). (3) Perform control experiments to assure that the observed line-broadening effect in ^{17}O NMR is caused *predominantly* by "chelation" rather than by other nonspecific effects such as pH, paramagnetic impurities, exchange processes, or viscosity. The results unequivocally show that the α-phosphate of ATP is involved in the chelation with Mg^{2+},[17] an issue that has been controversial for many years based on ^{31}P chemical shift data.[33] Except for the difficulty in accurate quantitation, the "quadrupolar broadening" should be a more direct way to study binding problems compared to the chemical shift effect. In this application, the τ_r is not expected to change dramatically upon chelation and the NQCC could be the major factor which is perturbed.

Figure 13 shows ^{17}O NMR spectra of [α-^{17}O]ATP, [β-^{17}O]ATP, [γ-^{17}O]ATP, [α-^{17}O]ADP, and [β-^{17}O]ADP, together with their corresponding Mg^{2+} complexes, obtained in the high-power probe of a Bruker CXP-300. Figure 14 shows the ^{17}O NMR spectra of some Co^{3+} complexes: the β,γ-bidentate complex of [α-^{17}O]ATP, the α,β-bidentate complex of [α-^{17}O]ADP, and the α,β-bidentate of [β-^{17}O]ADP. All samples and glasswares were treated with chelex prior to running. A ^{31}P NMR spectrum was taken after ^{17}O NMR experiments to ensure sample purity.

It is obvious from Fig. 13 that coordination with Mg^{2+} causes a line-broadening effect accompanied by a small upfield shift (2–6 ppm). We define the *R* value as a measure of line broadening,

$$R = (\Delta O_b - \Delta O_f)/\Delta O_f$$

where f and b designate free and bound states, respectively. The line widths measured should be corrected for artificial line-broadening (from exponential multiplication applied, or field inhomogeneity due to the nonspinning mode used), and for the broadening due to ^{17}O–^{31}P coupling,

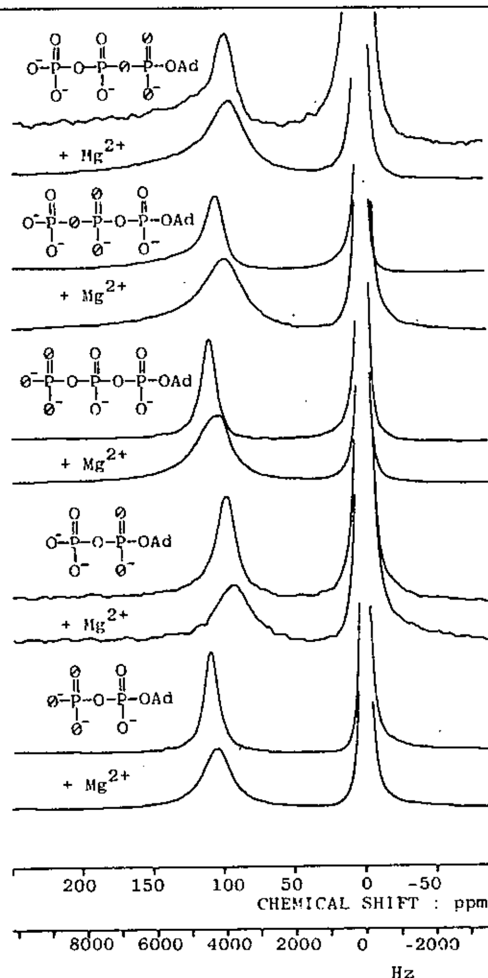


FIG. 13. ^{17}O NMR at 40.67 MHz, showing the effect of Mg^{2+} binding with [α-^{17}O]ATP, [β-^{17}O]ATP, [γ-^{17}O]ATP, [α-^{17}O]ADP, and [β-^{17}O]ADP. Sample conditions: 25 m*M* in D_2O, pD 7.8. Spectrometer (Bruker CXP-300) conditions: DE = 10 μsec, 4K data points, spectral width 50 KHz, unlocked, nonspinning, line broadening 100 Hz. Chemical shifts are referenced to external $H_2{}^{17}O$. The sharper signal is due to D_2O. [From Ref. 17 with permission.]

nisms or nonspecific factors to the line width becomes relatively insignificant. As shown by Eq. (3), the quadrupolar relaxation time T_q (and thus the linewidth ΔO) is directly related to $(NQCC)^2$ and τ_r (since $0 \leq \eta \leq 1$, the effect of η is relatively small). In the past 2 years we have tried, using limited instrumentation, to develop the ^{17}O NMR method for binding study of nucleotides in two aspects: In systems where τ_r is relatively constant, the change in ΔO can be related to the change in NQCC. In systems where the change in τ_r is large compared to the change in NQCC, the change in ΔO can be related to the change in τ_r. On the basis of Eq. (11), the $^{31}P(^{17}O)$ NMR method can be used complementarily to confirm the changes in ΔO, or to deduce the changes in ΔO when ^{17}O NMR signals are too broad to be detected.

By use of a low-field NMR spectrometer (1.879 T, ^{17}O at 10.85 MHz, ^{31}P at 32.4 MHz), we have first shown that Mg^{2+} causes the ^{17}O NMR signals of [γ-^{17}O]ATP and [β-^{17}O]ATP to "broaden," which in turn causes the corresponding ^{31}P NMR signals to "sharpen."[16] Since then, we have taken three steps[17]: (1) Investigate the Mg^{2+} effect of [α- and β-^{17}O]ADP and [α-, β-, *and* γ-^{17}O]ATP at different magnetic fields and different spectrometers. (2) Show that the metal-ion effect on ^{17}O NMR is *specific to sites of chelation* by use of substitution-inert Co^{3+} complexes of ADP (α,β-bidentate) and ATP (β,γ-bidentate). (3) Perform control experiments to assure that the observed line-broadening effect in ^{17}O NMR is caused *predominantly* by "chelation" rather than by other nonspecific effects such as pH, paramagnetic impurities, exchange processes, or viscosity. The results unequivocally show that the α-phosphate of ATP is involved in the chelation with Mg^{2+},[17] an issue that has been controversial for many years based on ^{31}P chemical shift data.[33] Except for the difficulty in accurate quantitation, the "quadrupolar broadening" should be a more direct way to study binding problems compared to the chemical shift effect. In this application, the τ_r is not expected to change dramatically upon chelation and the NQCC could be the major factor which is perturbed.

Figure 13 shows ^{17}O NMR spectra of [α-^{17}O]ATP, [β-^{17}O]ATP, [γ-^{17}O]ATP, [α-^{17}O]ADP, and [β-^{17}O]ADP, together with their corresponding Mg^{2+} complexes, obtained in the high-power probe of a Bruker CXP-300. Figure 14 shows the ^{17}O NMR spectra of some Co^{3+} complexes: the β,γ-bidentate complex of [α-^{17}O]ATP, the α,β-bidentate complex of [α-^{17}O]ADP, and the α,β-bidentate of [β-^{17}O]ADP. All samples and glasswares were treated with chelex prior to running. A ^{31}P NMR spectrum was taken after ^{17}O NMR experiments to ensure sample purity.

It is obvious from Fig. 13 that coordination with Mg^{2+} causes a line-broadening effect accompanied by a small upfield shift (2–6 ppm). We define the *R* value as a measure of line broadening,

$$R = (\Delta O_b - \Delta O_f)/\Delta O_f$$

where f and b designate free and bound states, respectively. The line widths measured should be corrected for artificial line-broadening (from exponential multiplication applied, or field inhomogeneity due to the nonspinning mode used), and for the broadening due to $^{17}O-^{31}P$ coupling,

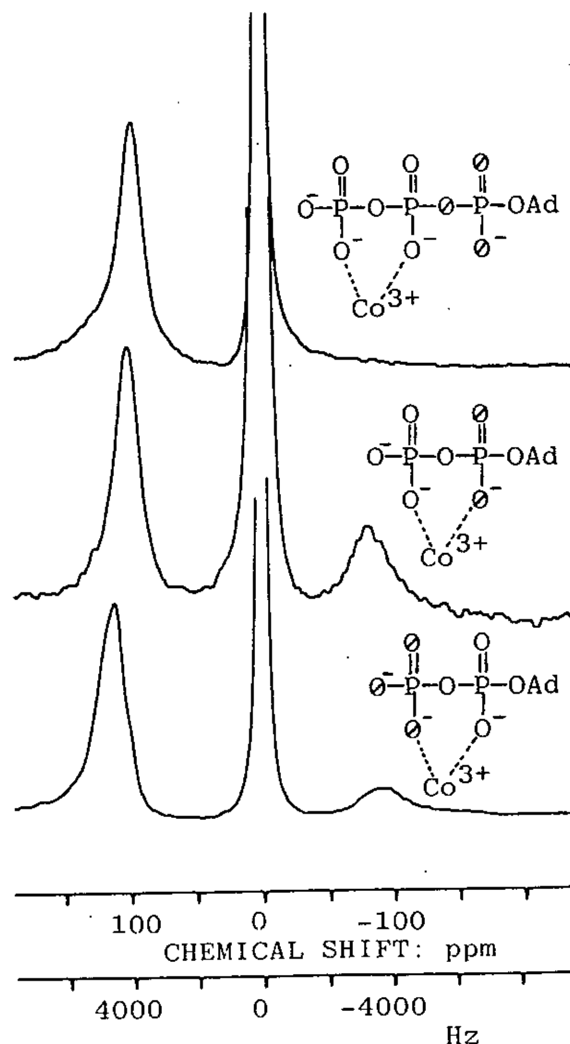


FIG. 14. ^{17}O NMR spectra of some Co^{3+} complexes at 40.67 MHz: the β,γ-bidentate of [α-^{17}O]ATP, the α,β-bidentate of [α-^{17}O]ADP, and the α,β-bidentate of [β-^{17}O]ADP. Sample and spectrometer conditions are same for Fig. 13. [From Ref. 17 with permission.]

TABLE III
SUMMARY OF ^{17}O NMR RESULTS OF Mg^{2+} AND Co^{3+} COMPLEXES[a]

	R Values[b]		Chemical shifts[d]			
Nucleotides	Mg^{2+} complex	Co^{3+} complex[c]	Free	Mg^{2+} complex	Co^{3+} complex	
[α-^{17}O]ATP	0.8–1.1	−0.1–0.3	96	94 (−2)	95 (−1)	—
[β-^{17}O]ATP	1.4–2.0	4.1	102	97 (−5)	105 (+3)	−81 (−183)
[γ-^{17}O]ATP	1.8–2.5	3.3–4.8	106	102 (−6)	115 (+9)	−89 (−195)
[α-^{17}O]ADP	1.2–1.8	2.0–4.2	97	91 (−6)	98 (+1)	−82 (−179)
[β-^{17}O]ADP	1.4–2.2	3.7–4.8	107	104 (−3)	113 (+6)	−89 (−196)

[a] Summarized from Tables I–III of Ref. 17.
[b] Obtained under various magnetic field strengths.
[c] Except for the Co^{3+} complex of [α-^{17}O]ATP, in which the ^{17}O is not directly coordinated, the *R* value refers to that of the upfield (broader) signal.
[d] Numbers in parentheses represent magnitudes of shift from the free nucleotide.

which is approximately 110 Hz in all cases.[81] The estimated error in *R* values is ±20%. The *R* values and chemical shifts of all Mg^{2+} complexes from Fig. 13 and from other experiments in different field strengths are summarized in Table III. The results indicate that the α-phosphate of ATP is also involved in the coordination with Mg^{2+}, but perhaps to a somewhat smaller extent than the β- and γ-phosphates. Since there are 17 possible stereoisomers for MgATP,[17] the above results can only assess the "macroscopic," not the "microscopic," structure of MgATP.

The results of Co^{3+} complexes in Fig. 14 are also summarized in Table III. They unequivocally establish that when there is no direct coordination, as in Fig. 14a, the ^{17}O NMR signal is neither shifted nor broadened to any appreciable extent. On the other hand, direct Co^{3+} coordination results in two signals, one slightly shifted downfield (1–9 ppm) and slightly broadened, the other greatly shifted upfield (180–200 ppm) and extensively broadened. The two signals are most likely due to $^{17}O{=}P{-}O^{-}\cdots Co^{3+}$ and $O{=}P{-}^{17}O^{-}\cdots Co^{3+}$, respectively, but unequivocal assignment is awaiting preparation of singly labeled species.

The following summary of experiments shows that most, if not all, nonspecific factors could have interfered with the results shown in Table III to only a small extent.

1. pH. All experiments were done at pH 7.6. Titration study of [$^{17}O_4$]P_i and [γ-$^{17}O_3$]ATP showed that the chemical shift of the ^{17}O

[81] J_{P-O} had been determined for some small and symmetrical molecules[16,22] but not for biophosphates. The values obtained for AMP, ADP, and ATP at various positions are approximately 110 ± 10 Hz at 95°C (J. A. Gerlt, P. C. Demou, and S. Mehdi, private communication).

signal varies with pH, but the ΔO is constant with $\pm 8\%$ from pH 2 to 10.

2. Viscosity. After the ^{17}O signal has been broadened by Mg^{2+}, addition of an equivalent or excess amount of EDTA can restore the signal. Therefore viscosity should not cause line-broadening upon metal chelation.
3. Paramagnetic impurities. In addition to the steps taken to ensure sample purity as mentioned above, we have separately studied the effect of Mn^{2+} on the ^{17}O NMR signal. We found that the ^{17}O NMR signal of [γ-^{17}O]ATP, being very broad, is relatively insensitive to the paramagnetic ion Mn^{2+}. In addition, Mn^{2+} causes some broadening on the solvent signal, which was not observed in the experiments with diamagnetic ions.
4. Exchange processes. If a medium or slow exchange process is responsible for the line-broadening caused by Mg^{2+}, it is expected to be dependent on magnetic fields. We have found that the R values are independent of magnetic field strength within experimental error ($\pm 20\%$). The Ca^{2+} ion, which is an example of extremely rapid exchange, also causes a line-broadening on [γ-^{17}O]ATP to approximately the same extent as Mg^{2+} does. The Co^{3+} complexes, which are in the slow exchange extreme, also show the line-broadening effect as shown in Table III. Thus, although it remains to be established whether the observed ^{17}O NMR signal of Mg^{2+} complexes represents the average of $^{17}O{=}P{-}O^{-}\cdots Mg^{2+}$ and $O{=}P{-}^{17}O^{-}\cdots Mg^{2+}$ or only one of them (the other might be too broad to be observed), the line broadening in Fig. 13 should not be dominated by chemical exchange processes.[85]

The complementary technique, $^{31}P(^{17}O)$ NMR, has also been used to substantiate the above ^{17}O NMR results. When Mg^{2+} causes the ^{17}O NMR signal to broaden, it also causes the $^{31}P(^{17}O)$ NMR signal to sharpen.[16] The results are consistent with the prediction of Eq. (11). If the Mg^{2+} line-broadening effect on ^{17}O NMR is solely due to an increase in the $^{31}P-^{17}O$ spin–spin coupling constant J upon binding, Eq. (11) would predict an increase rather than a decrease in ΔP. If the line-broadening effect on ^{17}O NMR is solely due to overlapping signals, it is not expected to cause a line sharpening in ^{31}P NMR.

MgATP is a very common substrate or intermediate in biological phosphoryl-transfer reactions. The ^{17}O NMR method provides a new and direct approach to define the coordination of MgATP. Whether such a method can be used in enzyme-bound states is yet to be established. We are approaching the enzyme binding problem by both ^{17}O NMR and $^{31}P(^{17}O)$ NMR. The ^{17}O NMR method is capable of determining line

broadening caused by a small percentage of enzymes.[82] Possibly, $^{31}P(^{17}O)$ NMR can be used to study the fully bound substrate. Figure 6 shows such an example. When [α- and β-^{17}O]ADP are bound to an excess of ribonuclease A (a small enzyme, MW ~ 14,000), the broad ^{31}P signals are dramatically sharpened. In this case, τ_r should be the major factor which changes upon binding. Figure 15 shows a qualitative application of the $^{31}P(^{17}O)$ method. In the "frozen" Li^{+} complex of phosphoglucomutase Ep·Li·Glc-1-P, the chemical shifts of both phosphate groups are shifted relative to Ep·Li and Glc-1-P. To make chemical shift assignments [$^{17}O_4$]Glc-1-P was used as a "marker". As shown in Fig. 15, the ^{31}P NMR signal of bound [$^{17}O_4$]Glc-1-P has not sharpened too much, which unequivocally assigns the upper field signal to the bound Glc-1-P.[83] In the extreme narrowing limit, it should be possible to deduce ΔO from the ΔP obtained by $^{31}P(^{17}O)$ NMR based on Eq. (11), and then deduce an approximate value for τ_r from ΔO based on Eq. (3). The extremely narrowing assumption may not be totally valid in macromolecules. In the "nonextreme narrowing condition," Eqs. (3) and (11) may not apply, and the ^{31}P relaxation may be dominated by the dipolar relaxation by ^{17}O. It remains to be established whether such a dipolar relaxation affects the ^{31}P relaxations in Figs. 6 and 15.

High-Resolution ^{17}O NMR: Chemical Shifts and Coupling Constants

While we were taking advantage of the fact that the quadrupolar relaxation causes significant line broadening such that other nonspecific factors contribute relatively insignificantly to ^{17}O linewidths, Gerlt pursued the application of ^{17}O NMR in an opposite approach. Unsatisfied by the low resolution ^{17}O NMR spectra of nucleotides first published,[16] Gerlt and coworkers have done two things to improve the resolution[84]: decoupling ^{31}P nuclei (by use of a specially made probe) to remove the line broadening due to $^{31}P-^{17}O$ coupling (~110 Hz),[81] and boiling the sample to remove the quadrupolar line broadening.[22] As a result, the ΔO of ^{17}O-labeled nucleotides can be reduced to < 150 Hz, and the chemical shifts and $^{31}P-^{17}O$ spin–spin coupling constants can be accurately determined. Figure 16 shows the ^{31}P-coupled and ^{31}P-decoupled ^{17}O NMR spectra of [α-^{17}O]ADP

[82] Whether such an approach is feasible also depends on the rate of exchange between free and bound nucleotides [T. J. Swift and R. Connick, *J. Chem. Phys.* **37**, 307 (1962)]. We have now observed that the ^{17}O NMR signals of [^{17}O]AMP, [α-^{17}O]ADP, and [β-^{17}O]ADP are broadened by 5–10% added ribonuclease A.

[83] W. J. Ray, Jr., G. I. Rhyu, and J. L. Markley, private communication.

[84] J. A. Coderre, S. Mehdi, P. C. Demou, R. Weber, D. D. Traficante, and J. A. Gerlt, *J. Am. Chem. Soc.* **103**, 1870 (1981).

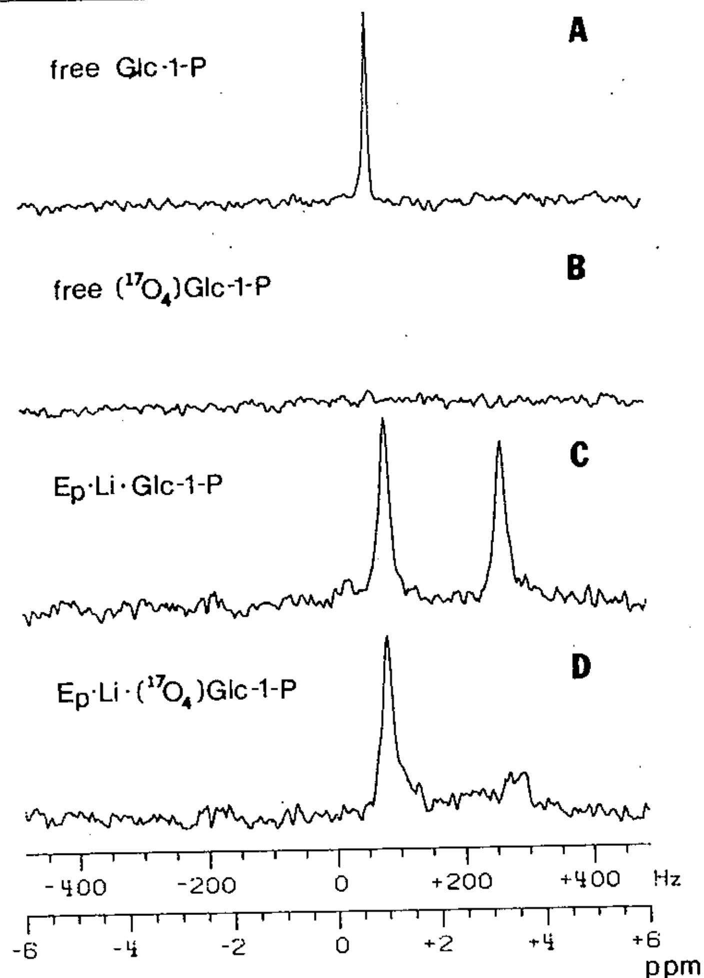


FIG. 15. ^{31}P NMR spectra (at 80.99 MHz) of A "free" Glc-1-P (0.4 m*M* Tris-Chloride buffer, pH 7.5); B "free" [$^{17}O_4$] Glc-1-P (conditions same as A); C Ep·Li·Glc-1-P, where Ep is the phosphoenzyme form of phosphoglucomutase (1 m*M* in 10% D_2O, 22 m*M* Tris-Chloride, 1 m*M* EDTA, 20°, pH 7.5); and (D) Ep·Li·[$^{17}O_4$]Glc-1-P (conditions same as C). Chemical shifts are referenced to external trimethyl phosphate. [From Ref. 83.]

and [β-^{17}O]ADP at 95°C in a magnetic field of 6.34 T. The signals and couplings are well resolved.

Although such a high temperature is not suitable for biological systems, the method can certainly help resolve overlapping signals and render the chemical shift and coupling constant information useful. An excel-

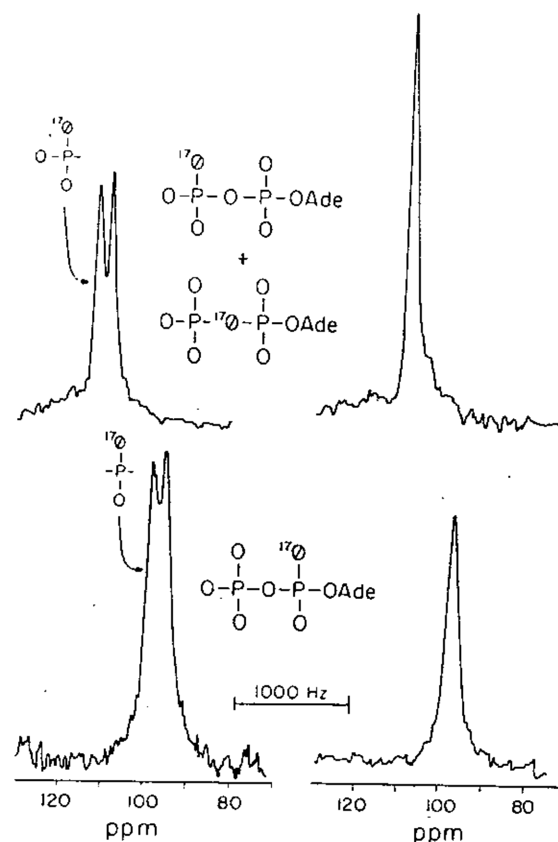


FIG. 16. ^{17}O NMR spectra (at 36.6 MHz, 95°) of singly labeled ADP. (a) A mixture of [β-$^{17}O_1$]ADP (37% ^{17}O) and [$\alpha\beta$-$^{17}O_1$]ADP (12% ^{17}O); (b) [α-$^{17}O_1$]ADP (32% ^{17}O). Left, ^{31}P-coupled spectra; Right, ^{31}P-decoupled spectra. [J. A. Gerlt, P. C. Demou, and S. Mehdi, unpublished experiments.]

lent example of application has already been achieved, in which they have resolved ^{17}O NMR signals due to the two diastereotopic oxygens in cyclic dAMP, as shown in Fig. 17. Comparison with single isomers reveals that the axial ^{17}O in the (R_p) isomer of cyclic [^{17}O,^{18}O]dAMP has a chemical shift of 92.8 ppm, whereas the equatorial ^{17}O in the (S_p) isomer resonate at 90.9 ppm. Thus, it is now possible to assign configuration based on the chemical shift of ^{17}O NMR, although an independent assignment is necessary to determine whether the pro-*R* or the pro-*S* oxygen resonates at higher field.

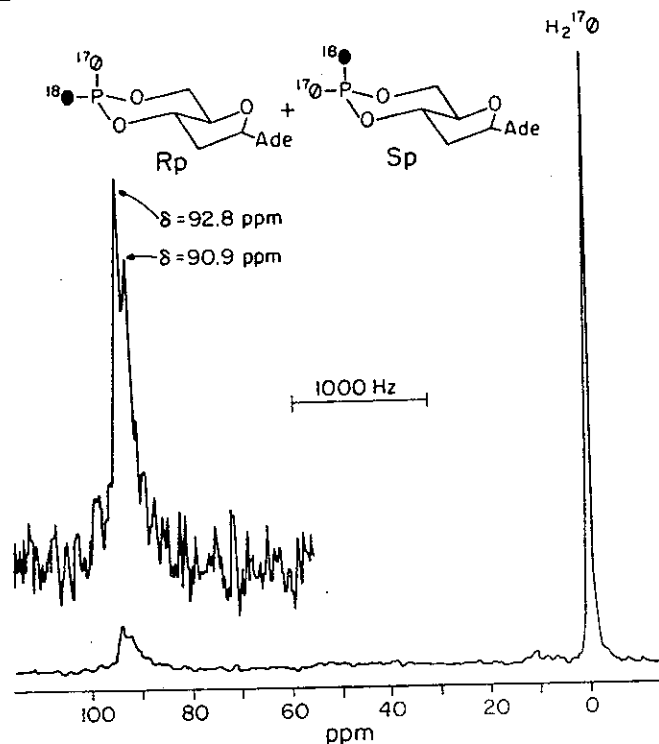


FIG. 17. ^{17}O NMR spectra (at 36.6 MHz, 95°) of cyclic [^{17}O,^{18}O]dAMP, racemic mixture of the (R_p) diastereomer (axial ^{17}O) and the (S_p) diastereomer (equatorial ^{17}O), ^{31}P-decoupled. [From Ref. 84 with permission.]

Related Magnetic Resonance Study Involving Oxygen Isotopes

The readers of this chapter may also be interested in three newly developed subjects: ^{17}O and ^{18}O effects on ^{13}C NMR, ^{17}O superhyperfine splitting in ESR of metal ions, and ^{17}O NMR of model membranes. A brief introduction will be given to each subject. Indeed, the latter two subjects represent two other important properties of the ^{17}O isotope, in addition to the quadrupolar line-broadening effect.

The isotope shift effect of ^{18}O in ^{13}C NMR has been investigated by

[85] The exchange rate of MgATP has been reported as 2×10^4 sec^{-1} (Ref. 80) or 1.2×10^3 sec^{-1} [H. Diebler, M. Eigen, and G. G. Hammes, *Z. Naturforsch., B: Anorg. Chem., Org. Chem., Biochem., Biophys., Biol.* **15B**, 554 (1960)]. The exchange process of MgATP is likely to approach the intermediate or slow side at high magnetic field.

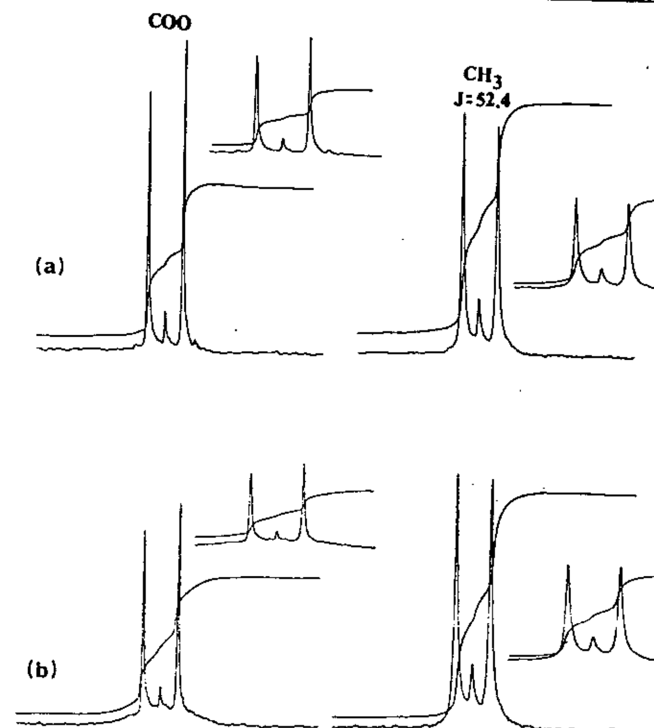


FIG. 18. ^{13}C NMR spectra (at 20.1 MHz, 1.88 T) showing the effect of ^{17}O in ^{13}C NMR. (a) [1,2-$^{13}C_2$]acetate (90 atom% ^{13}C); (B) [1,2-$^{13}C_2$, 1-$^{17}O_2$]acetate (90 atom% ^{13}C, 23 atom% ^{17}O). ^{17}O also causes a "line-broadening effect" on ^{13}C NMR signal, but the "ΔC" is only ~50 Hz in the carboxyl signal of (b). The intensity decrease caused by ^{17}O can be detected by integration of the "expanded" signal, as shown by the insets, but not by integrating the "unexpanded" signal. The "ΔO" of the same sample is 200 Hz.

several investigators.[6,9,86,87] The magnitude of shift has been shown to depend on structures and bond orders.[6,87] The method has not yet been widely used in biochemical problems, but application in biosynthetic problems has been achieved.[88] The ^{17}O nucleus also causes the ^{13}C NMR signal of the directly bonded ^{13}C nucleus to broaden. However, as shown in Fig. 18 for [1,2-$^{13}C_2$,1-$^{17}O_2$]acetate, the "broad" ^{13}C signal has a "ΔC" of only ~50 Hz. Thus $^{13}C(^{17}O)$ NMR may not be as useful as $^{31}P(^{17}O)$ NMR. However, the use of ^{17}O NMR for binding studies developed for nucleotides should be applicable to other substrates, such as amino acids, that can be labeled with ^{17}O at the carboxyl group.

[86] J. M. Risley and R. L. Van Etten, *J. Am. Chem. Soc.* **101**, 252 (1979).
[87] J. C. Vederas, *J. Am. Chem. Soc.* **102**, 374 (1980).
[88] J. C. Vederas and T. T. Nakashima, *J. Chem. Soc., Chem. Commun.* p. 183 (1980).

Another important property of ^{17}O is that it causes perturbation in the ESR spectra of paramagnetic metal ions due to superhyperfine coupling. Such an effect has been used in some metalloenzyme systems to identify ligands for the metal ion.[89] Complementary to our approach to investigate the effect of diamagnetic metal ions on the ^{17}O NMR of nucleotides, Reed and Leyh[35] have studied the effect of ^{17}O from ^{17}O-labeled nucleotides on the EPR spectra of the paramagnetic Mn^{2+} ion. Figure 19 shows that in the creatine kinase–MnADP–thiocyanate–creatine complex, the spectrum for [β-^{17}O]ADP exhibits an inhomogeneous broadening of its signal relative to those for unlabeled ADP. In this way, all six ligands of Mn^{2+} in the transition-state analog complexes of creatine kinase complexes have been identified. It is concluded that Mn^{2+} binds to all three phosphate groups in the transition state of the reaction.[35]

In addition to the two useful properties of ^{17}O described, a third should not be neglected. Equation (3) is valid only in the limit of rapid ($\omega^2\tau_c^2 << 1$) isotropic motion. It has been well established that in the solid or liquid crystalline state, the quadrupolar nucleus gives rise to a "quadrupolar splitting" in the NMR signal with the energy levels given by

$$E_m = -\gamma\hbar H^{\circ}m + \frac{e^2qQ}{4I(2I-1)}\frac{3\cos^2\theta - 1}{2}[3m^2 - I(I+1)] \quad (12)$$

where θ is the angle between principal axis of the electric field gradient and the applied field, and m is the quantum number for the z component of the angular momentum I.[2,90] In the liquid state, rapid reorientation of molecules averages the term $(3\cos^2\theta - 1)$ in Eq. (12) to zero. In the solid state, the quadrupolar term gives rise to quadrupolar splitting. For ^{17}O, the magnitude of splitting is

$$\Delta\nu = \frac{3}{40}\frac{e^2qQ}{h}(3\cos^2\theta - 1) \quad (13)$$

In liquid crystalline states, incomplete averaging results in a splitting smaller than that of a single crystal.

The quadrupolar splitting in ^2H NMR has now become the most pow-

[89] J. S. E. Deinum and T. Vanngard, *FEBS Lett.* **58,** 62 (1975); R. K. Gupta, A. S. Mildvan, T. Yonetani, and T. S. Srivastava, *Biochem. Biophys. Res. Commun.* **67,** 1005 (1975); R. K. Gupta, A. S. Mildvan, and G. R. Schonbaum, *ibid.* **89,** 1334 (1979); R. C. Bray, *J. Inorg. Biochem.* **11,** 355 (1979); S. P. Cramer, J. L. Johnson, K. V. Rajagopalan, and T. N. Sorrell, *Biochem. Biophys. Res. Commun.* **91,** 434 (1979); M. W. Makinen and L. C. Kuo, *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **39,** 1859 (1980).

[90] H. H. Mantsch, H. Saito, and I.C.P. Smith, *Prog. Nucl. Magn. Reson. Spectrosc.* **11,** 211 (1977).

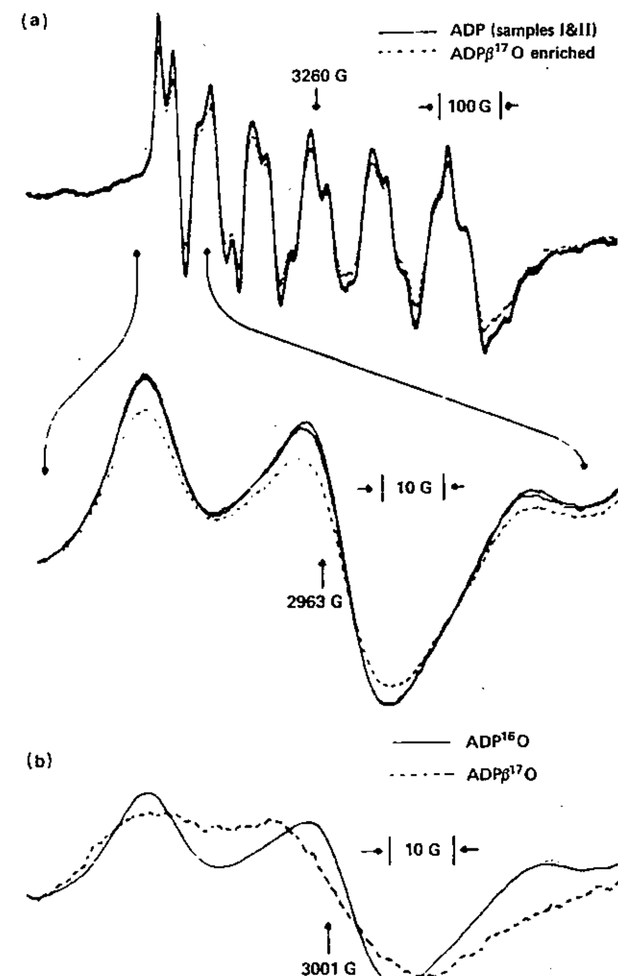


FIG. 19. (a) X-band EPR spectra for concentration-matched samples of the creatine kinase–MnADP–thiocyanate–creatine complex with normal ADP and with ADP enriched to 31% with ^{17}O in the β-phosphate. The solid curves represent spectra for two samples with normal ADP, and the dashed curve is for the sister sample with β-^{17}O-enriched ADP. The bottom curves are expansions of the region of the spectrum indicated by the arrows. (b) Difference spectrum (dashed curve) and spectrum for normal ADP (solid curve). The difference spectrum was obtained by subtracting 30% of the spectrum for the normal ADP sample from the spectrum from the β-^{17}O-enriched sample. The amplitude of the difference spectrum has been scaled to match that of the experimental spectrum. [From Ref. 35 with permission.]

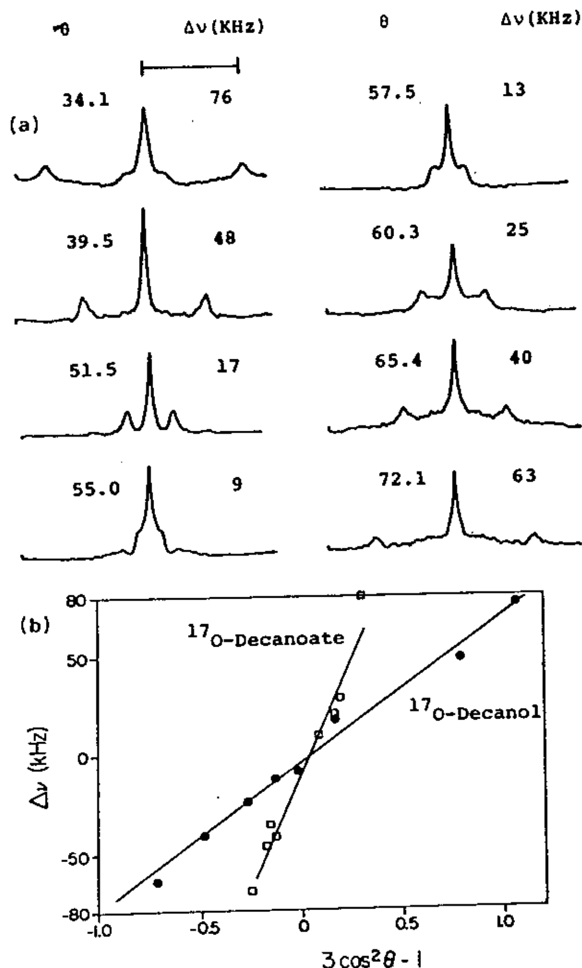


FIG. 20. (a) ^{17}O NMR spectra (at 48.8 MHz, 8.46 T) of ^{17}O-decanol in an oriented decanol–decanoate–water mesophase. The angle θ is the angle between the molecular axis (normal to the glass slides) and the magnetic field (z axis). (b) Plots of $\Delta\nu$ versus ($3 \cos^2\theta - 1$) for ^{17}O-decanol and ^{17}O-decanoate in the above lipid bilayer system. [M.-D. Tsai, T. M. Rothgeb, and E. Oldfield, unpublished experiments.]

erful probe in the investigation of membrane motions.[91–93] Figure 20 shows the ^{17}O NMR spectra of the [^{17}O]decanol/decanoate soap, which is

[91] E. Oldfield, N. Janes, R. Kinsey, A. Kintanar, R. W. K. Lee, T. M. Rothgeb, S. Schramm, R. Skarjune, R. Smith, and M.-D. Tsai, *Biochem. Soc. Trans.* **45,** 155 (1981).

[92] R. A. Kinsey, A. Kintanar, M.-D. Tsai, R. L. Smith, N. Janes, and E. Oldfield, *J. Biol. Chem.* **256,** 4146 (1981).

[93] J. Seelig, *Q. Rev. Biophys.* **10,** 353 (1977).

the first ^{17}O NMR study of lipid bilayer systems. Since the NQCC for ^{17}O is expected to be ~5–15 MHz,[94] which is beyond the limit of the spectral width, we have therefore produced samples oriented on glass slides, and carried out rotation plots near the "magic angle." The information obtained from the plot of quadrupolar coupling ($\Delta\nu$) vs. ($3 \cos^2 \theta - 1$) will certainly yield valuable new conformational and motional information when extended to phospholipid systems.

Acknowledgments

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[94] C. P. Cheng and T. L. Brown, *J. Am. Chem. Soc.* **102,** 6418 (1980).

[16] Chiral [^{16}O,^{17}O,^{18}O]Phosphoric Monoesters as Stereochemical Probes of Phosphotransferases

By STEPHEN L. BUCHWALD, DAVID E. HANSEN, ANNEMARIE HASSETT, and JEREMY R. KNOWLES

The stereochemical consequence of a replacement reaction at the phosphorus atom of a monoester of phosphoric acid (**1**) cannot be determined as long as the three unsubstituted oxygen atoms are indistinguishable from one another.[1,2] The equivalence of the three peripheral oxygens can be destroyed, however, in two ways—by elemental substitution or by isotopic replacement. The pioneering experiments in this field were done by Usher and Eckstein and co-workers,[3] who showed that the phosphorus

[1] To avoid confusion, the double bonds and formal charges on peripheral oxygen atoms of phosphoric esters will be omitted. Further, R and S configurational designations will be made according to the Cahn–Ingold–Prelog rules[2] on the basis that peripheral oxygen atoms are always singly bonded to phosphorus. Despite misleading statements in some texts, it should be remembered that the priority rules require[2] that the atomic *number* preferences be applied *to exhaustion,* before any cognizance is taken of atomic *weight*.

[2] R. S. Cahn, C. K. Ingold, and V. Prelog, *Experientia* **12,** 81 (1956); R. S. Cahn, C. K. Ingold, and V. Prelog, *Angew. Chem., Int. Ed. Engl.* **5,** 385 (1966).

[3] D. A. Usher, D. I. Richardson, and F. Eckstein, *Nature* (*London*) **228,** 663 (1970); D. A. Usher, E. S. Erenrich, and F. Eckstein, *Proc. Natl. Acad. Sci. U.S.A.* **69,** 115 (1972).

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