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NMR Methods Involving Oxygen Isotopes in Biophosphates

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1. INTRODUCTION

Biophosphate is an important class of biomolecules. It includes inorganic orthophosphate (P_i), small phosphomonoesters and phosphodiesters (nucleotides, sugar phosphates, coenzymes, etc.), nucleic acids (DNA, RNA), phospholipids, and others. These biophosphates are involved in many important biological processes such as energy storage and utilization, membrane transport, and genetic control.

The Fourier transform ^{31}P NMR technique has been used to investigate most, if not all, of the various types of biophosphates. The article by O'Neill and Richards (1980), among others, provides a very complete yet brief summary on the various experimental methods and approaches over the period 1970–1978. Some recent reviews (Nageswara Rao, Chapter 3 of this volume; Gorenstein, 1981; Cohn and Nageswara Rao, 1979; Hollis, 1979; Gadian *et al.*, 1979; Ugurbil *et al.*, 1979) also cover important recent progress on several specific subjects.

An important development in the NMR study of biophosphates since 1978 is the use of oxygen isotopes ^{17}O ($I = 5/2$, 0.037% natural abundance) and ^{18}O ($I = 0$, 0.204% natural abundance), which include the ^{18}O isotope effect in ^{31}P NMR, the ^{17}O quadrupolar effect in ^{31}P NMR, and the ^{17}O

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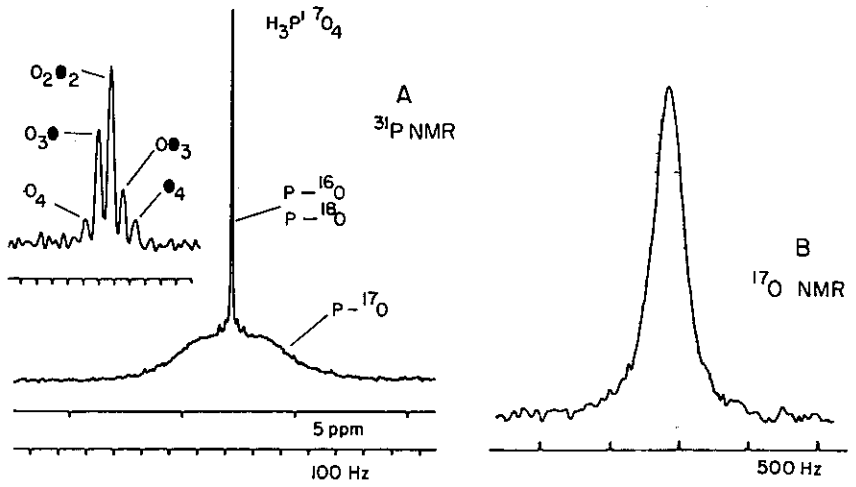


Figure 1. (*A*) The ^{31}P NMR spectrum of 50 m*M* $H_3P^{17}O_4$ (40 at. % ^{17}O) in D_2O, pD = 1.8, at 81.0 MHz. Spectral parameters: Acquisition time 4.1 sec, delay time 1.0 sec, spectral width 2 KHz, 70° pulse, line broadening 2.0 Hz, 1600 scans. The insets show the expanded spectrum of the sharp peak, processed with Gaussian multiplication (LB = −2, GB = 0.2). Chemical shift: 0.09 ppm downfield from 1 M H_3PO_4. (*B*) ^{17}O NMR spectrum of the same sample at 27.1 MHz. Spectral parameters: Acquisition time 0.0512 sec, spectral width 10 KHz, pre-acquisition delay 12 μsec, line broadening 10 Hz, 500 scans. Chemical shift: 80 ppm downfield from $H_2{}^{17}O$.

NMR of biophosphates. Figure 1A shows the ^{31}P NMR spectrum of $H_3P^{17}O_4$ (40 at. % ^{17}O). The spectrum consists of a "broad" signal due to the ^{31}P–^{17}O species and a "sharp" signal due to the residual non-^{17}O-labeled species. Since the ^{17}O-enriched water always contains some ^{18}O ($^{18}O/^{17}O = 0.67$ in this case), the "sharp" signal contains both ^{16}O and ^{18}O species, as shown by the expanded spectrum in the inset. In this compound, the ^{31}P NMR signal of the ^{18}O-labeled species is shifted upfield by 0.020 ppm per ^{18}O atom. The ^{17}O NMR spectrum of the same sample is shown in Figure 1B, which shows a broad signal with an apparent linewidth of 250 Hz. The spin–spin coupling between ^{31}P and ^{17}O is not resolved under this condition.

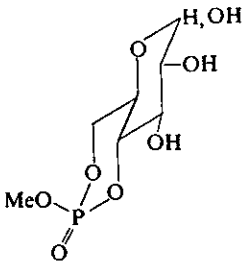
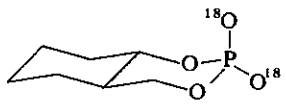
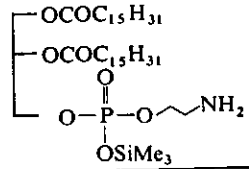
2. ^{18}O ISOTOPE SHIFTS IN ^{31}P NMR, $S_{^{31}P-^{18}O}$

Ramsey and Purcell (1952) first predicted an effect of isotope substitution on the magnetic shielding of nuclei. The effect has subsequently been widely observed in various systems, which have been summarized recently (Risley and Van Etten, 1980). With few exceptions (Kanazawa *et al.*, 1965; Fraenkel *et al.*, 1966), 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 compounds (Batiz–Hernandez and Bernheim, 1967).

The ^{18}O isotope shift effect in ^{31}P NMR, $S_{^{31}P-^{18}O}$, was first reported by Cohn and Hu (1978) and others (Lowe and Sproat, 1978; Lutz *et al.*, 1978). The magnitudes of the ^{18}O shift on the ^{31}P resonance of phosphate derivatives range from 0.015 to 0.040 ppm, and correlate well with the double-bond character of the P—O bond (Cohn and Hu, 1980; Cohn, 1982). Cohn (1982) has summarized the magnitudes of $S_{^{31}P-^{18}O}$ for a number of biophosphates. Table 1 extends such a list to cover some recently reported data. Some derivatives of biophosphates or their synthetic precursors are also included in Table 1. Since the data are collected from various reports which have different spectral resolution, some of the data in Table 1 may have an error of ±10% or larger.

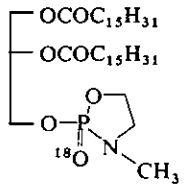
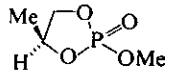
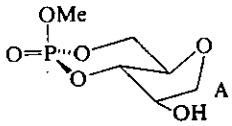
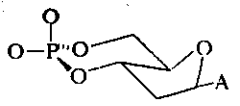
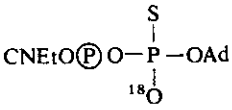
It seems proper to generalize the following statements concerning the magnitudes of ^{18}O isotope shifts in ^{31}P NMR, at least for the compounds in which the phosphorus has an oxidation number of +5. (a) The *S* value for a P=O double bond is 0.038–0.044 ppm, whereas that for a P—O single bond is 0.015–0.025 ppm. Bonds with a partial double bond character have *S* values proportional to the bond order (Cohn and Hu, 1980; Lowe *et al.*, 1979). (b) In case of multiple substitution, the magnitude of shift is generally additive. (c) The *S* values of phosphorothioates (in which an O is substituted by an S) are slightly greater than that of the corresponding phosphates.

Experimentally, resolution and quantitation are two important problems which dictate the capability of the ^{18}O isotope shift method in solving a specific biochemical problem. Both resolution and quantitation can be improved by a higher magnetic field, a better *S*/*N*, or a larger computer size. However, it should be realized that even under optimal conditions both the integrals and the *S* values may still have an error of ±5%. When peaks are not well resolved, the errors may increase to 10%–20%. Under such a condition various methods have been applied to improve the quantitation (e.g., spectral simulation) or the resolution (e.g., resolution enhancement). In general, a medium field (e.g., 81 MHz ^{31}P) is suitable to resolve most shifts. Aqueous samples are often treated with chelex-100 or EDTA to remove paramagnetic impurities. Two commonly used techniques for resolution enhancement are Gaussian multiplication and convolution difference (the CD command in the Bruker DISNMR program). Although the Gaussian multiplication is known to change the relative intensity of signals with different line shapes (Clin *et al.*, 1979), it may be safe to assume that the different peaks due to ^{18}O isotope shift have the same line shape. Of

Compound	Labeled position	Condition	$S_{^{31}P-^{18}O}$ (ppm)	Reference
$H_4P^{18}OO_3{}^+ClO_4{}^-$		0.2 *M*, D_2O	0.0188 ± 0.0007	Sammons *et al.* (1983)
$KH_2P^{18}O_4$		pH 2.1	0.0201 ± 0.0007	Sammons *et al.* (1983)
$KH_2P^{18}O_4$		pH 2.6	0.0200 ± 0.0011	Sammons *et al.* (1983)
$K_2HP^{18}O_4$		pH 8.6	0.0218 ± 0.0007	Sammons *et al.* (1983)
$(PhO)_3P^{18}O$		$CDCl_3$	0.0391 ± 0.0029	Sammons *et al.* (1983)
$(PhO)_2P^{18}OO$		pD 5.4	0.0293 ± 0.0007	Sammons *et al.* (1983)
$Ph_3P^{18}O$		$CDCl_3$	0.0399 ± 0.0007	Sammons *et al.* (1983)
$(CH_3{}^{18}O)_3P^{18}O$	P=O	$CDCl_3$	0.0392 ± 0.0029	Sammons *et al.* (1983)
$(CH_3O)_2PO^{18}O^-$			0.029	Lowe *et al.* (1979)
$(CH_3O)PO_2{}^{18}O^{2-}$			0.023	Lowe *et al.* (1979)
5′-AMP			0.025	Lowe and Sproat (1978a)
2′-deoxy AMP			0.032	Gerlt and Coderre (1980)
ADP	α		0.0286 ± 0.0015	Sammons *et al.* (1983)
	$\alpha\beta(P_\alpha)$	pH > 7.6	0.0166	Cohn and Hu (1980)
			0.0210	Sammons (1982)
	$\alpha\beta(P_\beta)$		0.0214	Sammons (1982)
	β		0.022	Cohn and Hu (1980), Sammons (1982)
ATP	$\alpha\beta(P_\alpha)$	pH > 7.6	0.0172	Cohn and Hu (1980)
	$\alpha\beta(P_\beta)$	pH > 7.6	0.0165	Cohn and Hu (1980)
	β	pH > 7.6	0.0281	Cohn and Hu (1980)
	$\beta\gamma(P_\beta)$	pH > 7.6	0.0165	Cohn and Hu (1980)
	γ	pH > 7.6	0.0220	Cohn and Hu (1980)
$Co(NH_3)_4ADP$, Δ isomer	α, R_p	pH 5.5	0.032	Sammons and Frey (1982)
Λ isomer	α, R_p	pH 5.5	0.018	Sammons and Frey (1982)
$Co(NH_3)_4ADP$, Δ isomer	**α, S_p**	**pH 5.5**	**0.020**	**Sammons and Frey (1982)**
Λ isomer	**α, S_p**	**pH 5.5**	**0.033**	**Sammons and Frey (1982)**
$Co(NH_3)_4dADP$, Δ isomer	α, R_p	pH 5.5	0.031	Coderre and Gerlt (1980)
Λ isomer	α, R_p	pH 5.5	0.016	Coderre and Gerlt (1980)
$Co(NH_3)_4dADP$, Δ isomer	α, S_p	pH 5.5	0.016	Coderre and Gerlt (1980)
Λ isomer	α, S_p	pH 5.5	0.030	Coderre and Gerlt (1980)
MgADP	α	pD 8.0	0.024	Sammons *et al.* (1983)
Ribose-1-P	P—^{18}O		0.027	Jordan *et al.* (1981)
	P—^{18}O—C		0.017	Jordan *et al.* (1981)
	P=^{18}O		0.041	Jarvest *et al.* (1980)
	P—^{18}O—CH_3		0.015	Jarvest *et al.* (1980)
	P—O	H_2O/D_2O	0.026	Gorenstein and Rowell (1980)
	Axial methlyl ester, P—OMe	$CDCl_3$	0.015	Gorenstein and Rowell (1980)
	P=O		0.040	Gorenstein and Rowell (1980)
	P=O		0.038	Bruzik and Tsai (1982)
	P—O—Si		0.018	Bruzik and Tsai (1982)

(Continued)

TABLE 1 (*Continued*)

Compound	Labeled position	Condition	$S_{^{31}P-^{18}O}$ (ppm)	Reference
		$CDCl_3$, 30°C	0.039 ± 0.0029	Bruzik and Tsai (unpublished)
	P=O		0.043	Buchwald and Knowles (1980)
	P—O—CH_3		0.018	Buchwald and Knowles (1980)
	Methyl ester			
	P=O		0.043	Jarvest *et al.* (1980)
	P—O—CH_3		0.017	Jarvest *et al.* (1980)
	Ethyl ester:			
	P=O		0.0418	Sammons (1982)
	P—O—Et		0.0192	Sammons (1982)
	**Ax. P—O**		**0.029**	**Gerlt and Coderre (1980)**
	Eq. P—O		0.032	Gerlt and Coderre (1980)
	Ethyl ester:			
	P—O		0.014	Gerlt and Coderre (1980)
	P=O		0.038	Gerlt and Coderre (1980)
5′-AMPS	α-^{18}O		0.0331 ± 0.0007	Sammons *et al.* (1983)
2′-UMPS			0.032	Gerlt and Wan (1979)
3′-UMPS			0.032	Gerlt and Wan (1979)
endo U > PS			0.041	Gerlt and Wan (1979)
exo U > PS			0.041	Gerlt and Wan (1979)
ADPαS	α-^{18}O		0.037	Webb and Trentham (1980a)
	αβ-^{18}O		0.021	Webb and Trentham (1980a)
	S_p isomer	pD 6.4	0.0363 ± 0.0045	Sammons *et al.* (1983)
	R_p isomer	pD 6.4	0.0363 ± 0.0045	Sammons *et al.* (1983)
$CNEtOP^{18}O_3$			0.0271	Sammons (1982)
$PhCH_2{}^{18}OPO_3$	P—O—C		0.016	Van Etten, R. L. (private communication)

[a] This table is an extension of the table of Cohn (1982) to cover some recent data, particularly those from our own laboratory which are used in the correlation between $J_{^{31}P-^{17}O}$ and $S_{^{31}P-^{18}O}$. It is, however, not an exclusive list of all reported data. The errors indicated come from the resolution used in acquiring data (Hz/point). When peaks are not well resolved, the isotope shift value is determined by line simulation for most data from our laboratory. Unless otherwise specified, the data from our laboratory were obtained at ambient temperature (25–30°C).

course any manipulation of the free induction decay leading to an improvement in resolution will result in a loss of signal-to-noise ratio.

In ^{31}P NMR, commonly used external references include 85% H_3PO_4, 1 *M* H_3PO_4 (in D_2O), trimethylphosphate, and others. In the earlier convention the "+" sign indicated an upfield shift. In recent years some authors have adopted the new IUPAC convention in which the "+" sign indicates a downfield shift. All previous publications from our laboratory used "+" sign for upfield shifts. However, we decided to follow the new IUPAC convention in this article and in all our future publications. All ^{31}P chemical shifts reported in this chapter are referenced to external 1 *M* H_3PO_4 in D_2O, or 85% H_3PO_4, unless otherwise specified.

3. ^{17}O NMR OF BIOPHOSPHATES

The article of Rodger *et al.* (1978) provides a very good summary of ^{17}O NMR work in various fields up to 1977. Due to the low natural abundance (0.037%) and the large quadrupole moment (-2.6×10^{-2} barns) of the ^{17}O nucleus, the work before the early 1970s had been limited to simple liquid compounds, particularly $H_2{}^{17}O$. Upon the development of the Fourier-transform technique, the research activity in ^{17}O NMR has increased steadily yet slowly.

In the biochemical area, the relaxation of $H_2{}^{17}O$ in various biological systems has been investigated most extensively (Zetter *et al.*, 1978; Kuntz and Kauzmann, 1974; Rose and Bryant, 1980). Recently ^{17}O NMR studies on amino acids (Valentine *et al.*, 1980; Pearson *et al.*, 1975) and nucleosides (Schwartz *et al.*, 1980) have been reported.

Tsai *et al.* (1980) first reported the ^{17}O NMR spectra of adenine nucleotides in a low magnetic field. The development since then is the subject of this and some later sections.

3.1. Chemical Shifts

For nuclei other than ^1H, nuclear shieldings are determined by the sum of a diamagnetic term, σ_d, and a paramagnetic term, σ_p, i.e., $\sigma = \sigma_d + \sigma_p$. For ^{17}O, the variation in σ_d, is relatively small compared to the variation in σ_p among different compounds (Kidd, 1967).

Some empirical correlations between $\delta^{17}O$ and the structure of organic compounds have been reported (Crandall and Centeno, 1979; Crandall *et al.*, 1979; Delseth *et al.*, 1980; Nguyen *et al.*, 1980). Beraldin *et al.* (1982) have shown that an electron enrichment in the oxygen atom is accompanied by a downfield ^{17}O NMR shift in aliphatic ethers, but an upfield ^{17}O NMR shift in carbonyl groups. However, the ^{17}O chemical shifts of biomolecules are less predictable, perhaps partially due to the involvement of solvent interaction such as H bonding. Although deprotonation of carboxyl groups invariably causes a downfield shift of ca. 10–20 ppm, Valentine *et al.* (1980) have indicated that the downfield shifts of the carboxyl group of glycine and alanine upon deprotonation (from the protonated amino acid to the zwitterionic form) contradict the prediction on the basis of paramagnetic screening effect. They explained the results by the effect of intermolecular hydrogen bonding with solvents. It has been established that hydrogen bonding to a carbonyl group causes an upfield shift of 30–50 ppm (Schwartz *et al.*, 1980; Reuben, 1969; Valentine *et al.*, 1980).

The ^{17}O chemical shifts of adenine nucleotides and their Mg^{2+} and Co^{3+} complexes have been reported by Tsai *et al.* (1980) and Huang and Tsai (1982). Recently Gerlt *et al.* (1982) have determined the chemical shifts of adenine nucleotides at elevated temperature or by decoupling the ^{31}P nucleus to obtain a better spectral resolution. These reported ^{17}O chemical shifts of adenine nucleotides, model compounds, and some ^{17}O-labeled phospholipids are listed in Table 2, which are referenced to external $H_2{}^{17}O$ (at ambient temperature), with the + sign indicating a downfield shift.

The chemical shifts of adenine nucleotides are, as expected, pH-dependent. Figure 2 shows the titration curves of inorganic phosphate (P_i), [γ-$^{17}O_3$]ATP, and Mg^{2+}/[γ-$^{17}O_3$]ATP. In these compounds and others ([$^{17}O_3$]AMP, [$^{17}O_2$]AMPS, and $H_2PS^{17}O_3^-$, deprotonation invariably causes a downfield shift of ca. 15–25 ppm (Huang and Tsai, 1982). Gerlt *et*

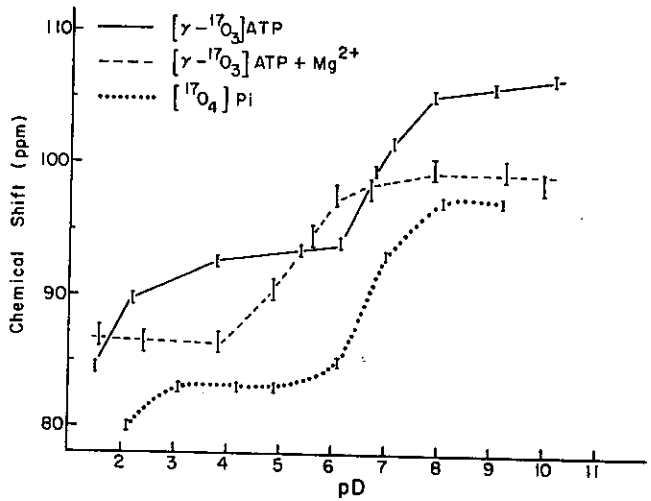
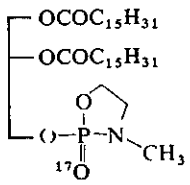
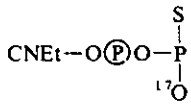
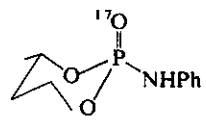


Figure 2. Plot of ^{17}O chemical shifts vs. pD for [$^{17}O_4$]Pi (25 m*M*), [γ-$^{17}O_3$]ATP (25 m*M*) and Mg^{2+}/[γ-$^{17}O_3$]ATP (25 m*M*). (From Huang and Tsai, 1982.)

TABLE 2
Chemical Shifts and Coupling Constants of P—O Groups[a]

Compound	Labeled position	Condition	$\delta^{17}O$ (ppm)	$J_{^{31}P-^{17}O}$ (Hz)	Reference
$H_4P^{17}OO_3{}^+ClO_4{}^-$		0.2 *M*, D_2O, 95°C	77.3	83.0 ± 2.4	Sammons *et al.* (1983)
$KH_2P^{17}O_4$		pH 2.1, 80°C		87.9 ± 2.4	Sammons *et al.* (1983)
$KH_2P^{17}O_4$		pH 2.6, 95°C	83.6	88.7 ± 2.4	Sammons *et al.* (1983)
$K_2HP^{17}O_4$		pH 8.6, 95°C	98.1	95.0 ± 2.4	Sammons *et al.* (1983)
$K_3P^{17}O_4$		pH 13.6, 95°C	114.2	96.6 ± 2.4	Sammons *et al.* (1983)
$(PhO)_3P^{17}O$		$CDCl_3$, 30°C	91.2	158.7 ± 2.4	Sammons *et al.* (1983)
$(PhO)_2P^{17}OO$		pD 5.4, 95°C	95	121 ± 2.4	Sammons *et al.* (1983)
$Ph_3P^{17}O$		$CDCl_3$, 30°C	43.3	160 ± 2.4	Sammons *et al.* (1983)
$(CH_3{}^{17}O)_3P^{17}O$	P=^{17}O	$CDCl_3$, 30°C	74	153.8 ± 2.4	Sammons *et al.* (1983)
	P—^{17}O—C	$CDCl_3$, 30°C	18		Tsai *et al.* (1980), Rodger *et al.* (1978)
$(CH_3O)_2PO^{17}O^-$	P—^{17}O	30°C	85.2	112	Gerlt *et al.* (1981)
		95°C	88.1	117	Gerlt *et al.* (1981)
$(CH_3O)PO_2{}^{17}O^{2-}$	P—^{17}O	30°C	98.5	98	Gerlt *et al.* (1981)
		95°C	101.7	104	Gerlt *et al.* (1981)
$P^{17}OCl_3$		THF, 30°C	210	207.5 ± 2.4	Sammons *et al.* (1983)
POF_3				184	Gray and Albright (1977)
$POBr_3$				201	Gray and Albright (1977)
		$CDCl_3$, 55°C	67	150	Bruzik and Tsai (unpublished)
Dipalmitoyl-phosphatidyl-choline	P—O Nonbridge	CH_3OD, 33°C	71		Bruzik and Tsai (unpublished)
		$CDCl_3$, 33°C	88		Bruzik and Tsai (unpublished)

AMP		**pD 7.8, 30°C**	**103**		**Huang and Tsai (1982)**
		95°C	100	98	Gerlt *et al.* (1981)
ADP	α	pD 7.8, 30°C	97		Huang and Tsai (1982)
		95°C	97.8	119	Gerlt *et al.* (1981)
		97°C	94.6	123 ± 2.4	Sammons *et al.* (1983)
	β	30°C	107		Huang and Tsai (1982)
		95°C	110.5	112	Gerlt *et al.* (1981)
ATP	α	30°C	96		Huang and Tsai (1982)
		95°C	97.9	105	Gerlt *et al.* (1981)
	β	30°C	102		Huang and Tsai (1982)
		95°C	105.0	119	Gerlt *et al.* (1981)
	γ	30°C	106		Huang and Tsai (1982)
		95°C	110.0	112	Gerlt *et al.* (1981)
	αβ		122		Tsai (1982)
	βγ		122		Tsai (1982)
Cyclic-dAMP	Axial	95°C	92.8	130	Goderre *et al.* (1981a)
	Equatorial	95°C	91.2	102	Goderre *et al.* (1981a)
	α, S_p isomer	pD 6.4, 97°C	138.7	148.0 ± 2.4	Sammons *et al.* (1983)
	α, R_p isomer	pD 6.4, 97°C	138.8	146.2 ± 2.4	Sammons *et al.* (1983)
AMPS	α	95°C	145.4	131.2 ± 2.4	Sammons *et al.* (1983)
	P=O	$CDCl_3$, 59°C	88.6	151.8 ± 2.4	Stec, W. J. (private communication)

[a] This table includes only the data from our own laboratory and from Gerlt's group, using an external H_2O reference. The result of Gerothanassis and Sheppard (1982) are not included since they have used a different reference (dioxan) and most of their data have also been obtained in the above two laboratories. For the data from our laboratory (Sammons *et al.*, 1983; Bruzik and Tsai, unpublished; Huang and Tsai, 1982), the coupling constant *J* was determined by the simulation, and the external H_2O reference was always at 30°C.

al. (1982) have also conducted a detailed titration study of adenine nucleotides and obtained similar results. The chemical shifts of the α- and β-phosphates of ATP are relatively constant in the pH range 3–10.

It is difficult at this stage to quantitatively interpret all chemical shift data in Table 2, particularly the effect of Co^{3+} binding (a 180–200 ppm upfield shift) and Mg^{2+} binding (a small upfield shift, < 10 ppm), which will be further discussed in Section 6.2. However, some qualitative order can be observed in Table 2, which, together with their possible implications, are discussed as follows. (a) On the basis of the observed order in ^{17}O chemical shifts,

$$P^{17}OO_3^{3-} > (CH_3O)P^{17}OO_2^{2-} > (CH_3O)_2P^{17}OO^- > (CH_3O)_3P^{17}O,$$

and the fact that protonation of a phosphoryl oxygen causes an upfield shift, Gerlt *et al.* (1982) suggested that the magnitude of the negative charge on phosphoryl oxygens is of importance in determining the ^{17}O chemical shift. The correlation between ^{17}O chemical shifts and charges on oxygen indicate that as the charge per oxygen decreases, the ^{17}O resonance moves upfield. (b) It was found by both Huang and Tsai (1982) and Gerlt *et al.* (1982) that the ^{17}O chemical shifts of fully de-ionized free nucleotides fall into the order

$$\alpha\text{-}^{17}O\text{-ATP} < \beta\text{-}^{17}O\text{-ATP} < \gamma\text{-}^{17}O\text{-ATP}, \quad \text{and} \quad \alpha\text{-}^{17}O\text{-ADP} < \beta\text{-}^{17}O\text{-ADP}.$$

Gerlt *et al.* (1982) suggested that these orders may again be a direct measure of the orders in the charge density on the phosphorly oxygens of ADP and ATP. The validity of such a correlation remains to be established, since it does not seem to be supported by the theoretical calculation on the charge densities of ADP and ATP (Boyd and Lipscomb, 1969). (c) The observation that the γ-^{17}O resonance of ATP is pH-sensitive while that of α- and β-^{17}O are quite insensitive to pH from 3 to 10 does support that the deprotonation of ATP at pH ~ 7 mainly occurs at the γ-phosphoryl group. On the basis of their detailed titration experiments and other literature data, Gerlt *et al.* (1982) suggested that the upfield chemical shift per charge neutralized is approximately constant, with the values of 52, 46, and 44 ppm, for P_i, monomethyl phosphate, and carboxylates, respectively. (d) Sulfur substitution of both AMP (at P_α) and ATP (at P_γ) causes a 40–50 ppm downfield shift. Protonation of AMPS also causes an upfield shift (146 ppm → 120 ppm, pK_a, 5.3), (Huang and Tsai, 1982).

3.2. ^{31}P–^{17}O Spin–Spin Coupling Constants, $J_{^{31}P-^{17}O}$

For a small and symmetrical compound such as $P^{17}OCl_3$, the ^{17}O NMR signal is split into a doublet by ^{31}P and the ^{31}P NMR signal is split into a sextet by ^{17}O (Figure 3A). In this case the spin–spin coupling constants $J_{^{31}P-^{17}O}$ can be measured directly from both ^{17}O NMR and ^{31}P NMR. For $P^{17}O(OCH_3)_3$ (Figure 3B), the ^{17}O NMR signal still shows a good coupling with ^{31}P, but the coupling pattern of the ^{31}P NMR signal is collapsing. In most larger or less symmetrical molecules, the ^{31}P–^{17}O coupling is obscured in both ^{17}O NMR and ^{31}P NMR (Figures 3C and 3D).

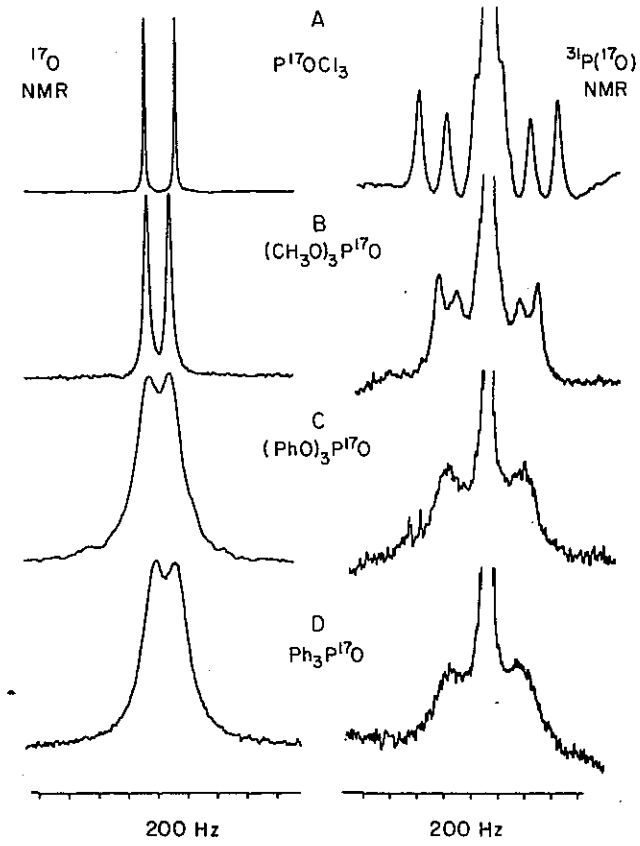


Figure 3. Line shapes of ^{17}O NMR (left, at 27.1 MHz) and $^{31}P(^{17}O)$ NMR (right, at 81.0 MHz). (*A*) $P^{17}OCl_3$, 51 atom % ^{17}O, in tetrahydrofuran, using acetone-d_6 for external lock; (*B*) $(CH_3O)_3P^{17}O$, 51 at. % ^{17}O, in $CDCl_3$; (*C*) $(PhO)_3P^{17}O$, 51 at. % ^{17}O, in $CDCl_3$; (*D*) $(Ph)_3P^{17}O$, 49 at. % ^{17}O, in $CDCl_3$. All spectra were run at 31°C and processed with 5 Hz line broadening (Sammons *et al.*, 1983).

However, as is discussed in Section 3.3, the linewidth of ^{17}O NMR signals decreases at elevated temperatures. When spectra of ^{17}O-labeled adenine nucleotides were taken at a near-boiling temperature (95°C), most ^{17}O–^{31}P splittings were resolved (Gerlt *et al.*, 1982; Gerothanassis and Sheppard, 1982). Table 2 lists $J_{^{31}P-^{17}O}$ of biophosphates together with some model compounds. It should be kept in mind, however, that in many ^{17}O NMR experiments the accuracy in the peak positions is no better than 5 Hz. In case of a partially resolved splitting, the error in the measured $J_{^{31}P-^{17}O}$ may be larger than 10 Hz, unless it is determined by line simulations.

As in the one-bond spin–spin coupling constant of other nuclei such as $^1J_{^{13}C-^{13}C}$ (Wehrli and Wirthlin, 1978), the one bond ^{31}P–^{17}O coupling constant $J_{^{31}P-^{17}O}$ seems to correlate with the π-character of the P—O bond. For example, Table 2 shows that the $J_{^{31}P-^{17}O}$ falls into the orders:

$$(CH_3O)_3P^{17}O > (CH_3O)_2P^{17}OO^- > (CH_3O)P^{17}OO_2^{2-} > P^{17}OO_3^{3-} > (HO)_4P^+$$

and

$$P^{17}OCl_3 > P^{17}OBr_3 > P^{17}O(OCH_3)_3 > P^{17}O(CH_3)_3$$

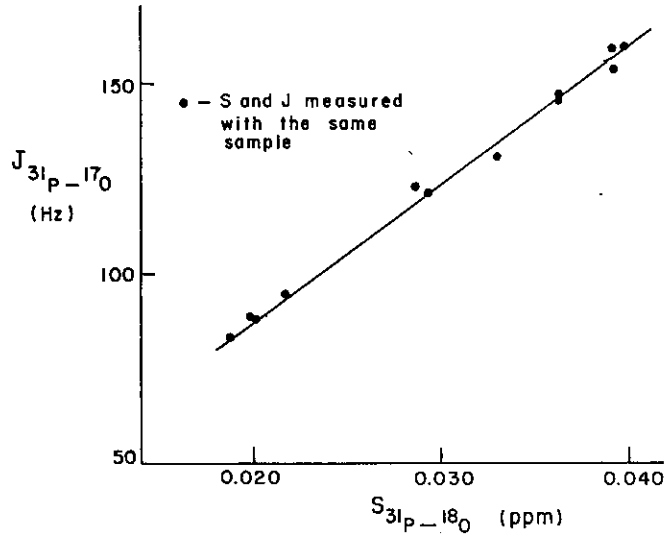


Figure 4. Correlation between $S_{^{31}P-^{18}O}$ (from Table 1) and $J_{^{31}P-^{17}O}$ (from Table 2), for the data which were obtained from our laboratory, using identical samples for the measurements of both S and J. When peaks overlap, the numbers are obtained by spectral simulation. (From Sammons *et al.*, 1983.)

(with $P^{17}OF_3$ out of order) (Gray and Albright, 1977). In general, a P=O double bond has $J > 150$ Hz whereas a P—O single bond has $J < 90$ Hz. For ^{17}O-labeled adenine nucleotides, the $J_{^{31}P-^{17}O}$ of nonbridging ^{17}O falls into the range 100–120 Hz.

Since both $J_{^{31}P-^{17}O}$ and $S_{^{31}P-^{18}O}$ are expected to be related to the bond order, and since they may also be affected simultaneously by other factors such as steric or inductive effect, we have tried to correlate the two parameters. As shown in Figure 4, there is an approximate linear relationship between $J_{^{31}P-^{17}O}$ and $S_{^{31}P-^{18}O}$ for the compounds for which we have measured both $J_{^{31}P-^{17}O}$ and $S_{^{31}P-^{18}O}$ under the same condition, and have resolved partially overlapped peaks by line simulation.

If such a correlation holds true, it should be possible to determine $J_{^{31}P-^{17}O}$ by measuring $S_{^{31}P-^{18}O}$ for those compounds which have broad ^{17}O NMR signals. Since most ^{17}O-enriched water also contains ^{16}O and ^{18}O, there is no need for additional synthesis of ^{18}O-enriched compounds. The following approximate equation can be derived from Figure 4: $J(\text{Hz}) = 3.65 \times 10^3\, S(\text{ppm}) + 14$.

3.3. Linewidths of ^{17}O Resonances

A nucleus with nuclear spin I greater than 1/2 possesses an electric quadrupole moment eQ. The dominant relaxation mechanism for quadrupolar nuclei comes from interactions of eQ with an electric field gradient eq at the nucleus and the modulation of these interactions by rotational motion (James, 1975). In the extreme narrowing conditions, i.e., very fast molecular motions with respect to resonance frequency, which is the case for small molecules in solution, the contribution of nuclear quadrupole relaxation to the relaxation rate can be expressed as (Abragam, 1961)

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

where e^2qQ/h is the nuclear quadrupole coupling constant (NQCC), η is the asymmetry parameter, and τ_r is the rotational correlation time.

Since T_q is generally smaller than 3 msec for biophosphates, the approximation $\Delta O \approx 1/\pi T_q$ can be justified, where ΔO is the linewidth of ^{17}O signals. Equation (1) becomes

$$\Delta O \approx \frac{12\pi}{125}\left(1+\frac{\eta^2}{3}\right)(\text{NQCC})^2\tau_r \qquad (2)$$

Thus, the linewidth ΔO is directly related to η, NQCC, and τ_r. Since $0 \leq \eta < 1$, the effect of $(1 + \eta^2/3)$ is not more than 33%. However, the "symmetry" can affect both η and NQCC, and the effect of the latter can be quite large. On the other hand, if NQCC can be kept constant, the ^{17}O linewidth can very well reflect changes in τ_r.

3.3.1. Effect of Nuclear Quadrupolar Coupling Constant (NQCC)

Cheng and Brown (1980) reported the following data for triphenyl phosphate measured by ^{17}O nuclear quadrupole double-resonance spectroscopy: for P=^{17}O, NQCC = 4.684 MHz, $\eta = 0.085$; for P—^{17}O-Ph (average of the three reported values), NQCC = 9.176 MHz, $\eta = 0.644$. These data, when fitted to equation (2) with an assumption that τ_r is constant, predict that $\Delta O_{P-O}/\Delta O_{P=O} = 4.37$.

Such a difference in NQCC is certainly responsible for the difference in ΔO between P=^{17}O and P—^{17}O-CH_3 in $(CH_3{}^{17}O)_3P^{17}O$ (Figure 5). The apparent ΔO in Figure 5 is 55 Hz for P=^{17}O and 250 Hz for P—^{17}O. After correcting for artificial line broadening and J_{P-O}, the observed ratio is $\Delta O_{P-O}/\Delta O_{P=O} \approx 3.5$.

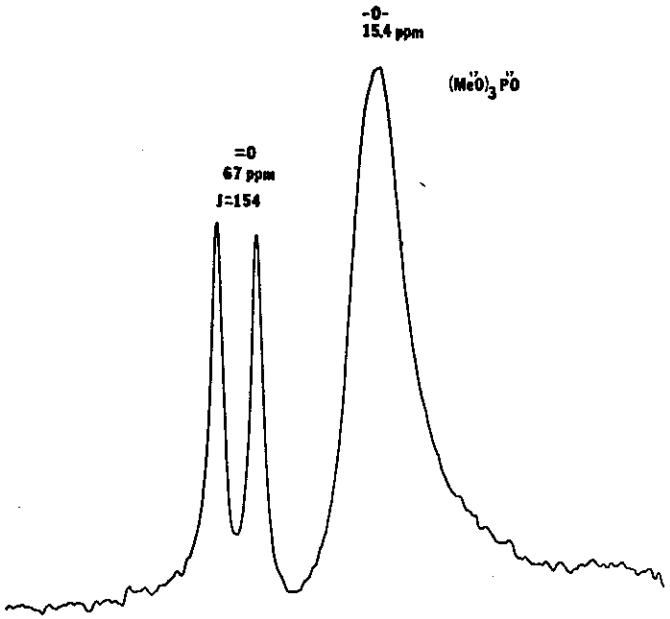


Figure 5. The ^{17}O NMR spectrum of $(CH_3{}^{17}O)_3P^{17}O$ (70 μmoles in 1.2 ml of $CDCl_3$) at 10.8 MHz. (From Tsai *et al.*, 1980.)

3.3.2. Effect of Rotational Correlation Time τ_r

The rotational correlation time τ_r is related to the molecular radius (a), the viscosity of the medium ($\bar{\eta}$) and the temperature (T) according to the Debye–Stokes theory (Abragam, 1961):

$$\tau_r = 4\pi a^3 \bar{\eta}/3kT \tag{3}$$

where k is the Boltzmann constant.

Since τ_r is directly related to ΔO according to equation (2), the NMR signal of ^{17}O "sharpens" when the viscosity $\bar{\eta}$ decreases or the temperature T increases (Rodger *et al.*, 1978; Schwartz *et al.*, 1980; Gerothanassis and Sheppard, 1982; Gerlt *et al.*, 1982). Since the viscosity $\bar{\eta}$ also decreases when temperature increases, a change in T from ambient (303 K) to 368 K causes a dramatic decrease ($\sim$ threefold) in τ_r, and accordingly in ΔO. By such a bold heating on ^{17}O-labeled nucleotides, they have improved the resolution of ^{17}O NMR and obtained relatively accurate values of $J_{^{31}P-^{17}O}$ and ^{17}O chemical shifts, some of which have been discussed in previous sections. Figure 6 shows such a temperature effect for the ^{31}P-decoupled ^{17}O NMR spectra of an equimolar mixture of ADP labeled with ^{17}O at both P_α and P_β obtained by Gerlt *et al.* (1981).

The effect of viscosity $\bar{\eta}$ on τ_r and ΔO can be shown by changing the solvent viscosity. For example, the ^{17}O NMR signal of $H_3P^{17}OO_3$

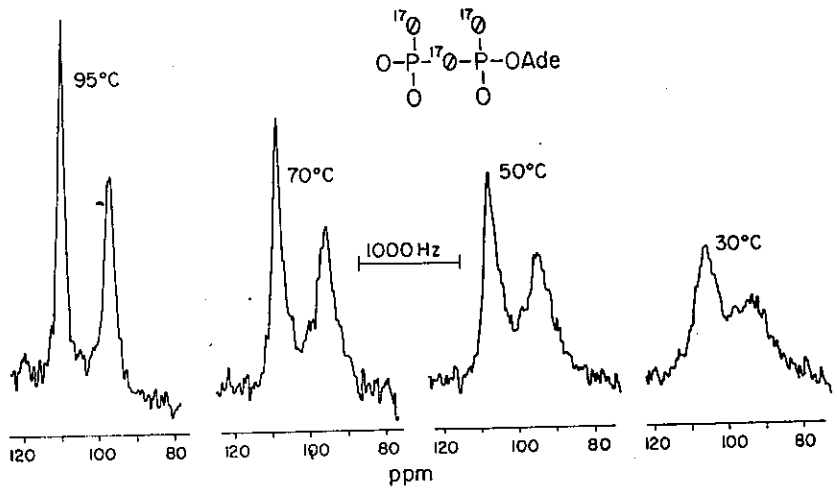


Figure 6. Phosphorus decoupled ^{17}O NMR spectra (at 36.6 MHz) of an equimolar mixture of [α-^{17}O]- and [β-^{17}O]-ADP as a function of temperature. (From Gerlt *et al.*, 1981.)

broadens when glycerol is added to increase the viscosity of solvents (see Figure 8 in Section 4.1).

3.3.3. Linewidths of Biophosphates

Before discussing the ^{17}O linewidths (ΔO) of biophosphates, it is necessary to address some experimental problems. The ^{17}O nucleus has a sensitivity of 2.91×10^{-2} in NMR relative to that of ^{1}H, 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) such 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. Indeed most ^{17}O NMR data reported earlier were obtained from unenriched compounds in the neat liquid form (Christ *et al.*, 1961). For the biophosphate work, however, an enriched sample is required for two 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 4–10 times broader than the solvent signal. The *S/N* ratio of the phosphate signal subsequently decreases.

For a 2-ml sample of 25 m*M* biophosphate enriched with 50 at. % ^{17}O, the total ^{17}O-containing biophosphate amounts to only 25 μmol, which is less than the $H_2{}^{17}O$ species present in 2 ml of water (40.7 μmol) due to natural abundance (0.037%). With such a concentration, a signal may not be detectable if it is too broad. A very short preacquisition delay (DE) (0–50 μsec) should be used for a broad signal. However, use of a short DE could result in a rolling base line or a distorted signal due to a pulse breakthrough (Canet *et al.*, 1976). In our experience, routine high-resolution NMR spectrometers are suitable for signals of < 1000 Hz, whereas the high-power probe of the Bruker CXP-300 has a much better capability to detect broader signals. Recently Gerothanassis and Sheppard (1982) have reported natural abundance ^{17}O NMR spectra of some inorganic phosphates and biophosphates, by employing a higher concentration of sample in ^{17}O-depleted water at a high temperature.

Most ^{17}O-labeled small biophosphate molecules (P_i, AMP, ADP, ATP, phospholipids, sugar phosphates, etc.) have a ΔO of < 1000 Hz at room temperatures, except that the ^{17}O at a P—O—P bridging position of nucleotides has a ΔO of > 1000 Hz. Binding of adenine nucleotides with diamagnetic metal ions causes a line broadening effect, which will be discussed in Section 6.2. Aggregation of phospholipids also results in the broadening of the ^{17}O NMR signal, as is discussed in Section 6.3. The ΔO of ATP follows the order α-$^{17}O > \beta$-$^{17}O > \gamma$-^{17}O and that of ADP follows the order α-$^{17}O > \beta$-^{17}O, which may be explained by a larger restriction in rotational motions for inner phosphates.

4. $^{31}P(^{17}O)$ NMR

4.1. ^{31}P–^{17}O Interactions in Small Biophosphates

When a dipolar nucleus (^{31}P in the present case) is bonded directly to a quadrupolar nucleus (^{17}O in the present case), the ^{31}P nucleus will also be relaxed by virtue of its spin–spin coupling with ^{17}O. This was termed "scalar relaxation of the second kind" by Abragam (1961). Such a scalar relaxation is dependent on the magnitudes of the longitudinal relaxation time of the quadrupolar nucleus (T_1, which is approximately equal to T_q under present conditions) and the spin–spin coupling constant $J_{^{31}P-^{17}O}$ (abbreviated as 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 (1964) have calculated the line shape of a dipolar nucleus coupled to a quadrupolar nucleus with $I = 5/2$, with different magnitudes of T_qJ as shown in Figure 7.

Such a theoretical treatment has not been verified experimentally, at least in the case of the ^{31}P–^{17}O interaction. However, the $^{31}P(^{17}O)$ NMR method which we have developed is based on such a scalar ^{31}P–^{17}O interaction. Figure 3 shows the ^{17}O and $^{31}P(^{17}O)$ NMR spectra of $P^{17}OCl_3$ (3A), $(CH_3O)_3P^{17}O$ (3B), $(PhO)_3P^{17}O$ (3C), and $Ph_3P^{17}O$ (3D). It can be seen in Figure 3 that as the ^{17}O NMR coupling pattern collapses

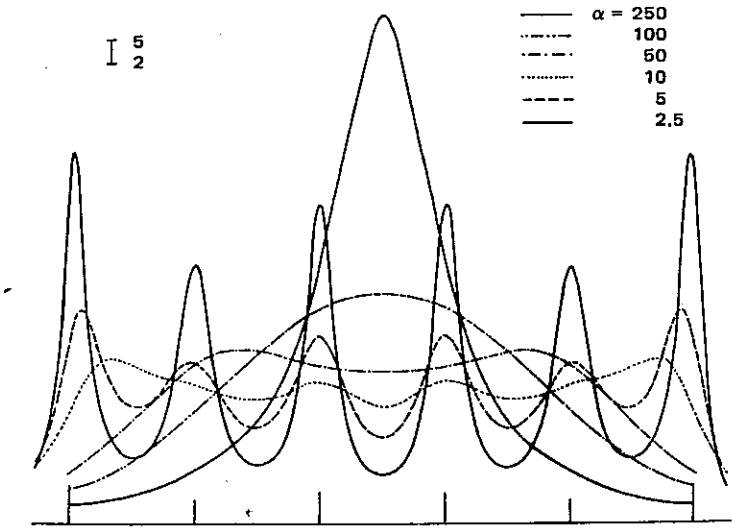


Figure 7. Calculated line shapes of a proton resonance directly coupled with a nucleus with $I = 5/2$, as a function of α, directly adapted from Suzuki and Kubo (1964). According to the authors' definition, $\alpha \approx 6.6/T_qJ$.

(decreasing J and/or increasing ΔO), the ^{31}P NMR coupling pattern also collapses. In all spectra the strong central peak is due to the residual unlabeled species. Although we have not been able to analyze the $^{31}P(^{17}O)$ spectra quantitatively, the experimental observation seems to fit the theoretical calculation at least qualitatively.

The compounds whose spectra are shown in Figure 3 are all symmetrical small molecules with a P=O double bond. These compounds have relatively long T_q and large J, thus showing fully or partially resolved ^{17}O and $^{31}P(^{17}O)$ NMR spectra. For biophosphate molecules, T_q is generally shorter (due to a larger molecular size and a small degree of symmetry) and J is generally smaller (due to a P—O bond with a smaller π-character). Therefore the ^{17}O NMR signals of biophosphates are broader and less well resolved, as shown in previous sections. Based on Figure 7, we would expect the $^{31}P(^{17}O)$ NMR signals of biophosphates to be a "broad singlet". Under this condition ($T_q J < 1$) the scalar relaxation will contribute to the relaxation of the dipolar nucleus accoring to (Lehn and Kintzinger, 1973; James, 1975; Abragam, 1961):

$$\frac{1}{T_{1sc}} = \frac{8\pi^2 J^2 I(I+1)}{3} \frac{T_q}{1+(\omega_P - \omega_O)^2 T_q^2} \tag{4}$$

$$\frac{1}{T_{2sc}} = \frac{4\pi^2 J^2 I(I+1)}{3} \left[T_q + \frac{T_q}{1+(\omega_P - \omega_O)^2 T_q^2} \right] \tag{5}$$

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 biophosphate molecules at the extreme narrowing limit ($\omega^2 \tau_c^2 \ll 1$), T_q is in the order of 10^{-2} to 10^{-4} sec. Since $\omega_P - \omega_O \sim 10^7$–$10^8$ Hz, $(\omega_P - \omega_O)^2 T_q^2 \gg 1$ and equation (4) and (5) can be reduced to

$$\frac{1}{T_{1sc}} \approx 0 \tag{6}$$

$$\frac{1}{T_{2sc}} \approx \tfrac{35}{3}\pi^2 J^2 T_q \tag{7}$$

Under this condition $1/T_2 \approx 1/T_{2sc}$ for ^{31}P, and $T_1 \approx T_2 \approx T_q$ for ^{17}O, which justifies the approximations $\Delta O \approx 1/\pi T_q$ and $\Delta P \approx 1/\pi T_{2sc}$. The following approximate relationship can be obtained from equation (7):

$$\Delta P\, \Delta O \approx \tfrac{35}{3} J^2 \tag{8}$$

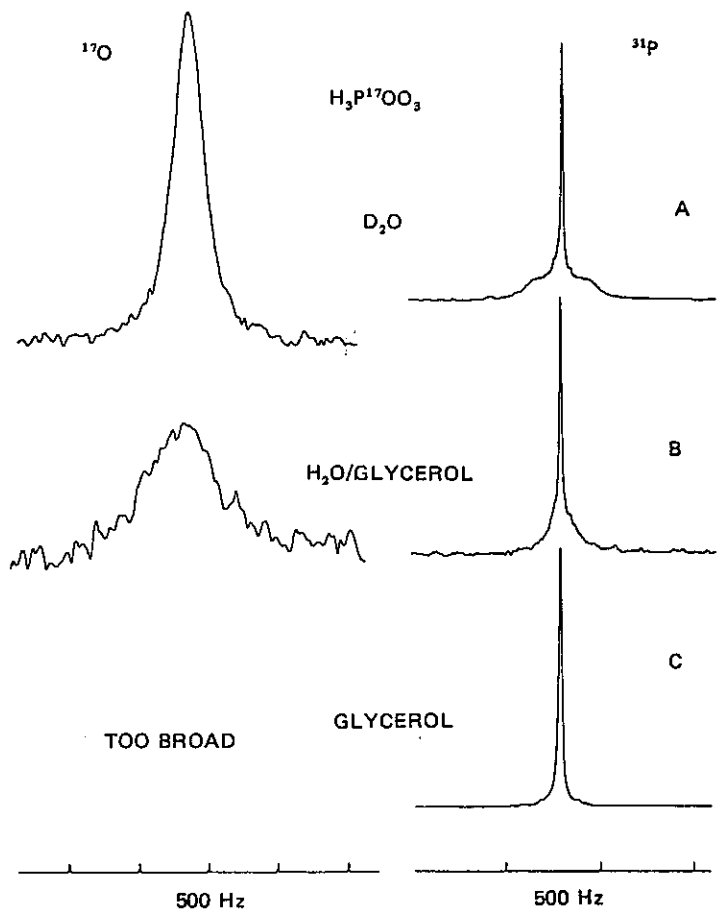


Figure 8. The ^{17}O NMR spectra (at 27.1 MHz) and $^{31}P(^{17}O)$ NMR spectra (at 81.0 MHz) of $H_3P^{17}OO_3$ (50 at. % ^{17}O) in D_2O (A), H_2O/glycerol (1 : 1 volume ratio) (B), and glycerol (C). All spectra were obtained at 30°C and processed with a line broadening of 20 Hz (^{17}O) and 4 Hz (^{31}P). (Sammons *et al.*, 1983)

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

Such an approximate inversely proportional relationship between ΔP and ΔO, or between T_q (of ^{17}O) and T_{2sc} (of ^{31}P), is illustrated by the $^{31}P(^{17}O)$ NMR signals of $H_3P^{17}OO_3$ in D_2O, H_2O/glycerol, and glycerol (Figure 8). As ΔO increases due to an increased viscosity, the ΔP decreases correspondingly.

The biochemical applicability of the $^{31}P(^{17}O)$ NMR method is not as

obvious as that of ^{17}O NMR or $^{31}P(^{18}O)$ NMR, thus deserves some explanation before real examples are presented in following sections.

The approximate equations (6)–(8) derived above are valid under the "extreme narrowing limit" and thus should be applicable for small biophosphate molecules in solution. Tsai *et al.* (1980) have shown that for adenine nucleotides with ^{17}O labeled at all possible positions, the $^{31}P(^{17}O)$ NMR signals are "broad". The case of $H_3P^{17}OO_3$/glycerol (Figure 8C), in which the $^{31}P(^{17}O)$ signal has sharpened to become almost indistinguishable from the $^{31}P(^{17}O)$ signal, should not occur in small biophosphates. Therefore, ^{17}O "quenches" the ^{31}P NMR signal of ^{31}P–^{17}O species, causing an apparent decrease in the intensity of a ^{31}P NMR signal.

Such a "line-broadening effect" of ^{17}O in ^{31}P NMR has been used to locate the position of a ^{17}O-label (Tsai, 1979), and to calculate the percent enrichment of ^{17}O (Huang and Tsai, 1982; Reed and Leyh, 1980). In addition, it has made possible analysis of the configuration of [^{16}O, ^{17}O, ^{18}O] phosphate monoesters and [^{16}O, ^{17}O, ^{18}O] thiophosphates by ^{31}P NMR (Section 5).

4.2. ^{31}P–^{17}O Interactions in Macromolecular Systems

The main thrust for the future application of the ^{31}P–^{17}O NMR method lies in macromolecular systems in which the ^{17}O NMR signal may be too broad to be detected. This aspect of application is still under development, and the following discussion should be considered tentative. In general, there are three possible cases:

(i) In E · S complexes, if τ_r increases by only 10^2 or less, which may be the case in small enzymes or if the phosphoryl group in the bound substrate has some internal rotational freedom, the approximations for small molecules are still valid. According to equation (8), the $^{31}P(^{17}O)$ signal should be "sharpened" dramatically, but probably not to the limit of extremely small ΔP as in $H_3P^{17}OO_3$/glycerol.

(ii) An intermediate situation which may occur is that if τ_r increases by 10^2–10^3, the extreme narrowing approximation may still be valid. In such a case, ΔO may increase from 500 Hz to 50–500 KHz, which is too broad to be detected. On the other hand, ΔP may decrease from 500 Hz to 5–0.5 Hz, and the ^{31}P–^{17}O signal cannot be distinguished from the ^{31}P–^{16}O signal, as in the case of $H_3P^{17}OO_3$ in glycerol (Figure 8).

(iii) On the other hand, if in E · S complexes τ_r increases by a factor of 10^4 or more (e.g., from 10^{-11} to 10^{-7} sec), the approximations $\omega^2\tau_c^2 \ll 1$ and $(\omega_P - \omega_O)^2 T_q \gg 1$ may not apply. In this "nonextreme narrowing" condition, the ^{17}O relaxation must be described by a sum of three decaying exponentials (Abragam, 1961), and the ^{31}P–^{17}O interaction can be even

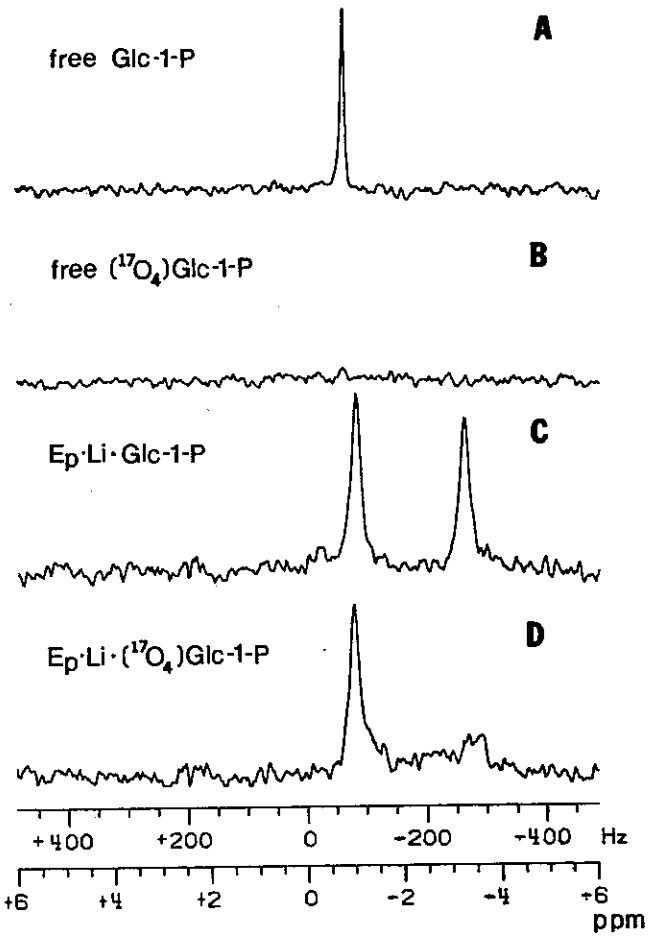


Figure 9. The ^{31}P NMR spectra of free and bound glucose-1-phosphate (Glc-1-P) at 80.99 MHz. (*A*) "Free" Glc-1-P in 2.5 ml D_2O containing 1 m*M* EDTA and 0.4 m*M* tris-Cl^- buffer, pH 7.5. Other NMR parameters: repetition time, 5 sec; spectral width, 2 KHz; memory size, 8 K; 1024 acquisitions. (*B*) "Free" [$^{17}O_4$]Glc-1-P. Sample conditions: same as in (*A*). Other NMR parameters: same as in (*A*). (*C*) Glc-1-P bound to E_p (the phosphoenzyme form of phosphoglucomutase) in the presence of Li^+. Sample conditions: 1.1 m*M* enzyme (0.8 m*M* E_p form) plus 0.9 m*M* Glc-1-P in 2.5 ml of 10% D_2O containing 20 m*M* LiCl, 22 m*M* tris-Cl^-, 1 m*M* EDTA, pH 7.5. Other NMR parameters: repetition time, 3.4 sec; spectral width, 10 kHz, memory size, 8 K; 4096 acquisitions. (*D*) [$^{17}O_4$]Glc-1-P bound to E_p (the phosphoenzyme form of phosphoglucomutase) in the presence of Li^+. Sample conditions: same as in (*C*). Other NMR parameters: same as in (*C*). For all spectra (*A–D*), 60° pulses were used (the 90° pulse was 30 μsec) and proton noise decoupling was used with high power (1.5 W) during acquisition and low power (0.5 W) off acquisition. Chemical shifts are referenced to trimethyl phosphate. A line broadening of 5 Hz has been applied. Temperature: 20°C. (Markley, J. L., Rhyu. G. I., and Ray, W. J., Jr., unpublished results.)

more complicated. It is quite possible that the ^{31}P relaxation will be dominated by the dipolar relaxation due to ^{17}O, which also results in the broadening of the ^{31}P NMR signal.

If a $^{31}P(^{17}O)$ NMR signal of an E · S complex or other macromolecular systems (such as nucleic acids, membranes) can be quantitatively analyzed, it could be useful in determining the τ_r of phosphate groups, or in monitoring changes in rotational motions due to conformational changes, metal ion coordination, etc.

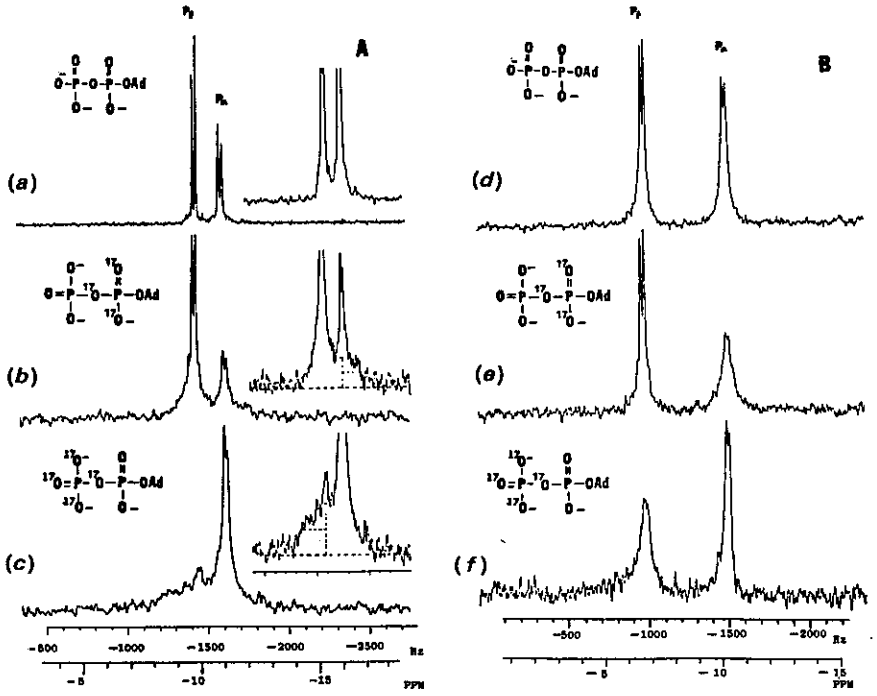


Figure 10. (*A*) The ^{31}P NMR spectra (at 145.7 MHz) of "free" ADP. Sample conditions: 2.5 ml of 0.12 *M* sodium acetate buffer, with 25% D_2O, pH 6.2, containing 10 mg EDTA. (*a*) 40 μmoles of unlabeled ADP, 200 scans; (*b*) 7.0 μmoles of [α-$^{17}O_2$, $\alpha\beta$-^{17}O]ADP, 1000 scans; (*c*) 7.0 μmoles of [β-$^{17}O_3$, $\alpha\beta$-^{17}O]ADP, 1000 scans. The insets represent the expanded "broad signals." A line broadening of 1.0 Hz has been applied to unlabeled ADP and 7.0 Hz to labeled ADP. Chemical shifts are referenced to external 85% H_3PO_4. Other NMR parameters: repetition time, 3.0 sec; spectral width, 6 kHZ; memory size, 16 K. $J_{\alpha\beta} = 20.6$ Hz; temperature: 25–26°C. (*B*) The ^{31}P NMR spectra (at 145.7 MHz) of "bound" ADP. Sample conditions: 2.5 ml of 0.12 *M* sodium acetate buffer, with 25% D_2O, pH 6.2, containing 10 mg EDTA, and 15 μmoles of the bovine pancreatic ribonuclease A. (*d*) 10 μmoles of unlabeled ADP, 1700 scans; (*e*) 10 μmoles of [α-$^{17}O_2$, $\alpha\beta$-^{17}O]ADP, 900 scans; (*f*) 10 μmoles of [$\beta^{17}O_3$, $\alpha\beta$-^{17}O]ADP, 600 scans. Other NMR parameters: same as (*A*). $J_{\alpha\beta} = 20.2$ Hz. Line broadening: 4.0 Hz. (Tsai, 1982.)

While such applications are not yet possible at the present stage, the ^{31}P–^{17}O interaction has been examined in some E · S complexes (Tsai, 1982; Markley *et al.*, 1982). Figure 9 shows that the $^{31}P(^{17}O)$ NMR signal of [$^{17}O_4$]Glc-l-P in the Ep · Li · [$^{17}O_4$]Glc-l-P complex (where Ep is the phosphoenzyme form of phosphoglucomutase, mw ~ 65,000) has not sharpened too much, which provides an unequivocal assignment of the upfield signal to Glc-l-P. On the other hand, the $^{31}P(^{17}O)$ NMR signal of E-P$^{17}O_3$

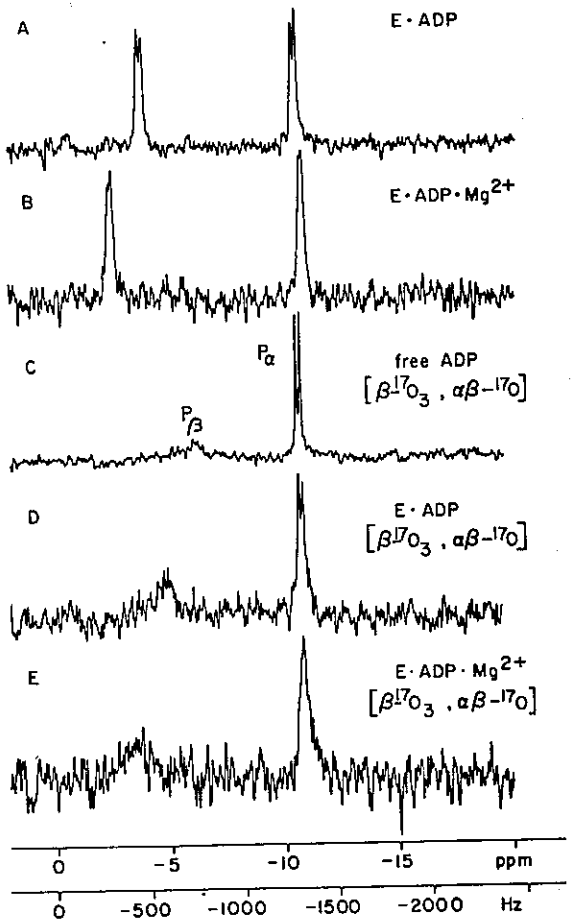


Figure 11. The ^{31}P NMR spectra (121.5 MHz) of ADP · arginine kinase (AK) complexes in 50 m*M* Hepes buffer (10% D_2O), pH 8.0. (*A*) 2.6 m*M* AK, 2.0 m*M* ADP, 0.67 m*M* EDTA. 4260 scans; (*B*) same as (*A*), 4.65 m*M* $MgCl_2$, 1530 scans; (*C*) free [β-$^{17}O_3$, $\alpha\beta$-^{17}O]ADP, 6.7 m*M* in D_2O, 458 scans; (*D*) 2.0 m*M* AK, 1.4 m*M* ^{17}O-ADP, 0.53 m*M* EDTA, 5000 scans; (*E*) same as (*D*), 4.74 m*M* $MgCl_2$, 8000 scans. Sample volumes: 1.5–2.0 ml. Line broadening: 5 Hz. Acquisition time: 1.36 sec. Temperature: 27°C. ^1H-decoupling. (Sammons *et al.*, 1983.)

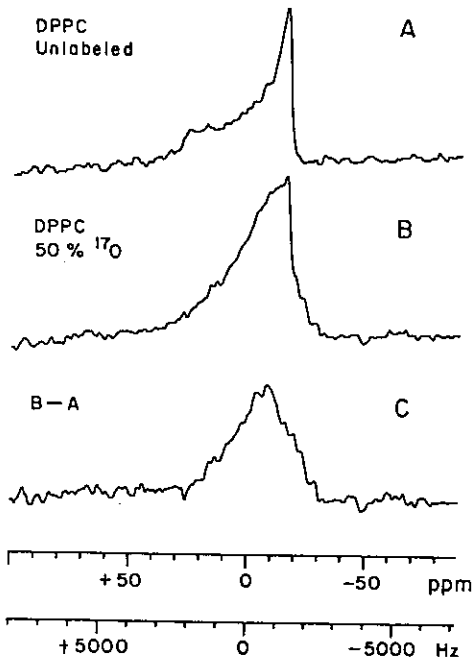


Figure 12. The ^{31}P NMR spectra (at 81.0 MHz) of unsonicated lipid bilayers. (*A*) dipalmitoylphosphatidylcholine (DPPC), unlabeled; (*B*) [$^{17}O_1$]DPPC, 50 at. % ^{17}O at phosphorus; (*C*) subtraction of (*A*) from (*B*). Sample conditions: 100 mg DPPC mixed with 1.5 ml D_2O by heating at 50°C. Spectral parameters: spectral width 25,000 Hz, ^1H decoupling (decoupler power 2.5 W), acquisition time 0.082 sec, 40,000 scans, line broadening 100 Hz, 45°C. (Sammons *et al.*, 1983.)

is greatly sharpened (G. I. Rhyu, W. J. Ray, and J. L. Markley, to be published). In the case of ADP bound to ribonuclease A (a small enzyme, mw 14,000), the $^{31}P(^{17}O)$ NMR signals of α- and β-^{17}O-ADP clearly have sharpened greatly, but not to the limit of extremely small ΔP (Figure 10). Since the gyration radius of ribonuclease A is only 18.3 A (Richards and Wyckoff, 1971), the bound ADP is more likely to be in category (i), or in the transition between categories (ii) and (iii). When ADP is bound to a larger enzyme, arginine kinase (mw 40,000), the $^{31}P(^{17}O)$ NMR signals appear to be "broader" than that of ribonuclease, as shown in Figure 11. Such a difference can only be explained by two possibilities: (1) ADP is bound to arginine kinase less tightly than to ribonuclease A, thus belongs to category (i); (2) ADP is bound to arginine kinase at least as rigidly as it is bound to ribonuclease. Due to an increased τ_r in the ADP · arginine kinase complex, it falls into category (iii) and experiences a larger dipolar broadening. The second explanation seems much more likely.

The ^{17}O-labeled phospholipids, when dispersed in H_2O, is certain to have $\tau_r \gg 10^{-7}$ sec and thus belongs to category (iii). As shown in Figure 12, the $^{31}P(^{17}O)$ NMR signal of unsonicated DPPC/D_2O is broadened, most likely by the dipolar relaxation by ^{17}O.

5. APPLICATIONS IN THE STEREOCHEMISTRY OF ENZYME REACTIONS AT PHOSPHORUS

5.1. Rationale of Configurational Analysis

The most fruitful application of the NMR methods involving oxygen isotopes has been in the stereochemical problems of enzyme-catalyzed reactions. The enzyme-catalyzed reactions involving a P—O bond cleavage can be categorized into the following types based on the stereochemistry involved:

$$ROPO_2OR' \text{ (prochiral)} \rightleftharpoons ROPO_2OR'' \text{ (prochiral)} \quad \text{(a)}$$

$$ROPO_2OR' \text{ (prochiral)} \rightleftharpoons ROPO_3 \text{ (pro-prochiral)} \quad \text{(b)}$$

$$ROPO_3 \text{ (pro-prochiral)} \rightleftharpoons R'OPO_3 \text{ (pro-prochiral)} \quad \text{(c)}$$

$$ROPO_3 \text{ (pro-prochiral)} \rightleftharpoons PO_4 \text{ (pro-pro-prochiral)} \quad \text{(d)}$$

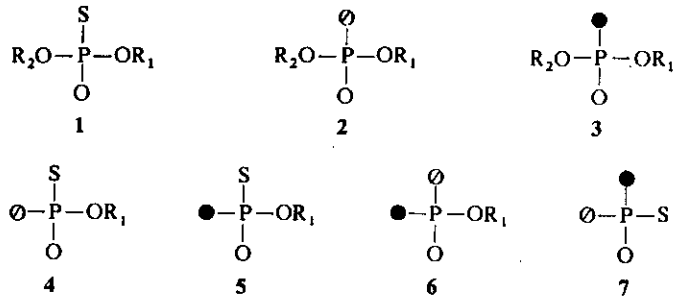
$$PO_4 \text{ (pro-pro-prochiral)} \rightleftharpoons PO_4 \text{ (pro-pro-prochiral)} \quad \text{(e)}$$

This subject has already been covered by several recent reviews (Knowles, 1980; Buchwald *et al.*, 1982; Tsai, 1982; Webb, 1982; Frey, 1982; Frey, *et al.*, 1982; Eckstein, 1979; Eckstein, *et al.*, 1982). The aim of this section is to illustrate the methodology rather than to elaborate the results and their mechanistic significance.

The general approach in elucidating the stereochemical course of an enzyme-catalyzed reaction involves the following steps: (1) synthesis of substrates chirally labeled (with ^{17}O, ^{18}O, or S) at phosphorus; (2) use of chirally labeled substrates to perform the reaction and isolate the product; and (3) determination of the absolute or relative configuration of the substrate and the product. It is the third step in which ^{17}O, $^{31}P(^{17}O)$, and $^{31}P(^{18}O)$ NMR methods have become indispensible tools.

A phosphodiester can be made chiral by substituting an oxygen with S (**1**), ^{17}O (**2**), or ^{18}O (**3**). A phosphomonoester can be made chiral by labeling with ^{17}O and S (**4**), ^{18}O and S (**5**), or ^{17}O and ^{18}O (**6**). The inorganic phosphate P_i can be made chiral by labeling with ^{17}O, ^{18}O, and S (**7**). (It is conventional to represent ^{18}O with ● and ^{17}O with Ø).

All types of chirally labeled compounds **1** to **7** have been used to elucidate the steric course of reactions (a) to (d) catalyzed by various enzymes. Examples of compound **1** include, among others, ATPαS and ATPβS developed by Eckstein (1978). Since there are several chiral centers in the ribose ring, the R_p and S_p isomers of ATPαS show different chemical shifts at P_α, (Sheu and Frey, 1977; Jaffe and Cohn, 1978), and those of ATPβS show different chemical shifts at P_β (Jaffe and Cohn, 1978). Thus



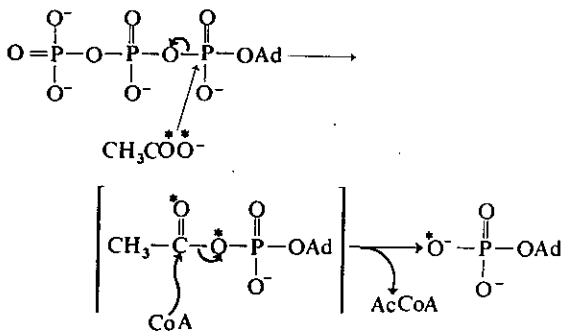
stereoisomers of type **1** can be distinguished simply based on ^{31}P chemical shifts, without using the NMR methods involving oxygen isotopes. The stereochemistry of several enzymes have been elucidated by such an approach which, however, is beyond the scope of this chapter.

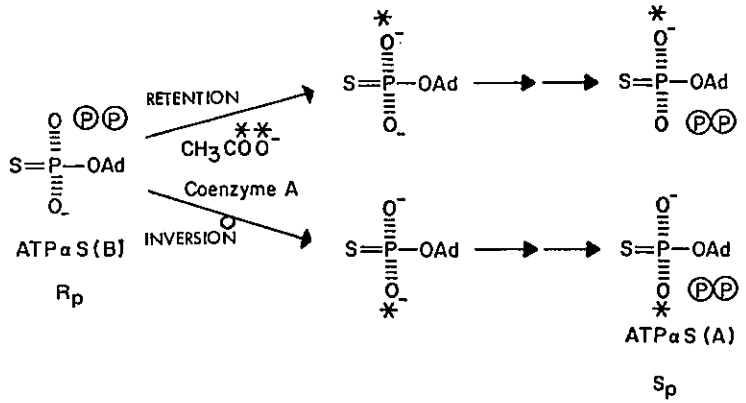
The configurational analysis of compounds **2** to **5** involves determining whether the labeled oxygen (^{17}O in **2** and **4**, ^{18}O in **3** and **5**) occupies the pro-R or pro-S position. In the case of cyclic [$^{17}O_1$]dAMP (type **2**), ^{17}O NMR has been used to distinguish the two prochiral oxygens (Coderre *et al.*, 1981a). In all other cases, the compound is first derivatized at one of the two oxygens. The position of ^{17}O or ^{18}O is then located by the $^{31}P(^{17}O)$ NMR or the $^{31}P(^{18}O)$ NMR method, respectively.

Compounds **6** and **7** contain both ^{17}O and ^{18}O. By a "substitution" and a "derivatization," the configuration can be analyzed by the *combination* of the $^{31}P(^{17}O)$ and the $^{31}P(^{18}O)$ NMR methods. Table 3 summarizes the chirally labeled biophosphates whose configuration have been determined by NMR methods involving oxygen isotopes.

5.2. Application of $^{31}P(^{17}O)$ NMR

The $^{31}P(^{17}O)$ NMR method was first used to elucidate the steric course of acetyl CoA synthetase-catalyzed reaction (Tsai, 1979) which is an example of the reaction type (b):



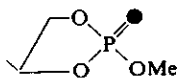


Scheme I

It was found that the enzyme 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. To determine the configuration of the ^{17}O-labeled AMPS (compounds type **4**), it is converted to (S_p)-ATPαS by stereospecific phosphorylation at the pro-R oxygen catalyzed by adenylate kinase, followed by a second phosphorylation catalyzed by pyruvate kinase (Sheu and Frey, 1977; Jaffe and Cohn, 1978). By such a conversion ^{17}O should be incorporated into the nonbridging position of (S_p)-ATPαS if the step of acetate activation proceeds with retention of configuration. On the other hand, ^{17}O should be located at the P-O-P bridging position if inversion occurs. A nonbridging ^{17}O at P_α should cause the P_α signal to broaden and decrease in ^{31}P NMR, whereas a bridging ^{17}O should quench both P_α and P_β signals.

Figure 13 shows the ^{31}P NMR spectra of unlabeled (S_p)-ATPαS (A), the synthesized (S_p)-[α-^{17}O, $\alpha\beta$-^{17}O] ATPαS (B), and the (S_p)-ATPαS obtained from the enzyme reaction (C). The ^{17}O isotope used was 20% enriched and the enrichment of ^{17}O-acetate was determined as 19%. In Figure 13B, the P_α signal decreases to $67 \pm 1\%$ and the P_β signal to $83 \pm 4\%$. In Figure 13C, the P_α signal decreases to $80 \pm 4\%$ and the P_β signal to $82 \pm 5\%$. Since both P_α and P_β have decreased in Figure 13C, these 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 (Tsai, 1979).

TABLE 3
Stereochemistry of Enzyme-Catalyzed Reactions Determined by ^{31}P NMR on the Basis of ^{17}O and ^{18}O Effects[a]

Enzyme	Substrate	Product	Final derivative	Method	Result	Reference
Type A: $ROPO_2$-OR′ ⇄ $ROPO_2$-OR″						
Phospholipase D	[^{18}O]DPPC (**9**)	[^{18}O]DPPE (**10**)	O-silylation	$^{31}P(^{18}O)$	Ret.	Bruzik and Tsai (1982)
Adenylate cyclase	Cyclic AMP (^{18}O)	ATP(α-^{18}O)	$Co(NH_3)_4ADP$	$^{31}P(^{18}O)$	Inv.	Coderre and Gerlt (1980)
Type B: $ROPO_2$-OR′ ⇄ $ROPO_3$						
Acetyl CoA synthetase	ATPαS (R_p, α-^{17}O)	AMPS (α-^{17}O)	ATPαS (S_p, α-^{17}O)	$^{31}P(^{17}O)$	Inv.	Tsai (1979)
Aminoacyl tRNA synthetases	ATPαS (α-^{18}O)	AMPS (α-^{18}O)	ATPαS (α-^{18}O)	$^{31}P(^{18}O)$	Inv.	Jaffe and Cohn (1979), Langdon and Lowe (1979)
cAMP Phospho-diesterase	cyclic dAMP (^{17}O, ^{18}O)	dAMP (α-^{16}O, ^{17}O, ^{18}O)	Cyclization + methylation	$^{31}P(^{17}O, ^{18}O)$	Inv.	Coderre *et al.* (1981b)
Exonuclease	TpNP (^{17}O, ^{18}O)	3′-TMP (^{16}O, ^{17}O, ^{18}O)	Same as above	$^{31}P(^{17}O, ^{18}O)$	Ret.	Mehdi and Gerlt (1981)
Phosphodiesterase (snake venom)	ATP (α-^{16}O, ^{17}O, ^{18}O)	AMP (α-^{16}O, ^{17}O, ^{18}O)	Same as above	$^{31}P(^{17}O, ^{18}O)$	Ret.	Jarvest and Lowe (1981a)
Nucleotide pyrophosphatase	ATP (γ-CNEt) (α-^{17}O, ^{18}O)	AMP (α-^{16}O, ^{17}O, ^{18}O)	Cyclization + ethylation	$^{31}P(^{17}O, ^{18}O)$	Ret.	Sammons (1982)
Nonspecific phosphohydrolase	*endo*-U > PS	UMPS (^{18}O)	Exo-U > PS (^{18}O)	$^{31}P(^{18}O)$	Inv.	Gerlt and Wan (1979)
Type C: RO-PO_3 ⇄ R′O-PO_3						
Creatine kinase	ATP (γ-^{16}O, ^{17}O, ^{18}O)	Creatine-P (^{16}O, ^{17}O, ^{18}O)		$^{31}P(^{17}O, ^{18}O)$	Inv.	Hansen and Knowles (1981)
Acid phosphatase	phenyl-P (^{16}O, ^{17}O, ^{18}O)	1-Phospho-propane-1,2-diol	Same as above	$^{31}P(^{17}O, ^{18}O)$	Ret.	Saini *et al.* (1981)
Hexokinase	ATP (γ-^{16}O, ^{17}O, ^{18}O)	Glucose-6-P (^{16}O, ^{17}O, ^{18}O)	Cyclization + methylation	$^{31}P(^{17}O, ^{18}O)$	Inv.	Lowe and Potter (1981a)
Pyruvate kinase	Phosphoenolpyruvate (^{16}O, ^{17}O, ^{18}O)	ATP (γ-^{16}O, ^{17}O, ^{18}O)	Same as above	$^{31}P(^{17}O, ^{18}O)$	Inv.	Lowe *et al.* (1981)
Phosphofructo-kinase	Fructose-1,6-diP (1-^{16}O, ^{17}O, ^{18}O)	ATP (γ-^{16}O, ^{17}O, ^{18}O)	Same as above	$^{31}P(^{17}O, ^{18}O)$	Inv.	Jarvest *et al.* (1981)
Phosphogluco-mutase	Glucose-1-P (^{16}O, ^{17}O, ^{18}O)	Glucose-6-P (^{16}O, ^{17}O, ^{18}O)	Same as above	$^{31}P(^{17}O, ^{18}O)$	Ret.	Lowe and Potter (1981b)
Polynucleotide kinase	ATP (γ-^{16}O, ^{17}O, ^{18}O)	3′, 5′-ADP (5′-^{16}O, ^{17}O, ^{18}O)	Same as above	$^{31}P(^{17}O, ^{18}O)$	Inv.	Jarvest and Lowe (1981b)
Phosphoglycerate kinase	Glycerate-1,3-diP (1-^{17}O, ^{18}O, S)	ATPγS (γ-^{17}O, ^{18}O)	S_p-ATPβS	$^{31}P(^{17}O, ^{18}O)$	Inv.	Webb and Trentham (1980a)
Type D: $ROPO_3$ ⇄ PO_4						
5′-Nucleotidase	AMPS (α-^{18}O)	Psi (^{16}O, ^{17}O, ^{18}O)	R_p-ATPβS	$^{31}P(^{17}O, ^{18}O)$	Inv.	Tsai and Chang (1980)
Myosin ATPase	ATPγS (γ-^{18}O)	Psi (^{16}O, ^{17}O, ^{18}O)	S_p-ATPβS	$^{31}P(^{17}O, ^{18}O)$	Inv.	Webb and Trentham (1980b)
Mitochondrial ATPase	ATPγS (γ-^{18}O)	Psi (^{16}O, ^{17}O, ^{18}O)	S_p-ATPβS	$^{31}P(^{17}O, ^{18}O)$	Inv.	Webb *et al.* (1980)
Sarcoplasmic reticulum ATPase	ATPγS (γ-^{17}O, ^{18}O)	Psi (^{16}O, ^{17}O, ^{18}O)	S_p-ATPβS	$^{31}P(^{17}O, ^{18}O)$	Ret.	Webb and Trentham (1981)
Ribosome-dependent GTPase	GTPγS (γ-^{17}O, ^{18}O)	Psi (^{16}O, ^{17}O, ^{18}O)	S_p-ATPβS	$^{31}P(^{17}O, ^{18}O)$	Inv.	Webb and Eccleston (1981)

[a] There are other excellent works on the stereochemistry of enzyme reactions, particularly in the earlier stage, which were done by mass spectral analysis.

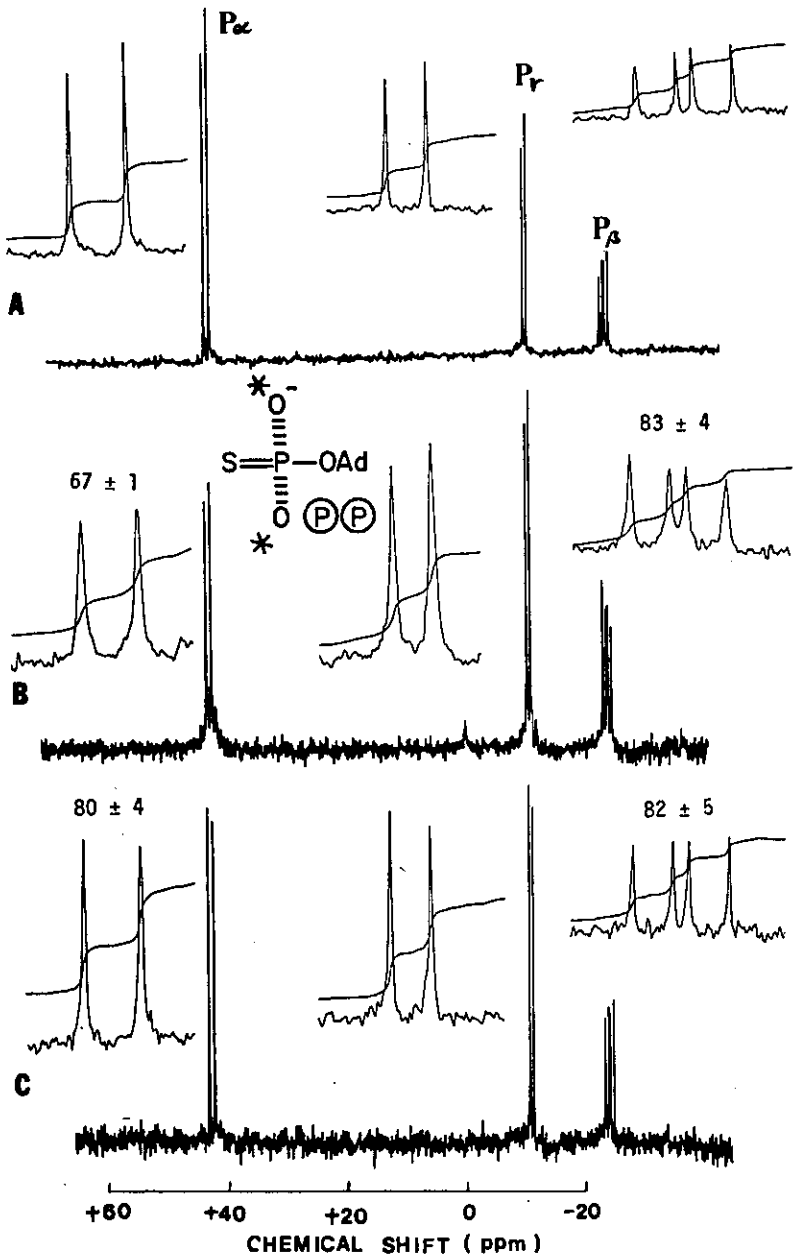
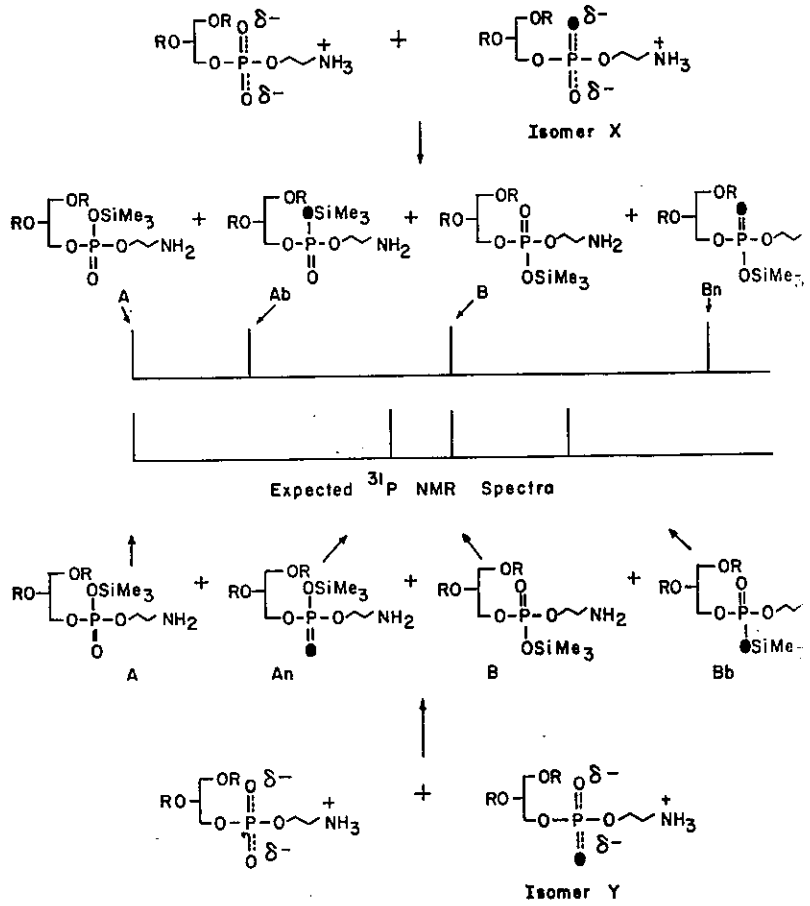


Figure 13. The ^{31}P NMR spectra (at 32.2 MHz) showing the results of acetyl-CoA synthetase. (*A*) Unlabeled (Sp)-ATPαS; (*B*) synthesized (Sp)-[α-^{17}O, αβ-^{17}O]ATPαS; (*C*) The (Sp)-ATPαS from [$^{17}O_2$] acetate. The insets represent the integrations of the corresponding signals. (From Tsai, 1979.)

5.3. Application of $^{31}P(^{18}O)$ NMR

Among the NMR methods involving oxygen isotopes, the $^{31}P(^{1}$ method has been most commonly used mainly because of its simplicity quantitation. The configuration of **3** and **5** can in principle be determi by first derivatizing at one of the two diastereotopic oxygens, followed determining the position of ^{18}O based on the magnitude of ^{18}O isot shift in ^{31}P NMR. As described in Section 2, a nonbridging ^{18}O ha larger π-character, thus a larger *S* value, than a bridging ^{18}O (P—O– does.

A recent example in this category is the determination of the "relati configuration of chirally labeled [$^{18}O_1$]dipalmitoylphosphatidyletha amine (DPPE) (Bruzik and Tsai, 1982), as illustrated in Scheme II. Sil



Scheme II

tion of an arbitrary isomer X of [$^{18}O_1$]DPPE (containing unlabeled DPPE) gives four different species: **A**, **Ab**, **B**, and **Bn**, where **A** and **B** (separated by 0.048 ppm in ^{31}P NMR) are the two diastereomers which result from silylation at the pro-R and the pro-S oxygen of unlabeled DPPE. **Ab** contains ^{18}O in a P—^{18}O—Si *b*ridge, while **Bn** contains a *n*onbridging ^{18}O, —P=^{18}O. On the other hand, the opposite isomer Y gives the four species **A**, **An**, **B**, and **Bb**. The isomers X and Y are therefore expected to show the ^{31}P NMR patterns shown in Scheme II. It should be noted that all formulas in Scheme II describe only *relative* configurations at phosphorus.

The observed ^{31}P NMR spectra (at 81.0 MHz) of the silylated products of two diastereomers of [$^{18}O_1$]DPPE, **8a** and **8b**, are shown in Figures 14A and 14B, respectively, which show a 1.45-Hz (0.018 ppm) and a 3.1-Hz (0.038 ppm) shift for bridge and nonbridge ^{18}O, respectively. The spectral analysis shows that **8a** contains 55% ^{18}O, 72% isomer X, and 28% isomer Y, whereas **8b** contains 63% ^{18}O, 17% isomer X, and 83% isomer Y. The [$^{18}O_1$]DPPE, **8a** and **8b** were methylated with CH_3I to give [$^{18}O_1$]dipalmitoylphosphatidylcholine (DPPC) (**9a** and **9b**, respectively) without affecting the configuration at phosphorus. Reaction of **9a** and **9b**

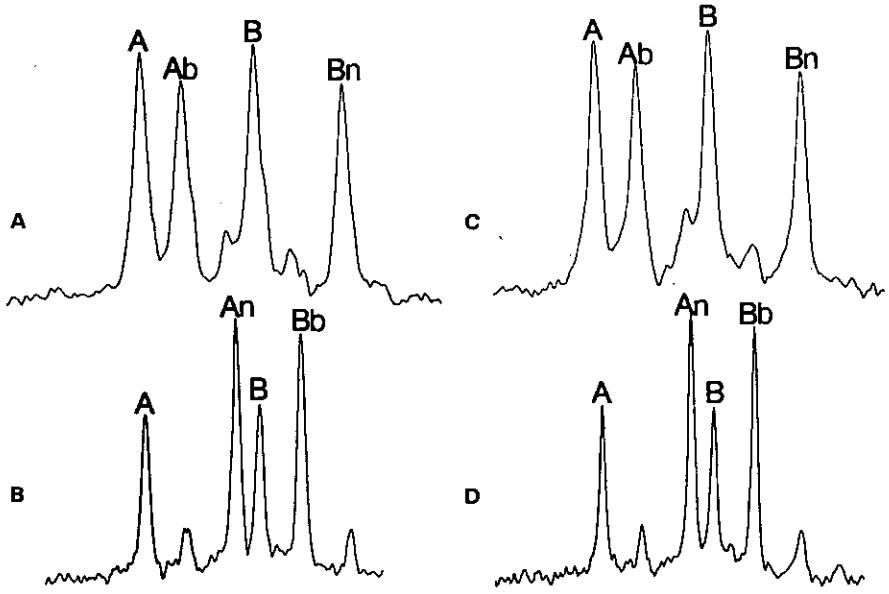
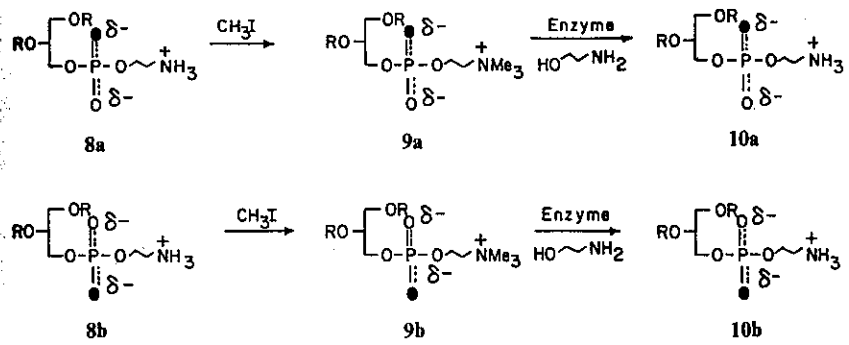


Figure 14. The ^{31}P NMR spectra (at 81.0 MHz) of the silylated products of DPPE from **8a** (*A*: 36 μmol, 500 scans line broadening 0.2 Hz), **8b** (*B*: 24 μmol, 110 scans, line broadening 0.1 Hz), **10a** (*C*: 10 μmol, 2000 scans, line broadening 0.2 Hz), and **10b** (*D*: 20 μmol, 3500 scans, line broadening 0.1 Hz). Spectral parameters: Spectral width 500 Hz, 16 K data points, ^1H decoupling, 60° pulse, repetition time 16 sec. (From Bruzik and Tsai, 1982.)



Scheme III

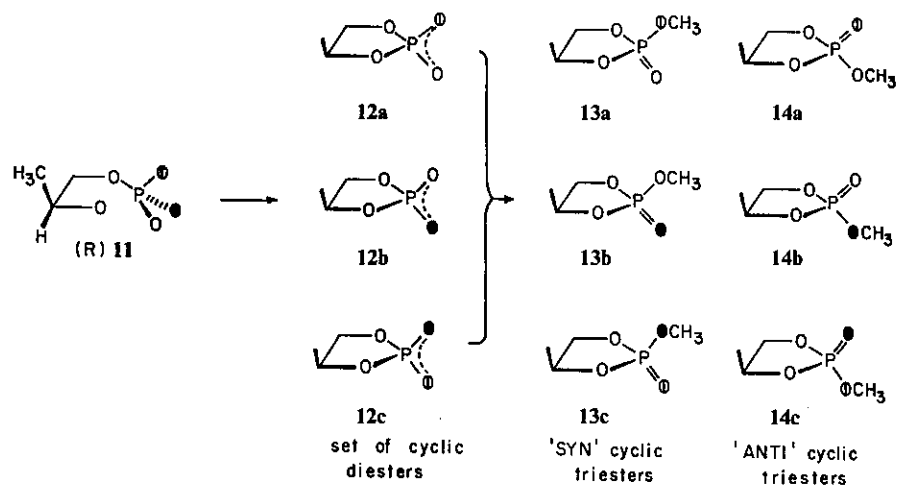
separately with ethanolamine in H_2O/ether catalyzed by phospholipase D gave DPPE **10a** and **10b**, respectively. The ^{31}P NMR spectra of the silylated products of **10a** and **10b** are shown in Figures 14C and 14D, respectively. The spectral analysis indicates that the transphosphatidylation proceeds with complete retention of configuration and without detectable oxygen exchange, as shown in Scheme III.

Other examples of **3** are [α-$^{18}O_1$]dADP, [α-$^{18}O_1$]ADP, and 3′,5′-cyclic[$^{18}O_1$]dAMP. Examples of **5** include: [$^{18}O_1$]AMPS, [β-$^{18}O_1$]ADPβS, and others. The configurational analysis of these chirally labeled biophosphates is summarized in Table 3. The stereochemical course of numerous enzyme-catalyzed reactions have been solved by use of these labeled compounds, most of which have been covered in other reviews (Knowles, 1980; Frey, 1982; Frey *et al.*, 1982; Tsai, 1982; Eckstein, *et al.*, 1982).

5.4. Combination of $^{31}P(^{17}O)$ and $^{31}P(^{18}O)$ NMR

Knowles and co-workers (Abbott *et al.*, 1978) first developed a mass spectral analysis to determine the configuration of a chiral [^{16}O, ^{17}O, ^{18}O]phosphomonoester. As shown in Scheme IV, [1(R)-^{16}O, ^{17}O, ^{18}O]phospho-(S)-propane-1,2-diol (**11**) is first "cyclized" to the 1,2-cyclic phosphate which consists of an equimolar mixture of three isotopically different species **12a**, **12b**, and **12c**. Methylation of the cyclic phosphate **12** occurs on either of the exocyclic oxygens and gives two sets ("syn" and "anti") of diastereomeric phosphotriesters **13** and **14**, respectively. **13** and **14** are then separated chromatographically and analyzed by metastable ion mass spectrometry.

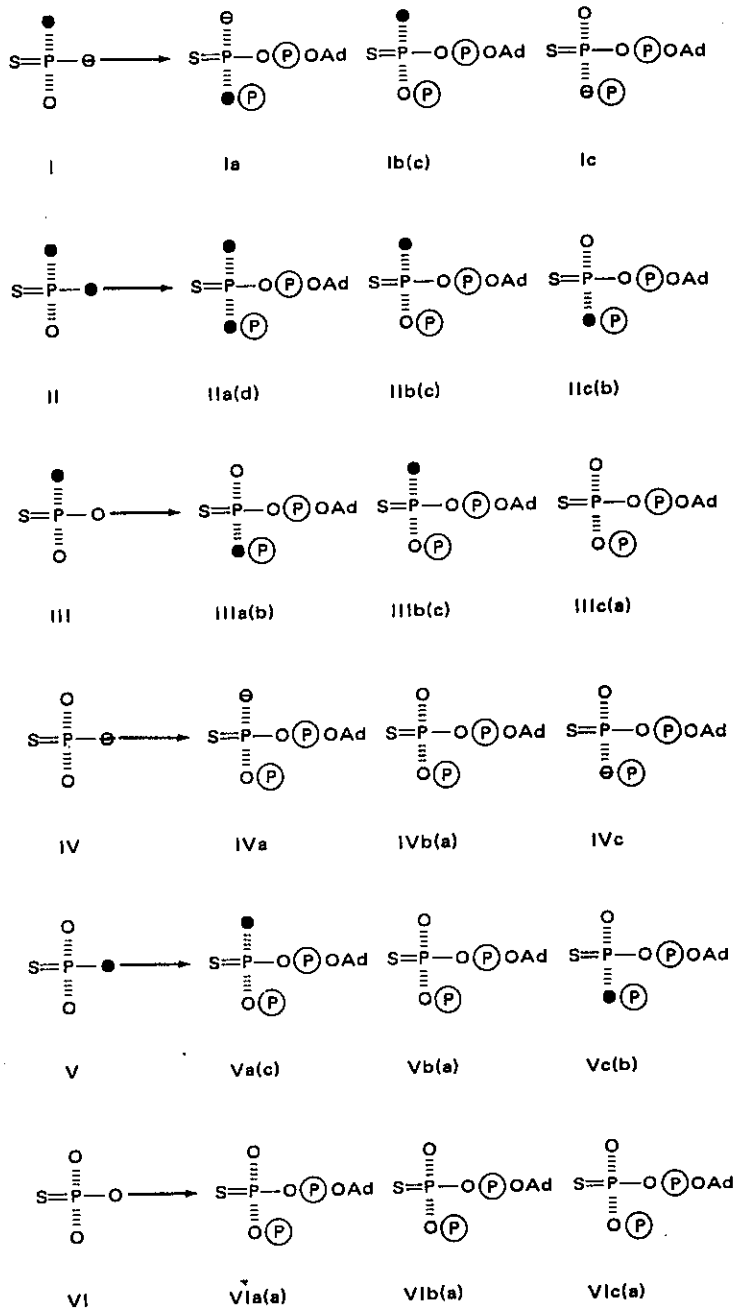
As the $^{31}P(^{18}O)$ and $^{31}P(^{17}O)$ NMR techniques became available later, it was obvious to a number of researchers that the two sets of triesters **13**



Scheme IV

and **14** can be distinguished by ^{31}P NMR. Each set of isomers consists of two species bearing an ^{17}O and a third species which contains only ^{16}O and ^{18}O. Since ^{17}O is expected to "quench" the ^{31}P NMR signal, only the species without ^{17}O (**13b** and **14b**) will show sharp ^{31}P NMR signals. In the "syn" isomer **13b**, the ^{18}O is nonbridging ($P{=}^{18}O$), thus causes a larger isotope shift ($S = 0.043$ ppm). In the "anti" isomer **14b**, the ^{18}O is located at the P—O—C bridging position and thus causes a small isotope shift ($S = 0.018$ ppm). An opposite pattern should be observed for the opposite isomer of **11**. In addition, the two diastereomers **13** and **14** show different chemical shifts, which allows a direct analysis of the mixture **13** and **14** without chromatographic separation. In reporting the ^{31}P NMR analysis of the configuration of **11** (Buchwald and Knowles, 1980), the authors described that the NMR method is "simpler both conceptually and practically."

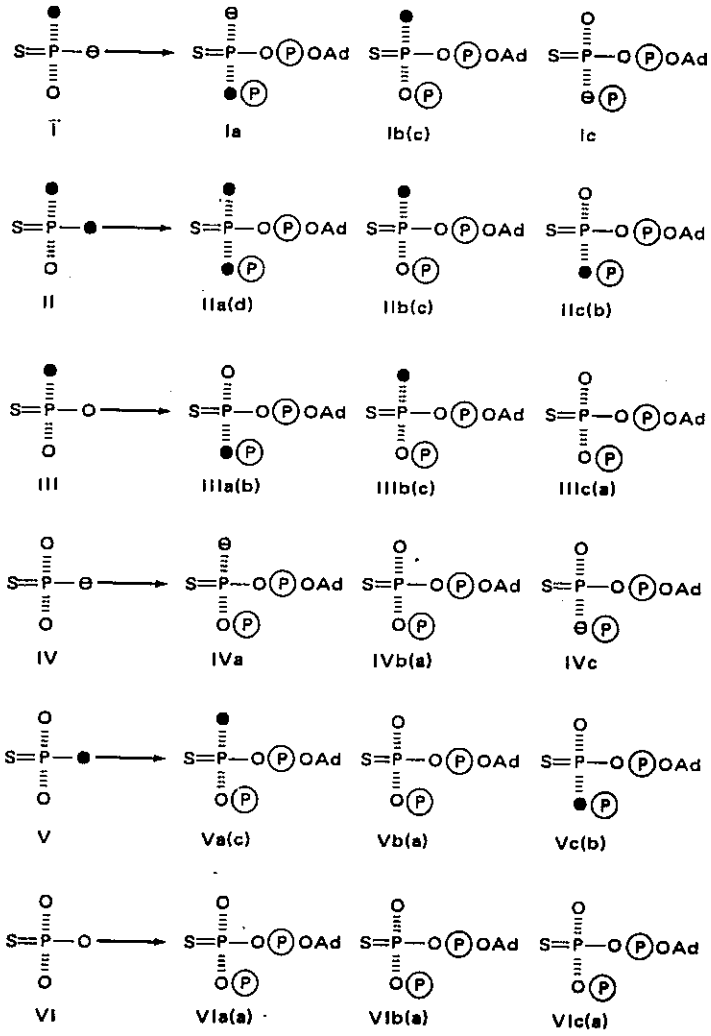
As summarized in Table 3, the configuration of more than 5 different [^{16}O, ^{17}O, ^{18}O] phosphomonoesters (compound **6**) and [^{16}O, ^{17}O, ^{18}O] thiophosphate (Psi) (compound **7**) have been determined by the combined use of $^{31}P(^{17}O)$ and $^{31}P(^{18}O)$ NMR independently, and perhaps also simultaneously, by a number of research groups. In each case there are three main steps: (1) substitution of one of the three oxygen isotopes with a procedure of known stereochemistry; (2) derivatization at one of the two diastereotopic oxygens; and (3) direct analysis by ^{31}P NMR. The stereochemical course of approximately 10–20 different enzyme-catalyzed reactions has been elucidated by such an approach during 1980–1981.



Scheme V

ERRATA

They are two errors in Scheme V on page 165, involving one of the oxygens in structures I and IV. A corrected version of Scheme V appears below:



Scheme V

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The above description has assumed a "pure" [^{16}O, ^{17}O, ^{18}O]-phosphomonoester or a pure [^{16}O, ^{17}O, ^{18}O]Psi. In reality, it is impossible to have such a 100% chirally labeled compound since the ^{18}O enrichment may not be 100%, and the ^{17}O isotope used ($H_2{}^{17}O$) always contains ^{16}O and ^{18}O. A chiral Psi sample actually contains 6 isotopic species (two of them are identical), as shown in the left column of Scheme V. Upon "substitution" and "derivatization" by the combined action of a number of enzymes (Tsai, 1982; Webb, 1982), each Psi species gives three ATPβS (B) (the Rp isomer) species, as shown in Scheme V. Fortunately, a careful examination reveals that there are only four different non-^{17}O-containing species **a**, **b**, **c**, and **d** and that all the non-chirally-labeled Psi species contribute *equally* to species **b** and **c**. Only the [^{16}O, ^{17}O, ^{18}O] Psi species gives specifically to **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 but not configuration.

Figure 15 shows the P_β signal of the ATPβS(B) obtained from PS $^{18}O_3{}^{3-}$ and two chiral Psi 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 4, where the F value is defined as the ratio b/c, the "purity" refers to the percentage of chirally labeled Psi species, and the "chirality" refers to the optical purity of chirally labeled Psi species. Buchwald *et al.* (1982) have proposed the "quality index", Q, as a measure of the quality of a specific experiment.

5.5. Use of ^{17}O NMR in Configurational Analysis

The ^{17}O NMR could be a direct method to distinguish the two diastereotopic oxygens in compounds **2** and **4**, or to distinguish a bridging and a nonbridging oxygen in the derivatives of **2** and **4**. However, such an approach is limited by the relatively low resolution of ^{17}O NMR. By applying a high temperature and decoupling the ^{31}P nucleus, Coderre *et al.* (1981a) have succeeded in resolving the signals of the axial and the equatorial ^{17}O of cyclic [^{17}O, ^{18}O]dAMP, as shown in Figure 16. The axial ^{17}O shows a downfield chemical shift (92.8 ppm) and a larger $J_{^{31}P-^{17}O}$ (130 Hz) than the equatorial ^{17}O (91.2 ppm, 102 Hz).

It should not be assumed, before more examples are available, that the axial ^{17}O always resonates at a lower field and gives a larger $J_{^{31}P-^{17}O}$. Contrary to the case of ^{17}O, Stec and co-workers (Stec *et al.*, 1972; 1976; Stec, 1974) have found that in the 1,3,2-dioxaphosphorinane ring system as well as in 3′,5′-cyclic nucleotide anilidates, the coupling constant $J_{^{31}P-X}$ is always larger when X (^1H, ^{13}C, ^{15}N, ^{19}F, or ^{77}Se) is in the equatorial

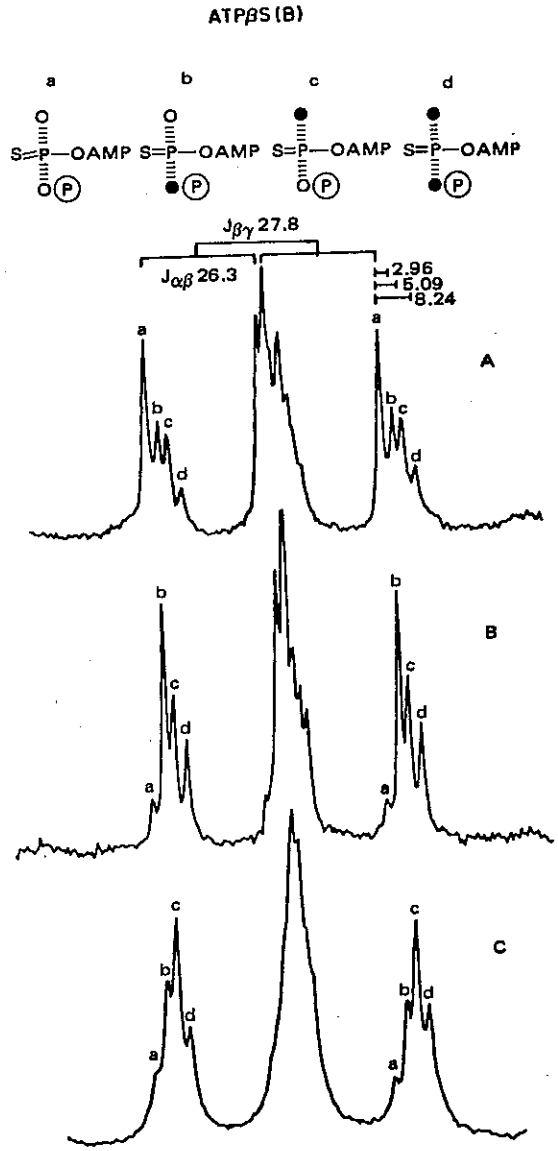

Figure 15. The P_β signals of the ^{31}P NMR spectra of the ATPβS(B) obtained from [$^{18}O_3$]P_{si}(A) and from the two chiral P_{si}(B, C). The sample (30 μmol) was dissolved in 2.5 mL of D_2O containing 10 mM EDTA and the spectra were recorded at 145.7 MHz at ambient temperature. The coupling constants and isotope shifts are expressed in hertz. The chemical shift of the P_β signal is −29.8 ppm from H_3PO_4. (From Tsai, 1980.)

TABLE 4
^{31}P NMR Analysis of the ATPβS(B) Derived from Chiral Thiophosphates

Psi samples	Intensity[a] (%) a	b	c	d	F value	Config.
A	41.3± 1.2	24.6± 0.1	22.1± 0.0	11.8± 1.2	1.11	
B	8.8± 0.5	42.8± 0.6	28.1± 0.5	20.3± 0.5	1.52	S
C	12.2± 0.5	26.5± 1.6	38.8± 0.1	22.4± 2.0	0.68	R
Calcd[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_α of 47.5% purity and 90% chirality expected based on isotopic enrichments.

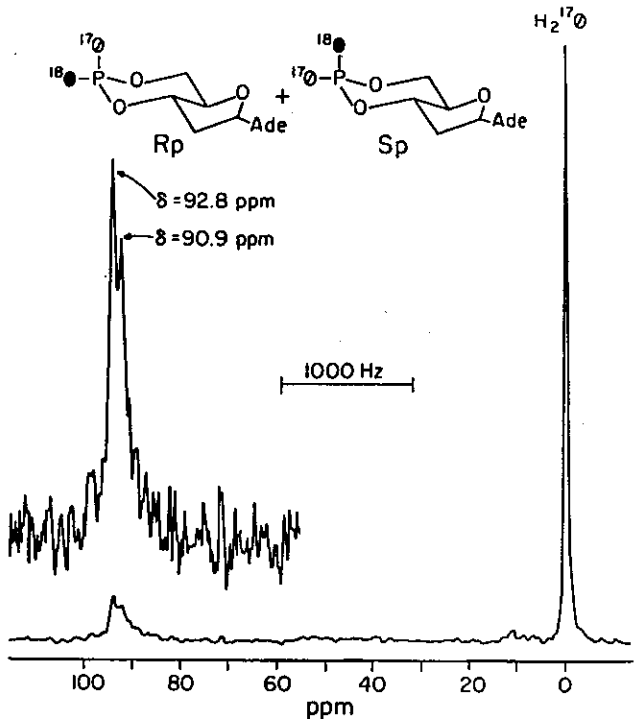

Figure 16. The ^{17}O NMR spectra (at 36.6 MHz, 95°C) of cyclic [^{17}O, ^{18}O]dAMP, racemic mixture of the (R_p)-diastereomer (axial ^{17}O) and the (S_p)-diastereomer (equatorial ^{17}O), ^{31}P decoupled. (From Coderre *et al.*, 1981a.)

position. In a series of 2-alkoxytetrahydropyrans, McKelvey (1981) have found that the ^{17}O in the axial alkoxyl group always resonates at a higher magnetic field than that of the equatorial alkoxyl group.

6. OTHER BIOCHEMICAL APPLICATIONS

6.1. Location and Quantitation of Oxygen or Phosphate

The ^{31}P(^{18}O) NMR technique has become a useful and convenient way to locate and quantitate the labeled oxygen. The NMR method is more informative than the commonly used mass spectral technique, but has some small drawbacks in the accuracy in quantitation, the quantity of sample, and the instrumental time required for analysis.

The oxygen exchange kinetics and use of ^{31}P(^{18}O) NMR in such problems have been reviewed elsewhere (Hackney *et al.*, 1980; Cohn, 1982; Tsai, 1982). In addition, the ^{31}P(^{18}O) NMR technique has also been used in determining the position of the bond cleavage catalyzed by several enzymes (Jordan *et al.*, 1979; Jordan *et al.*, 1981; Risley *et al.*, 1982; Salamone *et al.*, 1982) and the positional isotope exchange of ^{18}O (between bridging and nonbridging positions of ATP) catalyzed by pyruvate kinase (Lowe and Sproat, 1978b) and by carbamoyl-P synthetase (Raushel and Villafranca, 1980). Recently ^{17}O NMR has been used in finding that the hydrolysis of phosphomonoesters catalyzed by the Mn^{2+}-containing acid phosphatase involves the incorporation of oxygen from water into the product P (Kawabe *et al.*, 1981).

Such applications are straightforward since in all cases the analysis involves small molecules. A more difficult problem would be to locate the position of an oxygen isotope in macromolecular systems. For example, 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 free Glc-1-P. As shown in Figure 9D, the ^{31}P NMR signal of bound [$^{17}O_4$] Glc-1-P has not sharpened too much and thus unequivocally assigns the upper field signal to the bound Glc-1-P (W. J. Ray, G. I. Rhyu and J. L. Markley, private communication). In this example the ^{17}O-labeled phosphate is used as a "marker." Although under certain circumstances the ^{31}P(^{17}O) NMR signal may be indistinguishable from the ^{31}P(^{16}O) signal (Figure 8C), chances for this to occur in E · S complexes are very small. Thus the ^{31}P(^{17}O) NMR method provides a way to locate the labeled oxygen in E · S complexes (Figures 9–11) and in other macromolecular systems.

6.2. Metal–Nucleotide Interactions

6.2.1. Line Broadening Effect in ^{17}O NMR

The most important and ubiquitous metal ion in enzyme-catalyzed phosphoryl transfer reactions is Mg^{2+}. There is so far not a direct and unambiguous method to observe the binding of diamagnetic metal ions with nucleotides even in nonenzymatic systems. The previously used ^{31}P chemical shift method has been shown to be inadequate to define the coordination pattern of the MgATP complex (Jaffe and Cohn, 1978). The center of controversy is whether the α-phosphate of ATP is involved in binding. The use of ^{17}O NMR to study the binding of diamagnetic metal ions and nucleotides was first proposed by Tsai *et al.* (1980), who observed that Mg^{2+} causes the ^{17}O NMR signal of [γ-$^{17}O_3$]ATP to broaden.

The effect of diamagnetic metal ions on the ^{17}O NMR of nucleotides upon binding has later been unequivocally established by substitution-inert Co^{3+} complexes of ^{17}O-labeled ADP and ATP (Huang and Tsai, 1982).

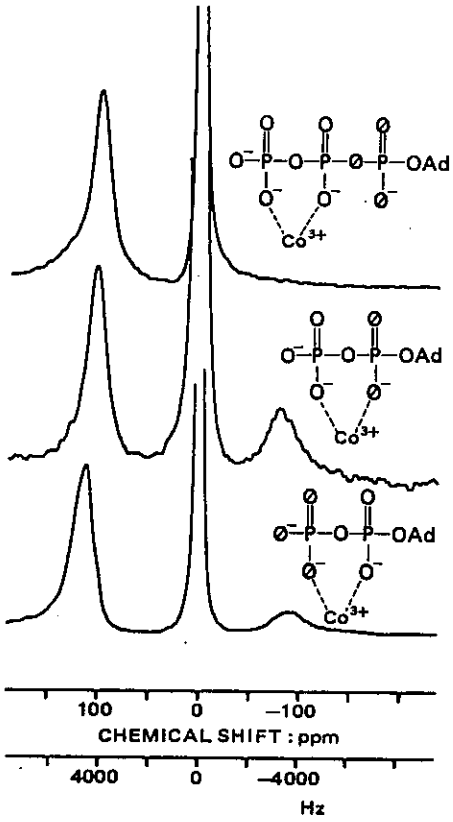

Figure 17. The ^{17}O NMR spectra of some Co^{3+} complexes at 40.67 MHz: the β,γ-bidentate of α-^{17}O-ATP, the α,β-bidentate of α-^{17}O-ADP, and the α,β-bidentate of β-^{17}O-ADP. Sample and spectrometer conditions are the same as that of Figure 19. (From Huang and Tsai, 1982.)

Since the binding patterns of CoADP and CoATP have been established (Cornelius *et al.*, 1977; Cleland, 1982), these compounds are excellent models to show the effect of binding. As shown in Figure 17A, coordination of Co^{3+} at the P_β and P_γ of ATP has little effect on the ^{17}O NMR signal of α-^{17}O-ATP. On the other hand, Figure 17B and 17C show that a direct coordination results in a downfield peak (shifted 1–9 ppm downfield, slightly broadened) and an upfield peak (shifted 180–200 ppm upfield, significantly broadened). By use of the separate diastereomers (Δ isomer and Λ isomer) of $Co(NH_3)_4$ ADP, where ADP is chirally labeled with ^{17}O at P_α, it has been unambiguously established that the upfield signal is due to $O{=}P{-}{}^{17}O^- \cdots Co^{3+}$ whereas the downfield signal is due to $^{17}O{=}P{-}O^- \cdots Co^{3+}$ (Sammons *et al.*, 1983), as shown in Figure 18.

Figure 19 shows the ^{17}O NMR spectra of ^{17}O-labeled ADP and ATP, together with the corresponding Mg^{2+} complexes. In all cases the sharper signal is due to the solvent (D_2O) and the broader signal due to nonbridging ^{17}O of nucleotides. No signal in the upfield region has been detected for Mg^{2+} complexes, even though the exchange rate of MgATP (2×10^4

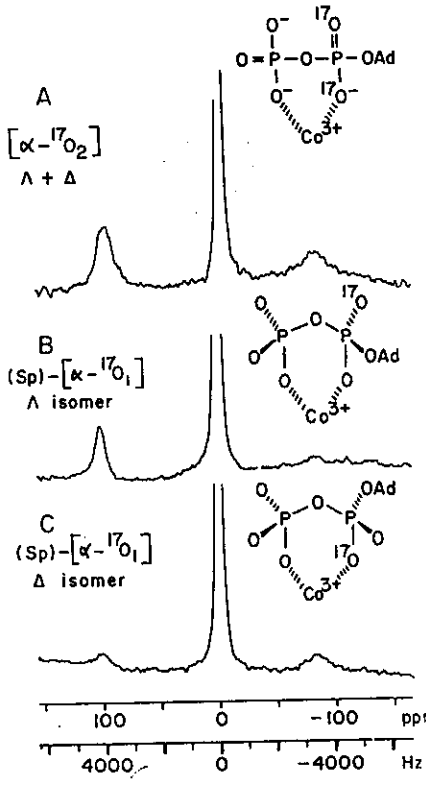

Figure 18. The ^{17}O NMR spectra (40.65 MHz) of ^{17}O-labeled $Co(NH_3)_4$ ADP (α,β-bidentate) showing the unequivocal assignments of the downfield peak to $P{=}{}^{17}O$ and the upfield peak to $P{-}{}^{17}O^- \cdots Co^{3+}$ (*A*) from [α-$^{17}O_2$]ADP, Λ isomer + Δ isomer; (*B*) from (S_p)-[α-$^{17}O_1$]ADP, Λ isomer; (*C*) from (S_p)-[α-$^{17}O_1$]ADP, Δ isomer. Sample conditions: (*A*) 12 m*M*, D_2O, pD 4.0; (*B*) and (*C*) 7 m*M*, 10% D_2O, pH 5.5. NMR conditions: spectral width 20,000 Hz, acquisition time 0.102 sec, 4 K data points, DE = 12 μsec. Line broadening 50 Hz, ^1H-decoupled, probe temperature 50°C. The Λ and Δ designations are those defined by Cornelius and Cleland (1978). 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. (From Sammons *et al.*, 1983.)

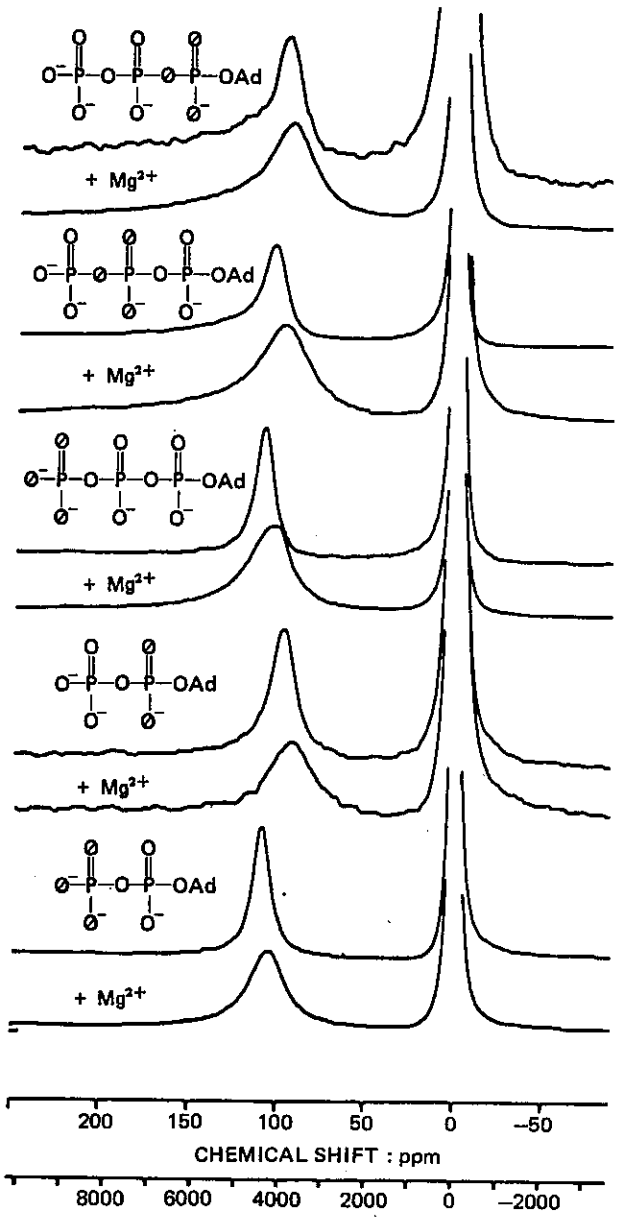

Figure 19. The ^{17}O NMR at 40.67 MHz showing the effect of Mg^{2+} binding with α-^{17}O-ATP, β-^{17}O-ATP, γ-^{17}O-ATP, α-^{17}O-ADP, and β-^{17}O-ADP. Sample conditions: 25 m*M* in D_2O, pD 7.8. Spectrometer (Bruker CXP-300) conditions: DE = 10 μsec, 4 K data points spectral width 50 kHz, unlocked, nonspinning, line broadening 100 Hz. Chemical shifts are referenced to external $H_2{}^{17}O$. The sharper signal is due to D_2O. (From Huang and Tsai, 1982.)

sec^{-1} from Bryant, 1972; 1.2×10^3 sec^{-1} from Diebler *et al.*, 1960) could be on the intermediate or slow-exchange side on the basis of the separation between the two signals in Co^{3+} complexes (ca. 8×10^3 Hz).

It remains to be unequivocally established whether the observed signal of Mg^{2+} complexes is an average of $^{17}O{=}P{-}O^- \cdots Mg^{2+}$ and $O{=}P{-}^{17}O^- \cdots Mg^{2+}$ or represents only one of them (the other signal might be too broad to be detected). However, there is enough evidence to suggest that it is most likely an average of two signals, and that the "line-broadening effect" of Mg^{2+} binding shown in Figure 19 is of the quadrupolar nature, as discussed by Huang and Tsai (1982) and by Sammons *et al.* (1983). A titration curve of ΔO vs. the ratio $[Mg^{2+}]/[ATP]$ (Figure 20) shows that the ΔO increases approximately linearly up to $[Mg^{2+}]/[ATP] = 1.0$. The line-broadening effect of MgADP does not show an appreciable dependence on the magnetic field from 1.88 to 8.45 T.

Huang and Tsai (1982) have defined the *R* value as a measure of the "line-broadening effect" in ^{17}O NMR:

$$R = \frac{\Delta O_b - \Delta O_f}{\Delta O_f}$$

where ΔO_f and ΔO_b represent the ^{17}O line widths of the free and bound nucleotides, respectively, which have been corrected for the artificially applied exponential multiplication and the broadening due to ^{31}P–^{17}O spin–

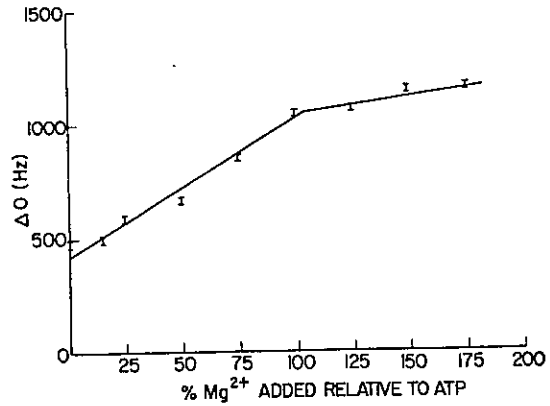

Figure 20. The ^{17}O linewidths of $[\gamma$-$^{17}O_3]$ATP as a function of $Mg(NO_3)_2$ concentration at low field (1.88T, 10.85 MHz). Sample condition: 25 m*M* in D_2O, pD 7.6. Spectrometer (Varian FT-80) condition: 1 K data points, sweep width 8 kHz, DE = 200 μsec, deuterium lock, recycle time 120 msec, line broadening 10 Hz. The ΔO in the plot have not been corrected for exponential multiplication and $J_{p\text{-}o}$. (From Huang and Tsai, 1982.)

TABLE 5
Summary of ^{17}O NMR Results of Mg^{2+} and Co^{3+} Complexes[a]

Nucleotides	R values[b]		Chemical shifts (ppm)[d]			
	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 Huang and Tsai (1982). Estimated errors in R values = ± 20%.
[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.

spin coupling. Table 5 summarizes the R values and chemical shifts of all Mg^{2+} and Co^{3+} complexes. The results suggest that Mg^{2+} interacts approximately equally with the P_α and P_β of ADP and the P_β and P_γ of ATP. The Mg^{2+} ion also interacts with the P_α of ATP, but to a somewhat smaller extent. These results establish the "macroscopic" structure of MgADP and MgATP but have not defined the "microscopic" structures.

6.2.2. Line Sharpening Effect in $^{31}P(^{17}O)$ NMR

Equation (8) predicts that if the ^{17}O NMR signal is "broadened" by Mg^{2+}, the $^{31}P(^{17}O)$ NMR signal should be "sharpened" if $J_{^{31}P-^{17}O}$ is constant. The $J_{^{31}P-^{17}O}$ of Mg^{2+} complexes is difficult to be directly measured. However, Figure 21 shows that the ^{18}O isotope shift $S_{^{31}P-^{18}O}$ for the P_α of MgADP is almost equal to that of free ADP, or the average of the S values of $^{18}O{=}P{-}O^- \cdots Co^{3+}$ and $O{=}P{-}^{18}O^- \cdots Co^{3+}$ in CoADP. It is therefore reasonable to expect that in the $^{31}P(^{17}O)$ NMR of MgADP, where the observed signal is an average of $^{17}O{=}P{-}O^- \cdots Mg^{2+}$ and $O{=}P{-}^{17}O^- \cdots Mg^{2+}$, the $J_{^{31}P-^{17}O}$ value is approximately equal to that of free ADP. Thus, a "line-sharpening effect" in $^{31}P(^{17}O)$ NMR can be expected. Figure 22 shows the $^{31}P(^{17}O)$ NMR spectra of α-^{17}O-ADP and its Mg^{2+} complex, in which a decrease in the apparent ΔP is observed for the Mg^{2+} complex. Such a "line-sharpening effect" in $^{31}P(^{17}O)$ NMR due to Mg^{2+} binding has been observed for α- and β-^{17}O-ADP and α-, β-, and γ-^{17}O-ATP, but a quantitative analysis is difficult due to the low sensitivity of the "broad" signal and the complication by multiple labeling. It has been suggested (Tsai *et al.*, 1980) that such a metal ion perturbation in $^{31}P(^{17}O)$ NMR may be useful in

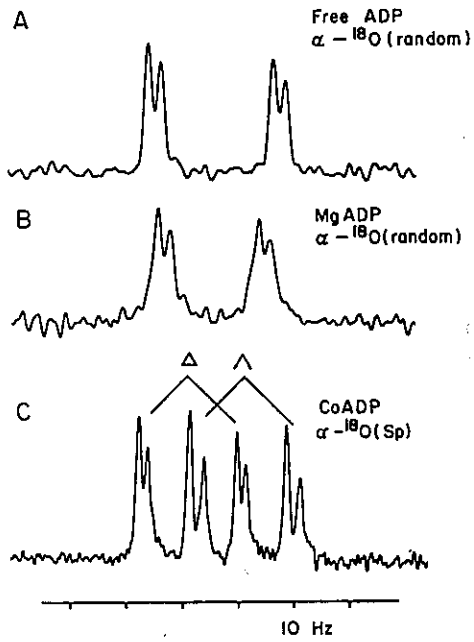

Figure 21. The P_α signals (^{31}P NMR at 81.0 MHz) of free ADP (*A*), MgADP (*B*), and $Co(NH_3)_4ADP$ (*C*), showing the ^{18}O isotope shift effect. The S values measured are: 0.026 ppm from free ADP, 0.024 ppm for MgADP, and 0.020 and 0.032 ppm for the Δ and Λ isomers of CoADP, respectively. (From Sammons and Frey, 1982; and Sammons *et al.*, 1983.)

enzyme · substrate complexes. On the basis of the discussion in this section and in Section 4.2, such an appplication would be very difficult if not impossible, unless the sensitivity of the method can be greatly improved. Attempts to observe the effect of Mg^{2+} on the $^{31}P(^{17}O)$ NMR signal of the ADP · arginine kinase complex have not been successful so far, as shown in Figure 12 (D and E).

6.3. Motional Problems of Phospholipids

The structure and physical property of phospholipids and the nature of protein–lipid interactions have been subjects of active research by use of various physical techniques in the past two decades. However, very few reports have been directed to the role of the phosphate group. The importance of the phosphate head group in biological functions has received more attention recently (Yeagle, 1978). On the basis of ^{13}C NMR results, Murari and Baumann (1981) have concluded that the aggregation of phospholipids involves interactions between polar head groups. The detailed conformation of the phosphate head group at various phases, however, remains to be defined by some additional parameters such as ^{17}O NMR as pointed out by Skarjune and Oldfield (1979).

Bruzik and Tsai (1982) have developed a synthetic procedure for phospholipids stereospecifically labeled with an oxygen isotope (^{17}O or ^{18}O) at

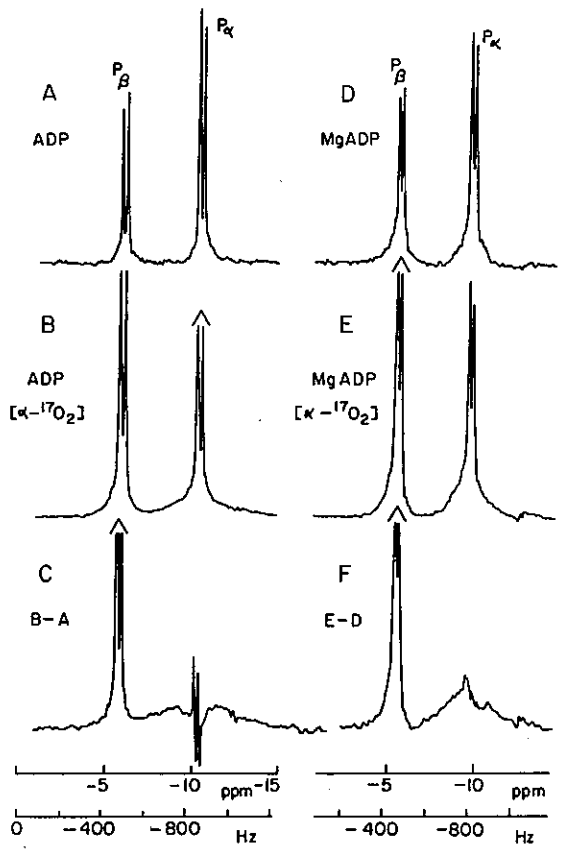

Figure 22. "Line sharpening effect" of Mg^{2+} binding in $^{31}P(^{17}O)$ NMR. (*A*) Free ADP; (*B*) free [α-$^{17}O_2$]ADP; (*C*) 100% ^{17}O-labeled ADP obtained by subtracting (*A*) from (*B*), (*D*) MgADP; (*E*) Mg[α-$^{17}O_2$]ADP; (*F*) 100% ^{17}O-labeled MgADP obtained by subtracting (*D*) from (*E*). Sample condition: 50 m*M* (*A*, *D*) and 25 m*M* (*B*, *E*) in D_2O, pD 7.9. NMR parameters: spectral frequency 81.0 MHz, spectral width 5,000 Hz, acquisition time 0.82 sec, repetition time 4 sec, line broadening 6 Hz, number of scans 9000 (*B*, *E*) 1800 (*A*), 600 (*D*), temp. 30°C. (From Sammons *et al.*, 1983.)

phosphorus. Such ^{17}O-labeled phospholipids are ideal for determining the conformation of the phosphate head group in various biological systems. However, the ^{17}O NMR signal of liquid crystalline phospholipids, which is expected to have a linewidth of several KHz and a quadrupolar splitting of hundreds of KHz, has not been detectable by most available NMR spectrometers. On the other hand, the ^{17}O NMR signal of [$^{17}O_1$] dipalmitoylphosphatidylcholine (DPPC) in solution is detectable, as shown in Figure 23. The DPPC is known to be "nonaggregated" in methanol but "aggregated"

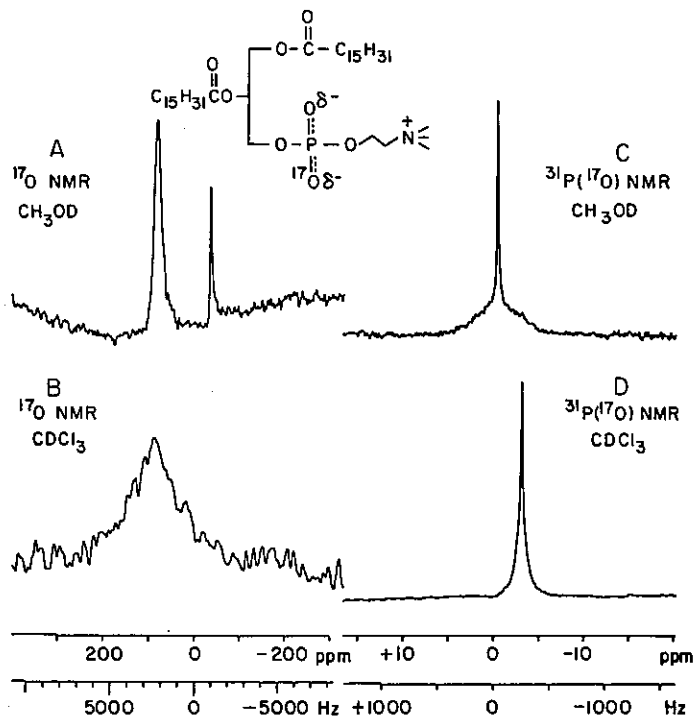

Figure 23. Effect of aggregation on the ^{17}O NMR (27.1 MHz) and $^{31}P(^{17}O)$ NMR (81.0 MHz) of [$^{17}O_1$]DPPC (100 mg in 1.5 ml solvent). (*A*) ^{17}O NMR in CH_3OD; (*B*) ^{17}O NMR in $CDCl_3$; (*C*) ^{31}P NMR in CH_3OD; (*D*) ^{31}P NMR in $CDCl_3$. Parameters for ^{17}O NMR: spectral width 20,000 Hz, acquisition time 0.051 sec, DE 5 μsec, line broadening 50 Hz, 16,000 scans (*A*), and line broadening 200 Hz, 40,000 scans (*B*); parameters for ^{31}P NMR: spectral width 3000 Hz, acquisition time 0.34 sec, acquisition delay 2 sec, line broadening 5 Hz. All spectra were taken at 33°C, with ^1H decoupling.

(forming reverse micelles) in chloroform (Murari and Baumann, 1981). As shown in Figure 23, aggregation of [$^{17}O_1$]DPPC causes a significant broadening in ^{17}O NMR and sharpening in $^{31}P(^{17}O)$ NMR. Such results suggest that the motion of the phosphate group is restricted due to molecular aggregation.

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NOTATION

P_i	Inorganic phosphate
P_{si}	Inorganic thiophosphate
PEP	Phosphoenolpyruvate
EDTA	Ethylenediaminetetraacetate
PP_i	Inorganic pyrophosphate
O	Oxygen-16
Ø	Oxygen-17
O	Oxygen-18
AMP	Adenosine 5′-monophosphate
ADP	Adenosine 5′-diphosphate
ATP	Adenosine 5′-triphosphate
AMPS	Adenosine 5′-thiophosphate
ADPαS	Adenosine 5′-(1-thiodiphosphate)
ATPαS	Adenosine 5′-(1-thiotriphosphate)
ADPβS	Adenosine 5′-(2-thiodiphosphate)
ATPβS	Adenosine 5′-(2-thiotriphosphate)
ATPγS	Adenosine 5′-(3-thiotriphosphate)
S/N	Signal/noise ratio
DE	Preacquisition delay
DPPE	Dipalmitoylphosphatidylethanolamine
DPPC	Dipalmitoylphosphatidylcholine
TpNP	Thymidine 3′-[(4-nitrophenyl)phosphate]
U > PS	Uridine 2′,3′-cyclic phosphorothioate
Glc-1-P	Glucose-1-phosphate

Diastereomers A and B are designated on the basis of their enzymatic activity (Eckstein and Goody, 1976)

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