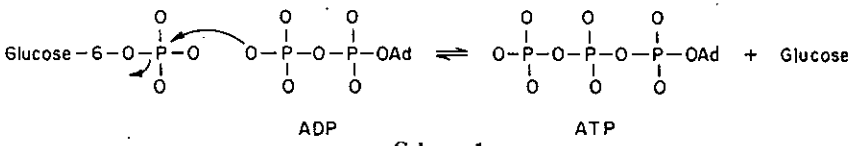
CHAPTER 6

Use of Chiral Thiophosphates and the Stereochemistry of Enzymatic Phosphoryl Transfer

Ming-Daw Tsai
Department of Chemistry
The Ohio State University
Columbus, Ohio

I. Stereochemical Problems Studied with Chiral Thiophosphates

The use of thiophosphate analogs of biophosphates in studying stereochemical problems was first introduced by Eckstein (1975) and subsequently widely applied to various problems. To illustrate the use of chiral thiophosphates in stereochemistry, consider the phosphoryl transfer reaction catalyzed by hexokinase (Scheme 1).[1] Three types of problems can be studied by



Scheme 1

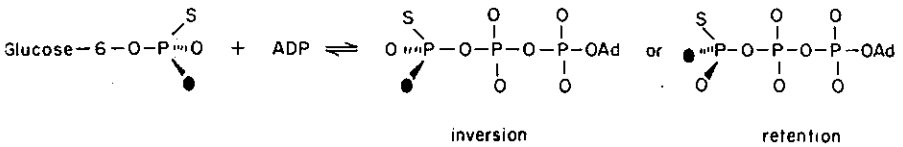
[1] To avoid confusion, the charges and double bonds on the oxygen and sulfur of phosphoryl group or thiophosphoryl groups are omitted throughout the text. The *R* and *S* configurational designations are made according to the Cahn–Ingold–Prelog rule (Cahn *et al.*, 1966), with the assumption that nonbridging oxygens and sulfurs are all singly bonded to phosphorus. According to the priority rules, the atomic number preferences should be applied to exhaustion before the atomic weight preferences are applied.

PHOSPHORUS-31 NMR
Principles and Applications

ISBN 0-12-391750-2

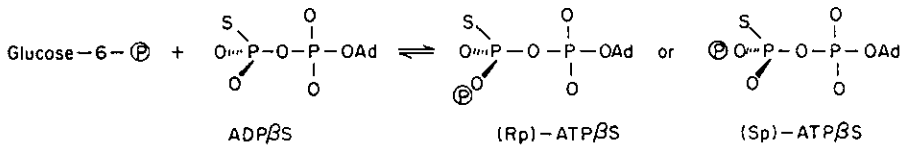
use of chiral thiophosphates:

1. Stereochemistry of the substitution at phosphorus (retention, inversion, or nonstereospecificity) can be investigated by use of chiral [^{18}O]thiophosphoryl groups (Scheme 2).



Scheme 2

2. By use of ADPβS,[2] the P_β of ADP becomes a prochiral center. In principle, enzymes can distinguish the two diastereotopic oxygens. Therefore, the phosphorylation may be stereospecific (Scheme 3).

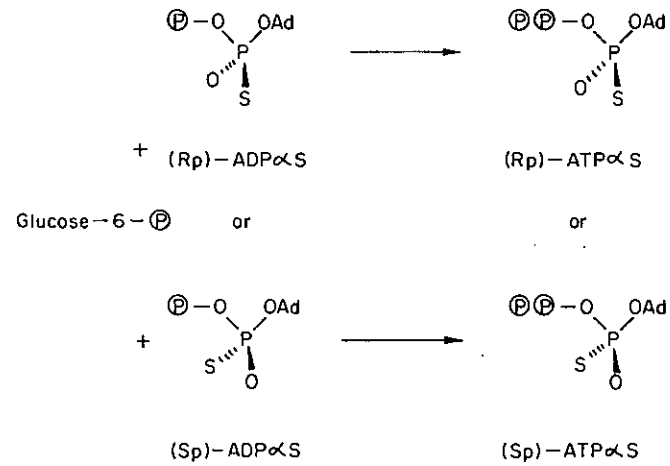


Scheme 3

3. By use of ADPαS, the P_α of ADP becomes a chiral center, and there are two possible isomers. Even though the α-phosphate of ADP is not directly involved in the reaction, the enzyme may be specific to one of the two isomers (Scheme 4).

The use of thiophosphates also has some disadvantages. They are in most cases poor substrates (0–10% of activity relative to the corresponding natural substrate) of enzymes and are relatively unstable. In addition, it may be argued that the stereochemical results obtained with thiophosphates are

[2] Abbreviations used: O, ^{16}O; ⊖, ^{17}O; ●, ^{18}O; P_i, inorganic phosphate; P_{si}, inorganic thiophosphate; AMP, adenosine 5′-monophosphate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate, AMPS, adenosine 5′-thiophosphate; ADPαS, adenosine 5′-(1-thiodiphosphate); ADPβS, adenosine 5′-(2-thiodiphosphate); ATPαS, adenosine 5′-(1-thiotriphosphate); ATPβS, adenosine 5′-(2-thiotriphosphate); ATPγS, adenosine 5′-(3-thiotriphosphate); CoA, coenzyme A; cAMPS, cyclic AMPS; GTPαS, guanosine 5′-(1-thiotriphosphate); GTPβS, guanosine 5′-(2-thiotriphosphate); $U_p(S)A$, uridinyl (3′→5′)-adenosyl-*O,O*-phosphorothioate; U > pS, uridine 2′,3′-cyclic phosphorothioate; UMPS, uridine 5′-thiophosphate; UDPαS, uridine 5′-(1-thiodiphosphate); UPTαS, uridine 5′-(1-thiotriphosphate); DPP_sE, dipalmitoylthiophosphatidylethanolamine; DPP_sC, dipalmitoylthiophosphatidylcholine; EDTA, ethylenediaminetetraacetic acid.



Scheme 4

not authentic. However, so far there has not been an example found in which the stereochemical result obtained with chiral phosphates (see Gerlt, Chapter 7) is different from that obtained with chiral thiophosphates.

The biochemical applications of thiophosphates have been covered in a number of reviews (Eckstein, 1979; Eckstein *et al.*, 1982; Frey, 1982; Frey *et al.*, 1982; Knowles, 1980; Buchwald *et al.*, 1982; Tsai, 1982). The purpose of this chapter is to illustrate the use of ^{31}P NMR[3] in these problems, which is based on three factors:

1. When a prochiral center becomes a chiral center because of sulfur substitution, the resultant two diastereomers give different ^{31}P chemical shifts.
2. Substitution of an oxygen by ^{18}O causes an isotope shift in ^{31}P NMR.
3. Substitution of an oxygen by ^{17}O causes a quadrupolar broadening on the ^{31}P-NMR signal.

Phosphorus-31 NMR is not the only method available for some problems, and this chapter does not intend to provide a comprehensive review of all chiral thiophosphate work.

[3] The ^{31}P chemical shifts described in this chapter are referenced to either 85% H_3PO_4 or 1 *M* H_3PO_4 as used in original references, which are different by only 0.3 ppm. The + sign always represents a downfield shift in this chapter, although the opposite convention has been used in most of the original references. It should be noted that the ^{31}P chemical shifts of most biophosphates are sensitive to pH, concentration, temperature, etc. In many cases the relative shifts are more important than the absolute chemical shifts.

II. ^{18}O Isotope Shifts in ^{31}P NMR

Figure 1 shows the ^{31}P-NMR spectrum of $H_3P^{17}O_4$ (40 atom % ^{17}O). The spectrum consists of a "broad" signal owing to the $^{31}P-^{17}O$ species and a "sharp" signal owing to the residual non-^{17}O-labeled species. Because ^{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 ^{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). Cohn (1982) and Tsai and Bruzik (1983) have summarized the magnitudes of $S_{^{31}P-^{18}O}$ for a number of biophosphates. Table I lists the S values for commonly used biophosphates, thiophosphates, and some model compounds. Because the data are collected from various reports that have different spectral resolution, some of the data in Table I may have an error of ± 10%, unless otherwise specified.

It seems proper to generalize the following statements concerning the

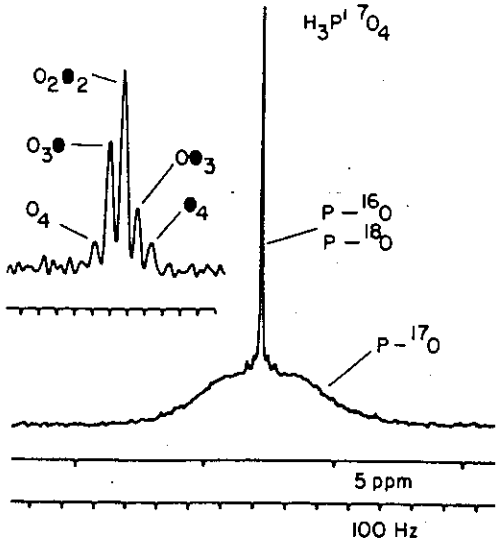
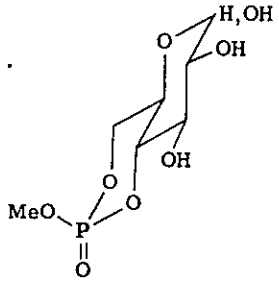


Fig. 1. ^{31}P-NMR spectrum of 50 mM $H_3P^{17}O_4$ (40 atom % ^{17}O, 27 atom % ^{18}O) in D_2O, pD 1.8 at 81.0 MHz. Spectral parameters: acquisition time 4.1 s, delay 1.0 s, spectra width 2 kHz, 70° pulse, line broadening 2.0 Hz, 1600 scans. The inset shows the expanded spectrum of the sharp peak, processed with Gaussian multiplication (LB = −2, GB = 0.2). Chemical shift 0.09 ppm downfield from 1 M H_3PO_4.

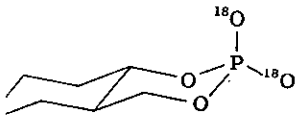
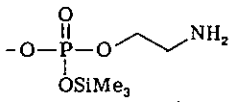
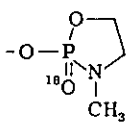
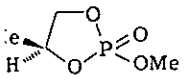
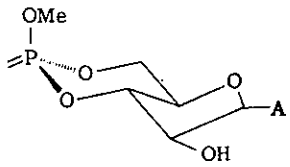
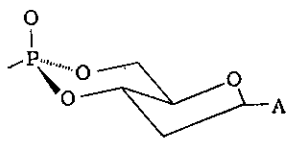
TABLE I

Magnitude of ^{18}O Isotope Shift in ^{31}P NMR[a]

Compound	Labeled position	Condition	$S_{^{31}O-^{18}O}$ (ppm)	References
$H_4P^{18}OO_3{}^+ClO_4^-$		0.2M, D_2O	0.0188 ± 0.0007	1
$KH_2P^{18}O_4$		pH 2.1	0.0201 ± 0.0007	1
$K_2HP^{18}O_4$		pH 8.6	0.0218 ± 0.0007	1
$(PhO)_3P^{18}O$		$CDCl_3$	0.0391 ± 0.0029	1
$(PhO)_2P^{18}OO$		pD 5.4	0.0293 ± 0.0007	1
$Ph_3P^{18}O$		$CDCl_3$	0.0399 ± 0.0007	1
$(CH_3{}^{18}O)_3P^{18}O$	P=O	$CDCl_3$	0.0392 ± 0.0029	1
$(CH_3O)_2PO^{18}O^-$			0.029	3
$(CH_3O)PO_2{}^{18}O^{2-}$			0.023	3
5′-AMP			0.025	5
2′-Deoxy-AMP			0.032	15
ADP	α		0.0286 ± 0.0015	1
	$\alpha\beta(P_\alpha)$		0.0210	9
	$\alpha\beta(P_\beta)$		0.0214	9
	β		0.022	4,9
ATP	$\alpha\beta(P_\alpha)$	pH > 7.6	0.0172	4
	$\alpha\beta(P_\beta)$	pH > 7.6	0.0165	4
	β	pH > 7.6	0.0281	4
	$\beta\gamma(P_\beta)$	pH > 7.6	0.0165	4
	γ	pH > 7.6	0.0220	4
$Co(NH_3)_4ADP$				
Δ isomer	α, R_p	pH 5.5	0.032	7
Λ isomer	α, R_p	pH 5.5	0.018	7
$Co(NH_3)_4ADP$				
Δ isomer	α, S_p	pH 5.5	0.020	7
Λ isomer	α, S_p	pH 5.5	0.033	7
$Co(NH_3)_4dADP$				
Δ isomer	α, R_p	pH 5.5	0.031	8
Λ isomer	α, R_p	pH 5.5	0.016	8
$Co(NH_3)_4dADP$				
Δ isomer	α, S_p	pH 5.5	0.016	8
Λ isomer	α, S_p	pH 5.5	0.030	8
MgADP	α	pD 8.0	0.026	1
Ribose-1-P	P—^{18}O		0.027	11
	P—^{18}O—C		0.017	11
				
	P=^{18}O		0.041	12,14
	P—^{18}O—CH_3		0.015	12,14

(Continue

TABLE I (*Continued*)

Compound	Labeled position	Condition	$S_{^{18}O(^{31}P)}$ (ppm)	References[a]
	P—O	H_2O/D_2O; $CDCl_3$	0.026	6
	Axial methyl ester			
	P—OMe		0.015	6
	P=O		0.040	6
$-OCOC_{15}H_{31}$	P=O		0.038	10
	P—O—Si		0.018	10
$-OCOC_{15}H_{31}$ 				
$-OCOC_{15}H_{31}$		$CDCl_3$, 30°C	0.039 ± 0.0029	2
$-OCOC_{15}H_{31}$ 				
	P=O		0.043	13
	P—O—CH_3		0.018	13
	Methyl ester			
	P=O		0.043	11,14
	P—O—CH_3		0.017	11,14
	Ethyl ester			
	P=O		0.0418	9
	P—OEt		0.0192	9
	Axial P—O		0.029	15
	Equatorial P—O		0.032	15
	Ethyl ester			
	P—OEt		0.014	15
	P=O		0.038	15
'-AMPS	α-^{18}O		0.0331 ± 0.0007	1
'-UMPS			0.032	16
'-UMPS			0.032	16
ido-U > PS			0.041	16
co-U > PS			0.041	16
DPαS	α-^{18}O		0.037	17
	$\alpha\beta$-^{18}O		0.021	17
NEtO Ⓟ O—P(S)—OAd (^{18}O)	S_p isomer	pD 6.4	0.0363 ± 0.0045	1
	S_p isomer	pD 6.4	0.0363 ± 0.0045	1

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:

1. 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 partial double-bond character have S values proportional to the bond order (Cohn and Hu, 1980; Lowe *et al.*, 1979).
2. In case of multiple substitution, the magnitude of shift is generally additive.
3. The S values of thiophosphates (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 that dictate the capability of the ^{18}O isotope-shift method in solving a specific biochemical problem. Even under optimal conditions both the integrals and the S values may still have an error of ±5%. 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 arising from the ^{18}O isotope shift have the same line shape. Of course, any manipulation of the free induction decay leading to an improvement in resolution will result in a loss of signal-to-noise ratio.

III. ^{17}O Quadrupolar Effects in ^{31}P NMR

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

[a] This table is an extension of the table of Cohn (1982) to cover some more recent data, particularly those from our own laboratory. It is, however, not an exclusive list of all reported data. Unless otherwise specified, the data from our laboratory were obtained at ambient temperature (25–30°C).

[b] References: 1. Sammons *et al.* (1983); 2. K. Bruzik and M.-D. Tsai (unpublished); 3. Lowe *et al.* (1979); 4. Cohn and Hu (1980); 5. Lowe and Sproat (1978); 6. Gorenstein and Rowell (1980); 7. Sammons and Frey (1982); 8. Coderre and Gerlt (1980); 9. Sammons (1982); 10. Bruzik and Tsai (1982); 11. Jordan *et al.* (1981); 12. Jarvest *et al.* (1980); 13. Buchwald and Knowles (1980); 14. Jarvest *et al.* (1981); 15. Gerlt and Coderre (1980); 16. Gerlt and Wan (1979); 17. Webb and Trentham (1980); 18. R. L. Van Etten (private communication).

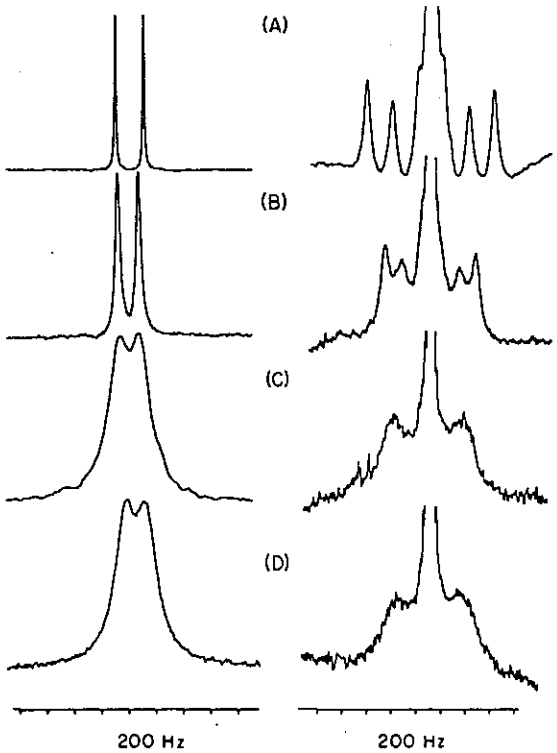


Fig. 2. Line shapes of ^{17}O NMR (left, at 27.1 MHz) and $^{31}P(^{17}O)$ NMR (right, at 81.0 MHz). (A) $P^{17}OCl_3$, 51 atom % ^{17}O, in tetrahydrofuran, using [$^2H_6$]acetone for external lock, δ = 210 ppm for ^{17}O, 2.5 ppm for ^{31}P. (B) $(CH_3O)P^{17}O$, 51 atom % ^{17}O, in $CDCl_3$, δ = 73.6 ppm for ^{17}O, 2.6 ppm for ^{31}P. (C) $(PhO)_3P^{17}O$, 51 atom % ^{17}O, in $CDCl_3$, δ = 91.2 ppm for ^{17}O, −17.9 ppm for ^{31}P. (D) $(Ph)_3P^{17}O$, 49 atom % ^{17}O, in $CDCl_3$, δ = 43.3 ppm for ^{17}O, 28.8 ppm for ^{31}P. All spectra were run at 31 °C and processed with 5-Hz line broadening. From Sammons *et al.* (1983).

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 $I = \frac{5}{2}$, with different magnitudes of T_qJ. Figure 2 shows the ^{17}O- and $^{31}P(^{17}O)$-NMR spectra of $P^{17}OCl_3$ (A), $(CH_3O)_3P^{17}O$ (B), $(PhO)_3P^{17}O$ (C), and $Ph_3P^{17}O$ (D). It can be seen in Fig. 2 that as the ^{17}O-NMR coupling pattern collapses (decreasing J and/or increasing ^{17}O linewidth ΔO), the ^{31}P-NMR coupling pattern also collapses. In all spectra the strong central peak is due to the residual unlabeled species.

The compounds whose spectra are shown in Fig. 2 are all symmetrical, small molecules with P=O double bonds. 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 (owing to a larger molecule size and a small degree of symmetry) and J generally smaller (owing to a P—O bond with a smaller π character). Therefore, the ^{17}O-NMR signals of biophosphates are broader and less well resolved. Based on Fig. 2, we would expect the $^{31}P(^{17}O)$-NMR signals of biophosphates to be a "broad singlet." Under this condition ($T_qJ < 1$), the scalar relaxation will contribute to the relaxation of the dipolar nucleus according to

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

and

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

(Lehn and Kintzinger, 1973; James, 1975; Abragam, 1961), where $I = \frac{5}{2}$, $1/T_{1sc}$ and $1/T_{2sc}$ are the contribution of scalar relaxation to the longitudinal and the transverse relaxations, respectively, of ^{31}P, $J = J_{^{31}P-^{17}O}$, 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 of the order of 10^{-2}–10^{-4} s. Because $\omega_p - \omega_o \simeq 10^7$–$10^8$ Hz, $(\omega_p - \omega_o)^2T_q^2 \gg 1$ and Eqs. (1) and (2) can be reduced to

$$\frac{1}{T_{1sc}} \simeq 0 \tag{3}$$

and

$$\frac{1}{T_{2sc}} \simeq \frac{35}{3}\pi^2J^2T_q \tag{4}$$

Under this condition $1/T_2 \simeq 1/T_{2sc}$ for ^{31}P, and $T_1 \simeq T_2 \simeq T_q$ for ^{17}O, which justifies the approximations $\Delta O \simeq 1/\pi T_q$ and $\Delta P \simeq 1/\pi T_{2sc}$. The following approximate relationship can be obtained from Eq. (4):

$$\Delta P\,\Delta O \simeq \tfrac{35}{3}J^2 \tag{5}$$

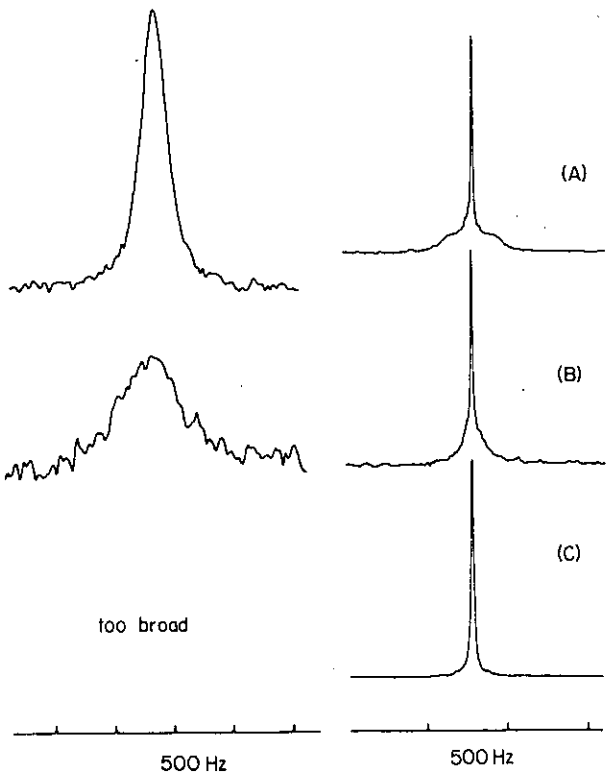


Fig. 3. ^{17}O-NMR spectra (left, at 27.1 MHz) and ^{31}P(^{17}O)-NMR spectra (right, at 81.0 MHz) of $H_3P^{17}OO_3$ (50 atom % ^{17}O) in D_2O (A), H_2O/glycerol (1 : 1 volume ratio) (B), and glycerol (C). All spectra were obtained at 30°C and processed with a line broadening of 20 Hz (^{17}O) and 4 Hz (^{31}P). From Sammons *et al.* (1983).

where ΔP and ΔO represent 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 (Fig. 3). As ΔO increases because of an increased viscosity, ΔP decreases correspondingly.

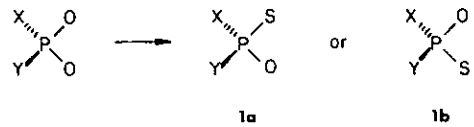
The approximate equations [(3)–(5)] derived are valid under the "extreme narrowing limit," and thus should be applicable for small biophosphate molecules in solution. Tsai *et al.* (1980) have shown that for adenine nucleotides with ^{17}O labeled at all possible positions, the ^{31}P(^{17}O)-NMR signals are "broad." The case of $H_3P^{17}O_4$/glycerol (Fig. 3C), in which the ^{31}P(^{17}O) signal has sharpened almost indistinguishably from ^{31}P(^{16}O) signal,

should not occur in small biophosphates in water. Therefore, ^{17}O "quenches" the ^{31}P-NMR signal of ^{31}P–^{17}O species, causing an apparent decrease in the intensity of the ^{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 VI).

IV. Prochiral Centers: ^{31}P NMR

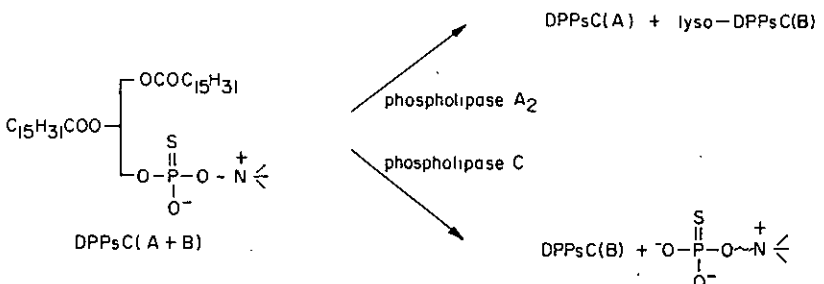
By a sulfur substitution, a prochiral phosphorus center (e.g., a phosphodiester) becomes a chiral center with two possible configurations (Scheme 5).



Scheme 5

Because most biophosphate molecules contain at least one chiral carbon center, the two isomers **1a** and **1b** are diastereomers and give distinguishable ^{31}P chemical shifts. Table II summarizes the chiral thiophosphates that belong to this category and the chemical shifts of the chiral phosphorus of these isomers.

The chiral thiophosphates **1a** and **1b** can be used for two types of studies: stereochemical course of enzymatic substitutions (type 1 in Section I) and stereospecificity of the two isomers as enzyme substrates (type 3 in Section I). A great number of enzyme reactions have been investigated by this approach and have been reviewed as described in Section I. An example in this category is the stereospecific hydrolysis of DPP_SC by phospholipases A_2 and C (Bruzik *et al.*, 1982). As shown in Scheme 6, when DPPsC(A + B)



Scheme 6

TABLE II

Phosphorus-31 Chemical Shifts of Diastereomeric Pairs of Chiral Thiophosphates

Compounds	Solvent	Chemical shifts[a] R_p	Chemical shifts[a] S_p	Reference
ADPαS	H_2O/D_2O, pH 7.3	41.7	42.1	Jaffe and Cohn (1978)
	D_2O	40.61	40.96	Sheu and Frey (1977)
ATPαS	D_2O	42.74	42.97	Sheu and Frey (1977)
ATPβS	H_2O/D_2O, pH 8.1	30.0	29.9	Jaffe and Cohn (1978)
UDPαS	D_2O	40.39	40.84	Sheu *et al.* (1979)
UTPαS	D_2O	42.62	42.39	Sheu *et al.* (1979)
GTPαS	D_2O	42.23	42.55	Connolly *et al.* (1982)
GTPβS	D_2O	28.27	28.27	Connolly *et al.* (1982)
cAMPS	H_2O	54.27	53.22	Eckstein *et al.* (1974)
	pH 9	55.66	54.05	Gerlt *et al.* (1980)
U > pS		74.8 (endo)	76.1 (exo)	Usher *et al.* (1972)
Up(S)A	D_2O	56.1	55.5	Burgers and Eckstein (1979)
DPPsE[b]	$CDCl_3$	59.61(A)	59.47(B)	Orr *et al.* (1982)
DPPsC[c]	$CDCl_3$	56.12(A)	56.07(B)	Bruzik *et al.* (1983)
	CH_3OD	60.822(A)	60.801(B)	Bruzik *et al.* (1983)
	D_2O/Triton X-100	57.133(A)	57.205(B)	Bruzik *et al.* (1983)

[a] Only the chemical shift of the chiral phosphorus is listed. In some of the original references reporting the chemical shift, the absolute configuration was not assigned. The assignments listed are based on some later reports which are not cited.

[b] The absolute configuration is unknown. Isomers A and B are arbitrarily defined.

[c] The absolute configuration is unknown. Isomers A and B are defined as in Bruzik *et al.* (1982).

(which gives two ^{31}P-NMR signals in $CDCl_3$, as shown in Fig. 4A; the isomer resonating at lower field was defined as isomer A) was digested with phospholipase A_2 from bee venom, only isomer B was specifically hydrolyzed to lyso-DPPsC(B). Figure 4B,C shows the ^{31}P-NMR spectra of the unreacted DPPsC(A) and the pure DPPsC(B) obtained from reacylation of lyso-DPPsC(B), respectively. On the other hand, phospholipase C is specific to isomer A. Figure 4D shows the ^{31}P-NMR spectra of DPPsC after partial hydrolysis by phospholipase C from *Bacillus cereus*. The requirement of a specific configuration at phosphorus in the phospholipase C catalysis is to be expected since the reaction involves a P—O bond cleavage. However, the stereospecificity observed for phospholipase A_2 is surprising because it hydrolyzes the C-2 ester but not the phosphodiester, and it can tolerate substitution of the choline side chain by other groups.

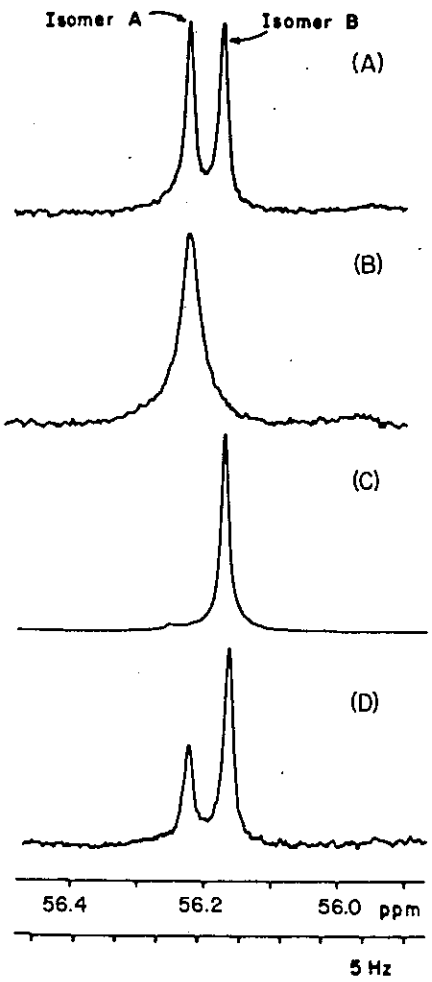


Fig. 4. ^{31}P-NMR spectra (81.0 MHz) of DPP_SC (10 mM in $CDCl_3$). (A) Mixture of diastereomers from chemical synthesis. (B) Pure isomer A recovered from hydrolysis by phospholipase A_2, (C) Pure isomer B (containing 3% isomer A) obtained from acylation of the product of phospholipase A_2 hydrolysis, lyso-DPP_SC. (D) DPP_SC after partial hydrolysis by phospholipase C. NMR parameters: spectral width 1000 Hz, acquisition time 4.1 s, ^{1}H decoupling, line broadening 0.1 Hz, pulse width 12 μs (90° pulse at 20 μs). From Bruzik *et al.* (1982).

V. Pro-Prochiral Centers: ^{31}P(^{18}O) NMR or ^{31}P(^{17}O) NMR

By a sulfur substitution, a pro-prochiral phosphorus center (e.g., a phosphomonoester) becomes a prochiral center (Scheme 7).

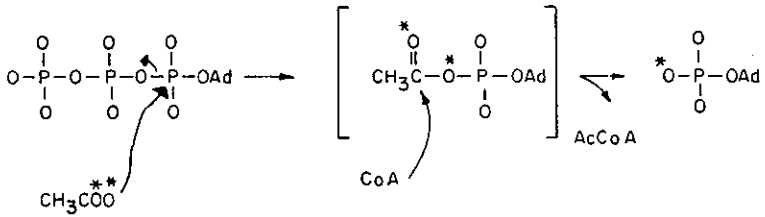
2

Scheme 7

As described in Section I, when 2 is phosphorylated enzymatically, the reaction is most likely stereospecific because enzymes can differentiate between the two diastereotopic oxygens. Because the prochiral center becomes a chiral center after phosphorylation, the two isomers can be identified based on ^{31}P chemical shifts.

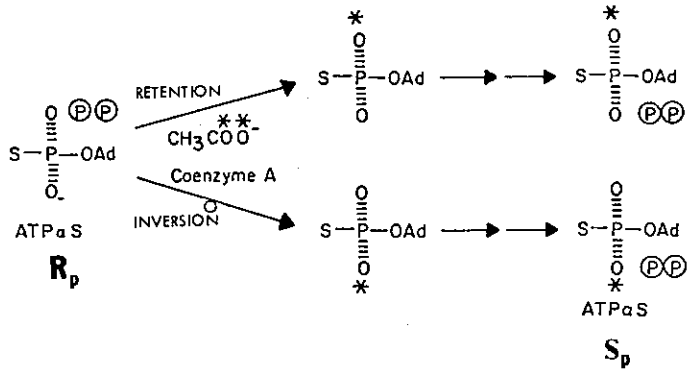
If 2 is made chiral by an ^{17}O or ^{18}O label, the configuration of 2 can be determined by stereospecifically phosphorylating one of the two oxygens, followed by determining whether the labeled oxygen is located at the P—O—P briding position or the P—O nonbridging position. If ^{17}O is used, a bridging ^{17}O should cause a broadening (and a decrease in the apparent intensity) of both ^{31}P-NMR signals, whereas a nonbriding ^{17}O should exert the effect to only one ^{31}P signal. If ^{18}O is used, a nonbridging ^{18}O should cause a larger isotope shift on the ^{31}P signal than a bridging ^{18}O.

The $^{31}P(^{17}O)$-NMR method has been used to elucidate the steric course of acetyl-CoA synthetase-catalyzed reaction (Scheme 8) (Tsai, 1979). The



Scheme 8

enzyme was found to be specific to (R_p)-ATPαS but not to (S_p)-ATPαS. As shown in Scheme 9, when (R_p)-ATPαS and [^{17}O]acetate are used as sub-



Scheme 9

strates, 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-la-

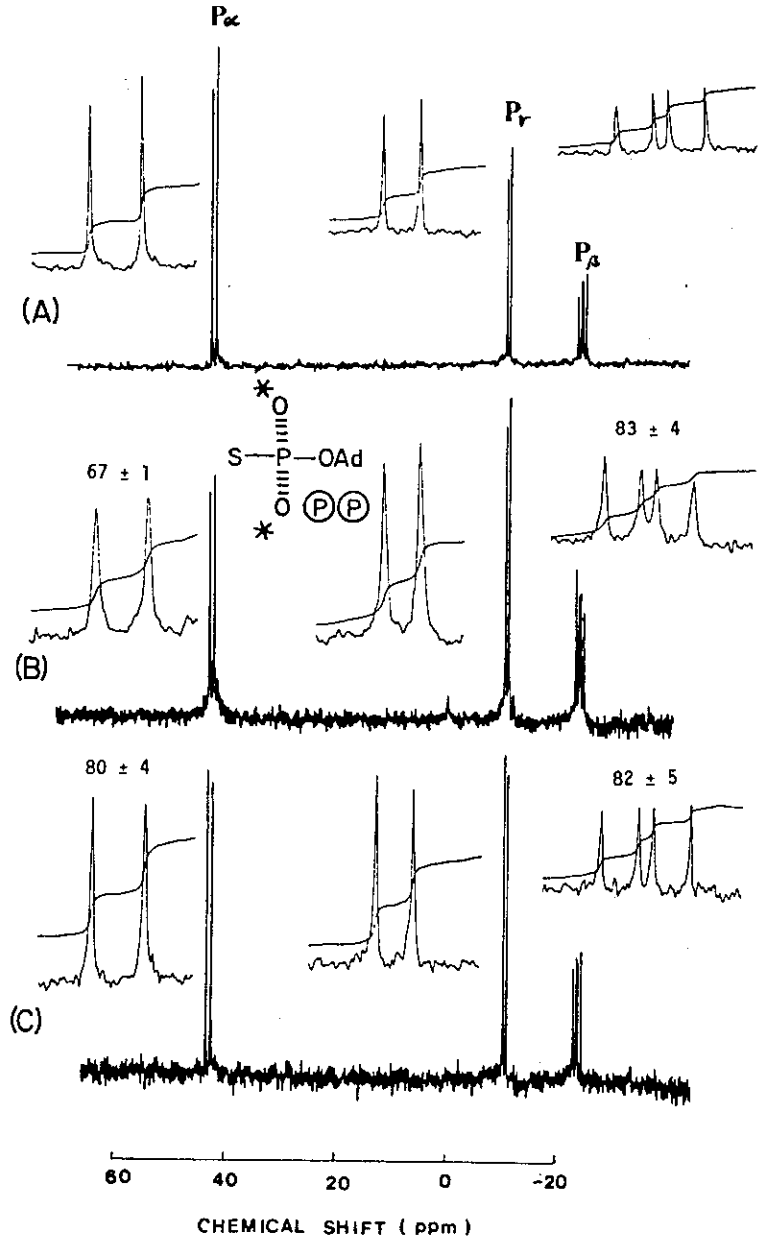


Fig. 5. ^{31}P-NMR spectra (at 32.2 MHz) showing the results of acetyl-CoA synthetase. (A) Unlabeled (S_p)-ATPαS. (B) Synthesized (S_p)-[α-^{17}O, αβ-^{17}O]ATPαS. (C) (S_p)-ATPαS from [$^{17}O_2$]acetate. The insets represent the integrations of the corresponding signals. From Tsai (1979).

beled AMPS, 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 5 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 Fig. 5B, the P_α signal decreases to 67 ± 1% and the P_β signal to 83 ± 4%. In Fig. 5C, the P_α signal decreases to 80 ± 4% and the P_β signal to 82 ± 5%. Because both P_α and P_β have decreased in Fig. 5C, the results indicate that ^{17}O must be located at the bridging position, and the reaction catalyzed by acetyl-CoA synthetase must proceed with inversion of configuration (Tsai, 1979).

The same problem could have been solved by use of ^{18}O isotope shifts in ^{31}P NMR, which may be advantageous over the ^{17}O method in terms of quantitation but requires a high-resolution and a higher-field instrument. Indeed, our original idea was to use ^{17}O NMR to differentiate the bridging and nonbridging ^{17}O, which was not successful with a low-field spectrometer used earlier; but it has now been shown to be feasible at higher magnetic field and higher temperature (Tsai, 1982; Gerlt *et al.*, 1982; Gerothanassis and Sheppard, 1982). On the other hand, a number of stereochemical studies in this category made use of ^{18}O-labeling and mass spectroscopy, which gives relatively accurate quantitation but requires derivatization and/or degradation of the product.

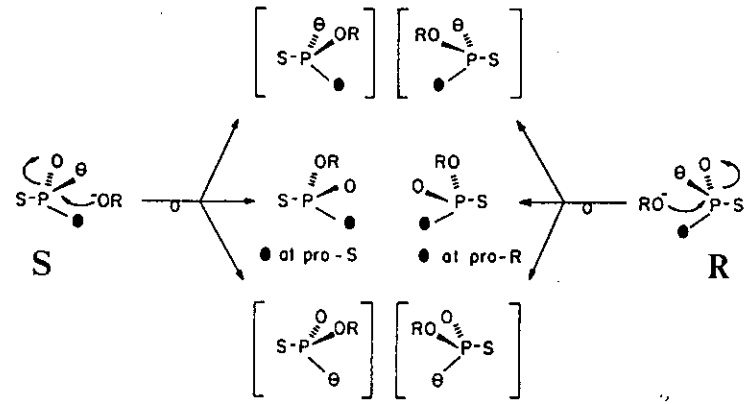
There are, however, no alternatives in the configurational analysis of the chiral [^{16}O,^{17}O,^{18}O]thiophosphate (P_{si}) discussed in the next section. The only method available is a ^{31}P-NMR method based on the combined effects of ^{17}O and ^{18}O.

VI. Pro-Pro-Prochiral Centers: $^{31}P(^{18}O)$ NMR and $^{31}P(^{17}O)$ NMR

Hydrolysis of phosphomonoesters generates inorganic phosphate (P_i), which contains a pro-pro-prochiral phosphorus center. To make a P_i chiral, it is necessary to make use of all three stable oxygen isotopes (^{16}O,^{17}O,^{18}O) and sulfur, as shown in Scheme 10.

Scheme 10

The rationale of configurational analysis for chiral P_{si} is illustrated by Scheme 11. The same principle applies to chiral phosphate monoesters



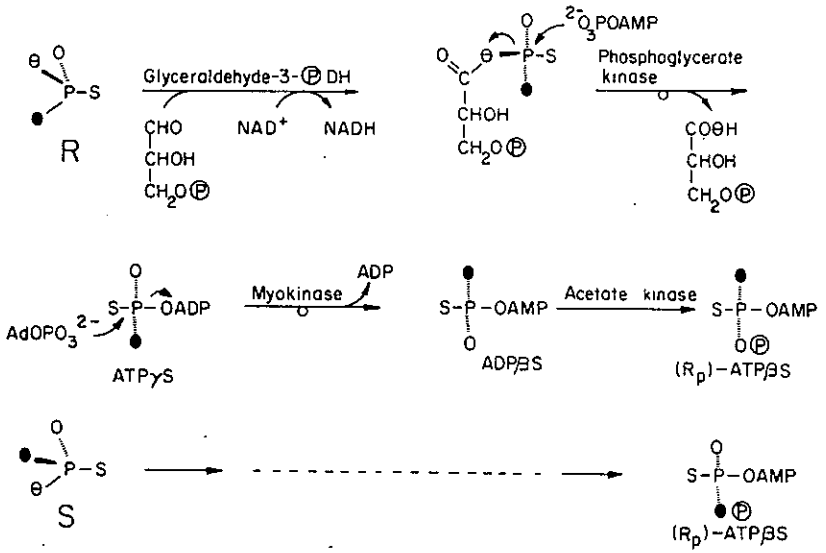
Scheme 11

where the P—S bond is replaced by P—OR. Displacement of one of the three oxygen isotopes of (S_p)-[^{16}O,^{17}O,^{18}O]P_{si} by a nucleophile (RO^-) gives a mixture of three inseparable, isotopically different species. Among them, two (those in brackets) contain an ^{17}O isotope, which should quench the corresponding ^{31}P-NMR signals. Only the species that contains only ^{16}O and ^{18}O (^{18}O at the pro-*S* position) should give a sharp, unquenched ^{31}P-NMR signal. Analogously, the (*R*)-[^{16}O,^{17}O,^{18}O]P_{si} should give correspondingly a non-^{17}O-containing species with ^{18}O at the pro-*R* position.

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

To illustrate the application and configurational analysis of chiral [^{16}O,^{17}O,^{18}O]thiophosphate, we describe the stereochemical study of 5′-nu-

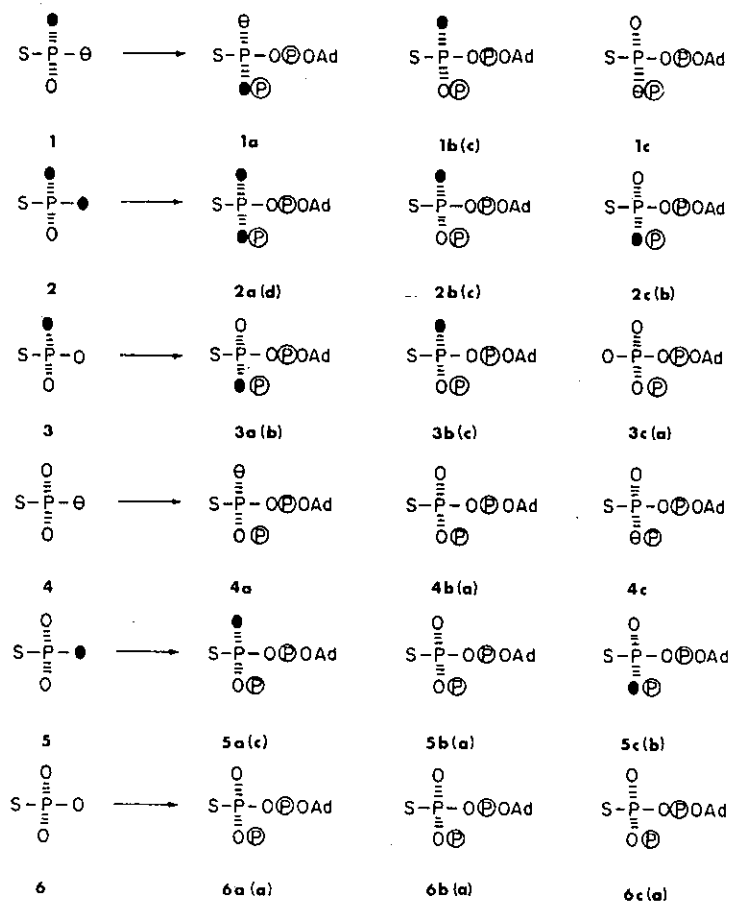
cleotidase (Tsai and Chang, 1980; Tsai, 1980). The venom 5′-nucleotidase catalyzes hydrolysis of AMP to adenosine and P_i but does not catalyze transphosphorylation or $P_i \rightleftarrows H_2O$ oxygen exchange (Koshland and Springhorn, 1956). We have first synthesized (R_p)-[^{18}O]AMPS and (S_p)-[^{18}O]AMPS of known configuration. Hydrolysis of these two isomers in $H_2{}^{17}O$ gave two chiral [^{16}O,^{17}O,^{18}O]P_{si} enantiomers with unknown configuration. The two main steps required were available separately in the literature (Eckstein, 1977), as shown in Scheme 12. The stereochemical course of



Scheme 12

each step in Scheme 12 had been elucidated separately (Richard and Frey, 1978; Richard *et al.*, 1978) except that of phosphoglycerate kinase, which was elucidated by Webb and Trentham (1980) by use of synthesized chiral P_{si} of known configuration on the basis of the same NMR analysis discussed next.

According to Scheme 12, the (R_p)-chiral P_{si} should give (R_p)-ATPβS with ^{18}O located specifically at the β-nonbridging position. The (S_p)-enatiomer should give (R_p)-ATPβS with ^{18}O at the $\beta\gamma$-bridging position. It is known that a bridging ^{18}O should cause a smaller isotope shift in ^{31}P NMR than a nonbridging ^{18}O. On this basis the configuration can be determined. However, Scheme 12 only shows the species that will give an unquenched ^{31}P-NMR signal. In reality, each chiral P_{si} species should give a mixture of three (R_p)-ATPβS species (**1a**, **1b**, and **1c** in Scheme 13). In addition, it is impossible to have a chiral P_{si} of 100% purity. A chiral P_{si} sample actually contains up to six isotopic species, as shown in the left column of Scheme 13



Scheme 13

(two of them are identical species); each of them gives three (R_p)-ATPβS species. Fortunately, a careful examination of Scheme 13 reveals that there are only four different non-^{17}O-containing species **a**, **b**, **c** and **d**, and that all the nonchirally labeled P_{si} species contribute *equally* to species **b** and **c**. Only the [^{16}O,^{17}O,^{18}O]P_{si} species gives specifically **b** or **c**, depending on whether the configuration is *S* or *R*, respectively. The amounts of species **a** and **d** have to do with isotopic enrichments but not configuration.

Figure 6 shows the P_β signals of the (R_p)-ATPβS obtained from $PS^{18}O_3^{3-}$ and the two chiral P_{si} enantiomers. The signal contains two overlapping doublets owing to ^{31}P–^{31}P coupling. Each half of a doublet contains four lines arising from the four species **a**, **b**, **c**, and **d**. The results are summarized in Table III, where the *F* value is defined as the ratio **b**/**c**, the purity refers to the percentage of chirally labeled P_{si} species, and the chirality refers to the

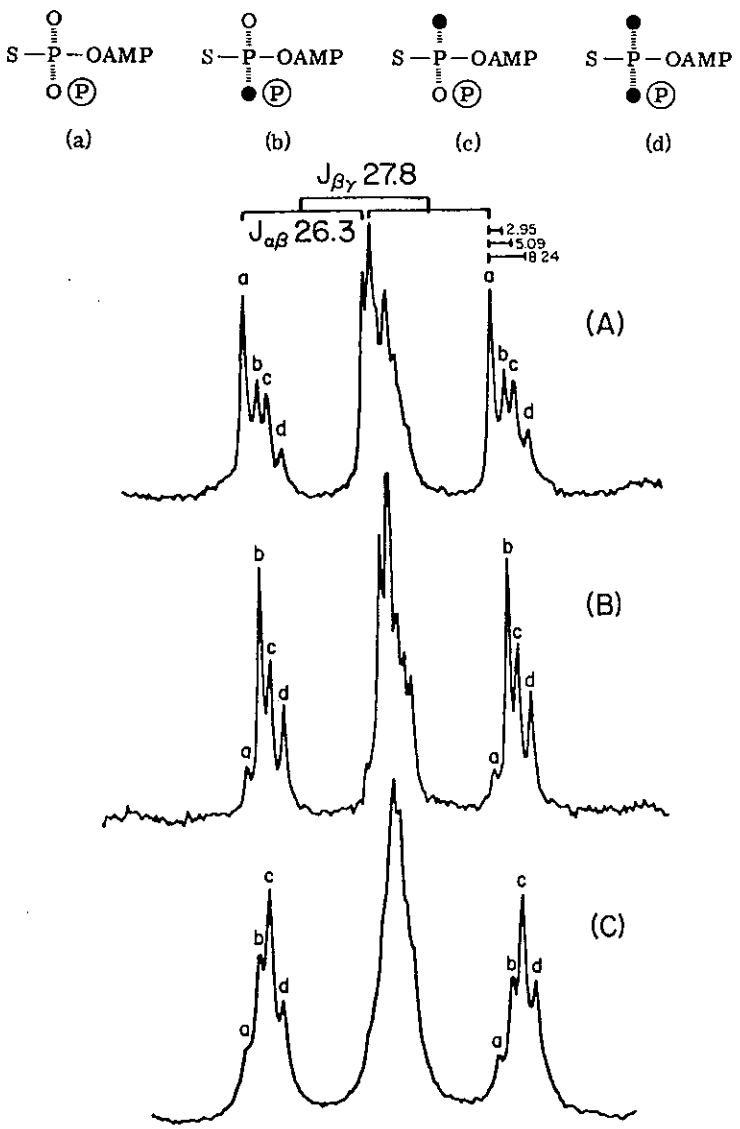


Fig. 6. P_β signals of the ^{31}P-NMR spectra of the (R_p)-ATPβS obtained from [$^{18}O_3$]P_{si} (A) and from the two chiral P_{si} (B, C). The sample (30 μmol) was dissolved in 2.5 ml of D_2O containing 10 mM EDTA and the spectra recorded at 145.7 MHz at ambient temperature. The coupling constants and isotope shifts are epxressed in Hertz. The chemical shift of the P_β signal is 29.8 ppm downfield from H_3PO_4. From Tsai (1980).

TABLE III

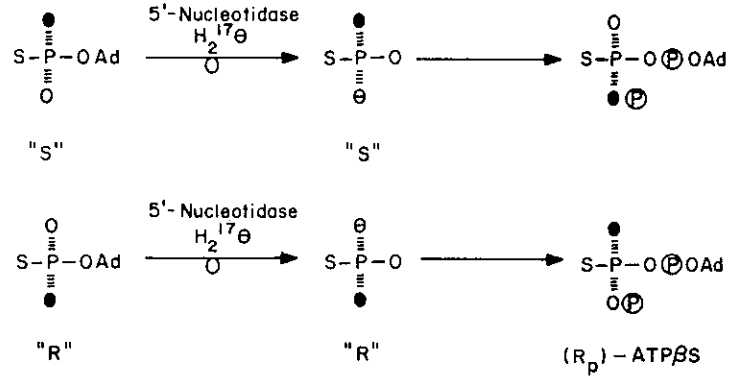
^{31}P-NMR Analysis of the (R_p)-ATPβS Derived from Chiral Thiophosphates

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

[a] Obtained from peak-height measurements for the P_β signal of ATPβS. The errors represent deviations between the two nonoverlapping halves of the two doublets.

[b] Calculated for chiral P_{si} of 47.5% purity and 90% chirality expected based on isotopic enrichments.

optical purity of chirally labeled P_{si} species. The results indicate that 5′-nucleotidase catalyzes the hydrolysis of AMPS with inversion of configuration, as shown in Scheme 14. The causes for the deviations of the observed F values from theoretical values have been discussed (Tsai, 1980).



Scheme 14

By such a ^{31}P-NMR analysis, Webb and Trentham have also elucidated the stereochemical course of a number of nucleoside triphosphatases (Webb, 1982). The ^{31}P-NMR analysis based on the combined effects of ^{17}O and ^{18}O is the only method available for configurational analysis of chiral [^{16}O,^{17}O,^{18}O]P_{si}, and is also a commonly used method for configurational analysis of chiral [^{16}O,^{17}O,^{18}O]phosphomonoesters, which are covered by Gerlt (Chapter 7.)

Acknowledgments

The work from the author's laboratory was supported by research grants GM 29041 and GM 30327 from the National Institutes of Health, and PCM 8140443 from the National Science Foundation.

References

Abragam, A. (1961). "The Principles of Nuclear Magnetism." Oxford Univ. Press, London and New York.
Bruzik, K., and Tsai, M.-D. (1982). *J. Am. Chem. Soc.* **104,** 836–865.
Bruzik, K., Gupte, S. M., and Tsai, M.-D. (1982). *J. Am. Chem. Soc.* **104,** 4682–4684.
Bruzik, K., Jiang, R.-T., and Tsai, M.-D. (1983). *Biochemistry* **22,** 2478–2486.
Buchwald, S. L., and Knowles, J. R. (1980). *J. Am. Chem. Soc.* **102,** 6601–6603.
Buchwald, S. L., Hansen, D. E., Hassett, A., and Knowles, J. R. (1982). *In* "Methods in Enzymology," Vol. 87, pp. 279–301. Academic Press, New York.
Burgers, P. M. J., and Eckstein, F. (1979). *Biochemistry* **18,** 592–596.
Cahn, R. S., Ingold, C. K., and Prelog, V. (1966). *Angew. Chem., Int. Ed. Engl.* **5,** 385–415.
Clin, B., de Bony, J., Palanne, P., Biais, J., and Lemanceau, B. (1979). *J. Magn. Reson.* **33,** 457–463.
Coderre, J. A., and Gerlt, J. A. (1980). *J. Am. Chem. Soc.* **102,** 6594–6597.
Cohn, M. (1982). *Annu. Rev. Biophys. Bioeng.* **11,** 23–42.
Cohn, M., and Hu, A. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 200–203.
Cohn, M., and Hu, A. (1980). *J. Am. Chem. Soc.* **102,** 913–916.
Connolly, B. A., Romaniuk, P. J., and Eckstein, F. (1982). *Biochemistry* **21,** 1983–1989.
Eckstein, F. (1975). *Angew. Chem. Int. Ed. Engl.* **14,** 160–166.
Eckstein, F. (1977). *Biochim. Biophys. Acta* **483,** 1–5.
Eckstein, F. (1979). *Acc. Chem. Res.* **12,** 204–210.
Eckstein, F., Simonson, L. P., and Bär, H.-P. (1974). *Biochemistry* **13,** 3806–3810.
Eckstein, F., Romaniuk, P. J., and Connolly, B. A. (1982). *In* "Methods in Enzymology" Vol. 87, pp. 197–212. Academic Press, New York.
Frey, P. A. (1982). *Tetrahedron* **38,** 1541–1567.
Frey, P. A., Richard, J. P., Ho, H.-T., Brody, R. S., Sammons, R. D., and Sheu, K.-F. (1982). *In* "Methods in Enzymology" Vol. 87, pp. 213–235. Academic Press, New York.
Gerlt, J. A., and Coderre, J. A. (1980). *J. Am. Chem. Soc.* **102,** 4531–4533.
Gerlt, J. A., and Wan, W. H. Y. (1979). *Biochemistry* **18,** 4630–4637.
Gerlt, J. A., Coderre, J. A., and Wolin, M. S. (1980). *J. Biol. Chem.* **255,** 331–334.
Gerlt, J. A., Demou, P. C., and Mehdi, S. (1982). *J. Am. Chem. Soc.* **104,** 2848–2856.
Gerothanassis, I. P., and Sheppard, N. (1982). *J. Magn. Reson.* **46,** 423–439.
Gorenstein, D. G., and Rowell, R. (1980). *J. Am. Chem. Soc.* **102,** 6165–6166.
Huang, S.-L., and Tsai, M.-D. (1982). *Biochemistry* **21,** 951–959.
Jaffe, E. K., and Cohn, M. (1978). *Biochemistry* **17,** 652–657.
James, T. L. (1975). "Nuclear Magnetic Resonance in Biochemistry," pp. 48–49. Academic Press, New York.
Jarvest, R. L., Lowe, G., and Potter, B. V. L. (1980). *J. Chem. Soc., Chem. Commun.* pp. 1142–1145.

Jarvest, R. L.,Lowe, G.,and Potter, B. V. L. (1981). *J. Chem. Soc.,Perkin Trans. 1* pp. 3186–3195.
Jordan, F., Salamone, S. J., and Wang, A. (1981). *ACS Symp. Ser.* **171,** 585–589.
Knowles, J. R. (1980). *Annu. Rev. Biochem.* **49,** 877–919.
Koshland, D. E., Jr., and Springhorn, S. S. (1956). *J. Biol. Chem.* **221,** 469–476.
Lehn, J. M., and Kintzinger, J. P. (1973). *In* "Nitrogen NMR" (M. Witanowski and G. A. Webb, eds.), pp. 80–161. Plenum, New York.
Lowe, G., and Sproat, B. S. (1978). *J. Chem. Soc., Chem. Commun.*, pp. 565–566.
Lowe, G., Potter, B. V. L., Sproat, B. S., and Hull, W. E. (1979). *J. Chem. Soc., Chem. Commun.* pp. 733–735.
Lutz, O., Nolle, A., and Staschewski, D. Z. (1978). *Z. Naturforsch, A* **33,** 380–382.
Orr, G. A., Brewer, C. F., and Heney, G. (1982). *Biochemistry* **21,** 3202–3206.
Reed, G. H., and Leyh, T. S. (1980). *Biochemistry* **19,** 3472–3480.
Richard, J. P., and Frey, P. A. (1978). *J. Am. Chem. Soc.* **100,** 7757–7758.
Richard J. P., Ho, H. T., and Frey, P. A. (1978). *J. Am. Chem. Soc.* **100,** 7756–7757.
Sammons, R. D., and Frey, P. A. (1982). *J. Biol. Chem.* **257,** 1138–1141.
Sammons, R. D., Frey, P. A., Bruzik, K., and Tsai, M.-D. (1983). *J. Am. Chem. Soc.* **105,** 5455–5461.
Sammons, R. D. (1982). Ph.D. Dissertation, Ohio State University, Columbus.
Sheu, K. F. R., and Frey, P. A. (1977). *J. Biol. Chem.* **252,** 4445–4448.
Sheu, K. F. R., Richard, J.-P., and Frey, P. A. (1979). *Biochemistry* **18,** 5548–5556.
Suzuki, M., and Kubo, R. (1964). *Mol. Phys.* **7,** 201–209.
Tsai, M.-D. (1979). *Biochemistry* **18,** 1468–1472.
Tsai, M.-D. (1980). *Biochemistry* **19,** 5310–5316.
Tsai, M.D. (1982). *In* "Methods in Enzymology" Vol. 87, pp. 235–279. Academic Press, New York.
Tsai, M.-D., and Bruzik, K. (1983). In "Biological Magnetic Resonance" (L. J. Berliner and J. Reuben, eds.), Vol. 5, pp. 129–181. Plenum, New York.
Tsai, M.-D., and Chang, T. T. (1980). *J. Am. Chem. Soc.* **102,** 5416–5418.
Tsai, M.-D., Huang, S. L., Kozlowski, J. F., and Chang, C. C. (1980). *Biochemistry* **19,** 3531–3536.
Usher, D. A., Erenrich, E. S., and Eckstein, F. (1972). *Proc. Natl. Acad. Sci. U. S. A.* **69,** 115–118.
Webb, M. R. (1982). *In* "Methods in Enzymology" Vol. 87, pp. 301–316. Academic Press, New York.
Webb, M. R., and Trentham, D. R. (1980). *J. Biol. Chem.* **255,** 8629–8632.