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STEREOCHEMISTRY OF ENZYMATIC REACTIONS OF PHOSPHATES

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1. INTRODUCTION

1.1 *Chiral phosphates*

The stereochemical course of any chemical reaction is determined by the structure of the transition state of that reaction, or in multistep reactions by the structures of transition states and intermediates. Inasmuch as mechanisms are defined by transition states, reaction mechanisms and stereochemistry are closely related, and knowledge of the stereochemical course of a reaction is recognized as one of the most useful kinds of information for distinguishing among possible mechanisms.

The phosphoric acid molecule **1** is tetrahedral though not a perfect tetrahedron because of the inequivalence of the double bonded oxygen and the hydroxyl groups. The oxygen atoms in a collection of phosphoric acid molecules become equivalent by the process of proton exchange, but the oxygen atoms do not themselves exchange rapidly; and in phosphates such as phosphate mono-, di- and trianions their rates of exchange are so small as to be negligible for most purposes. This is also true of phosphorothioates and phosphoramidates. Phosphates can, therefore, be chiral molecules when the substituents of tetrahedral phosphorus differ in the physical sense. Examples of simple chiral phosphates are **2**–**4**, all of which have been synthesized. Note the symbols ● for ^{18}O and ⓘ for ^{17}O in **3** and **4** which will be used repeatedly in this report. Note also that **3** and **4** are shown as the free acids but can and do exist as the

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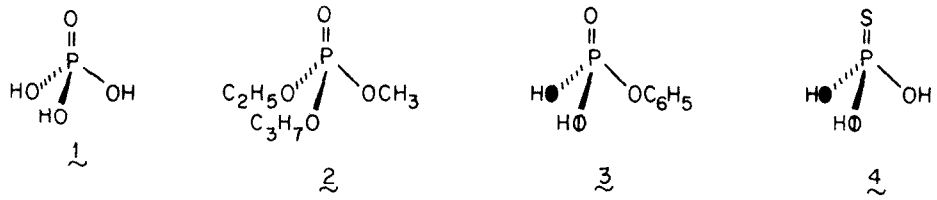


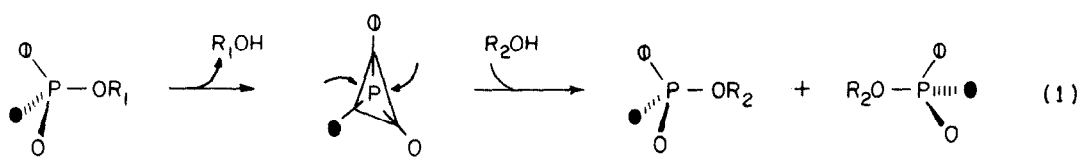
anionic phosphates resulting from the ionization of their protons. In aqueous solutions they exist as the mono-, di-, or trianions at pH's above 1; in fact, phosphorothioates are hydrolytically stable only as di- and trianions, i.e. at pH's above 7.

Compound **2** is chiral by virtue of the different alkyl substituents on the four oxygens, whereas **3** and **4** are chiral by virtue of the substitution of heavy oxygen isotopes and sulfur for oxygen 16. Molecules of phosphoric acid, **1**, may individually be chiral when the three hydroxyl groups are $-^{16}OH$, $-^{17}OH$ and $-^{18}OH$ (or $-\overset{1}{O}-H$, $-\overset{2}{O}-H$, $-\overset{3}{O}-H$) but no collection of such molecules comprising a real sample can all have the same chirality because the rapid exchange of protons among the four oxygen atoms makes them equivalent. Exchange of protons, fractional bond orders in anionic forms, and the involvement of double bonds which shift positions in fully protonated species complicate the assignment of R, S configurational symbols to chiral phosphates. These difficulties are avoided by ignoring protons, double bonds, and negative charges when assigning configurational symbols. For this reason they are not included in most structural formulas depicting chiral phosphorus centers. Double bonds are retained in fully alkylated chiral phosphoric esters.

Inasmuch as chiral phosphates can in principle be synthesized, their use in stereochemical studies of the reactions of phosphates is possible, and the information obtained should be as useful in mechanistic research as comparable information in carbon chemistry. This research has awaited the development of methodologies for synthesising the appropriate molecules and assigning their configurations. Such methodologies have recently been developed for the specialized purpose of determining the stereochemical courses of enzymatic substitution at phosphorus in biological phosphates. This report reviews the recent advances in this field. The proliferation of work in the field over the past ten and especially the past four years precludes a complete discussion in this space of all of the published research. This report, therefore, deals with representative investigations which exemplify the methodologies developed for enzymatic stereochemical experiments.

1.2 *Stereochemical consequences of substitution in phosphates*

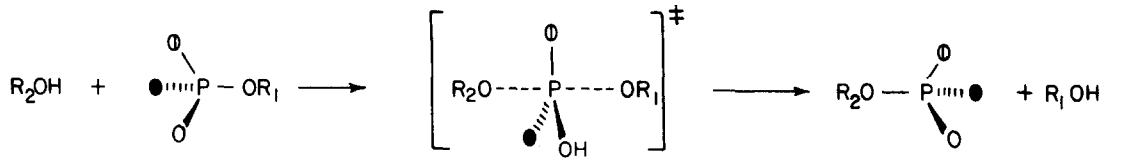
Associative and dissociative mechanisms of nucleophilic substitution at phosphorus in phosphates have been proposed based on detailed studies of non-enzymatic reactions.[1-3] These are the chemical mechanisms that may be considered for enzymatic reactions;[4] as in carbon chemistry, stereochemistry is one means of distinguishing between them. The dissociative mechanism represented by eqn (1) is a S_N1



(1)

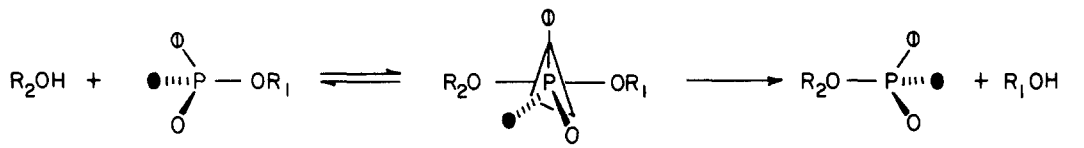
mechanism, in which the phosphate undergoing substitution expels the leaving group in the rate limiting step, producing a planar electrophilic metaphosphate as an intermediate. The intermediate is captured by the nucleophile in a second fast step. The mechanism is analogous to the S_N1 mechanism in carbon chemistry, where the metaphosphate is the electrophilic intermediate analogous to the carbonium ion. Since metaphosphate is planar and can capture the nucleophile at either face, this mechanism will result in the loss of configuration in chirally substituted phosphates.

Three associative mechanisms that differ with respect to the occurrence and decomposition of pentacovalent trigonal bipyramidal intermediates have been proposed. One, exemplified by eqn (2), is a concerted S_N2 mechanism in which the displacing nucleophile attacks from the side opposite the leaving group and displaces it in a single step. Substitution by this mechanism would proceed with compulsory inversion of configuration at a chiral phosphorus center.



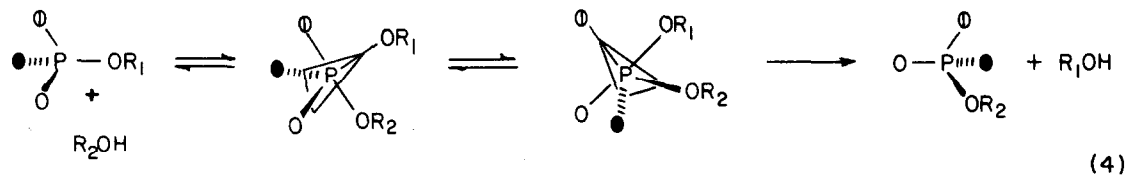
(2)

Two other associative mechanisms differ from the foregoing in that they involve *intermediates*, and they differ from each other in the manner in which the intermediates are produced and decomposed. The simpler mechanism is one in which the attacking nucleophile approaches from the side opposite the leaving group, forming a bond to the phosphorus atom. The resulting pentacovalent trigonal bipyramidal intermediate shown in eqn (3) has the displacing nucleophile and the leaving group in the apical positions



(3)

and the other three substituents in the equatorial plane. The intermediate decomposes to products by the departure of R_1OH from its apical position. The stereochemical course dictated by this mechanism is as in eqn (2), inversion. In the other mechanism, illustrated in eqn (4), the leaving group is in the equatorial



(4)

plane while the attacking nucleophile is in an apical position of the first intermediate. In the formation and decomposition of such intermediates the attacking nucleophile enters at an apical position of the intermediate and the leaving group departs from an apical position. The leaving species R_1OH cannot, therefore, leave from the first intermediate in equation 4, since the R_1O– group is in the equatorial plane. The expulsion of R_1OH must be preceded by a pseudorotatory rearrangement to a second intermediate in which the two apical groups of the first intermediate become equatorial and two of the equatorial groups, specifically including $-OR_1$, become apical. The leaving group R_1OH then departs from its apical position in the second intermediate. The overall stereochemical course dictated by this mechanism involves retention of configuration at chiral phosphorus.

The pseudorotation in eqn (4) is governed by rules which correlate a large body of kinetic and spectroscopic data on a large variety of phosphorus compounds. The rules of pseudorotation are presented and discussed by Westheimer in two penetrating reviews of the literature dealing with pentacovalent phosphorus compounds and their pseudorotatory rearrangements.[1,3] According to these rules, the first intermediate in equation 4 can undergo pseudorotation in two ways. In the one shown, ^{18}O serves as the pivot, remaining equatorial in the second intermediate. The ^{16}O may also serve as the pivot, however, and in this case (not shown) the second intermediate has ^{16}O equatorial and ^{18}O apical. This difference is not reflected in the overall stereochemical course which involves retention of configuration by either pathway.

In over thirty stereochemical studies of enzymatic substitution at phosphorus in biological phosphates the findings uniformly indicate that each substitution proceeds by an in-line mechanism which inverts the configuration at a chiral phosphorus center.[4,5] This is consistent with eqns (2) and (3) but not with the adjacent attack mechanism of eqn (4), which has not been observed in enzymatic reactions. The metaphosphate mechanism of eqn (1) is not ruled out, however, because of the high probability that interactions of such an intermediate with the active site of an enzyme, which would protect it from capture by water, would also control the stereochemical course and prevent racemization. To explain the data on the basis of eqn 1 it must be postulated that all such interactions lead to in-line attack and inversion of configuration.

Since each enzymatic substitution proceeds with inversion, the overall stereochemical course provides information concerning the number of substitution steps in the catalytic pathway. An important question in phosphoryl group transfer reactions is whether catalysis involves a double or single displacement of the phosphoryl group. A few reactions follow double displacement pathways such as that represented by eqns (5) and (6), in which the group –XH is an enzymic nucleophile such as the ϵ-NH_2 of lysine, the β-OH of a serine, or the imidazole group of histidine. The symbol Ⓟ designates

$$\text{E–XH} + \text{A–Ⓟ} \rightleftharpoons \text{E–X–Ⓟ} + \text{AH} \tag{5}$$

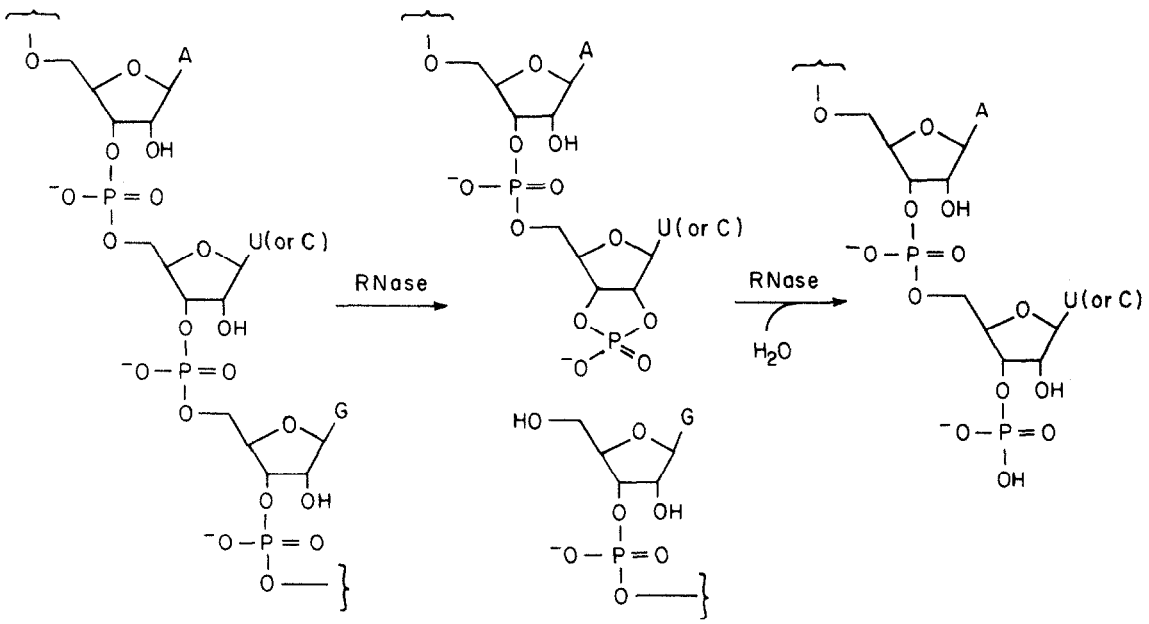
$$\text{E–X–Ⓟ} + \text{BH} \rightleftharpoons \text{E–XH} + \text{B–Ⓟ} \tag{6}$$

the phosphoryl or phosphoryl ester group being transferred from a molecule A to another molecule B. In the single displacement the reactants A–Ⓟ and BH are brought together by the enzyme and the phosphoryl group is transferred directly from A–Ⓟ to BH to produce AH and B–Ⓟ without covalent catalysis by an enzymic nucleophile. The double displacement pathway can be assigned when the intermediate E–X–Ⓟ can be isolated and structurally and kinetically characterized. However, in the majority of cases such a species cannot be detected or isolated. In these cases the only basis upon which to assign a single displacement pathway has until recently been failure to detect a double displacement. The usual nonstereochemical evidence could not provide positive indication of a single displacement. Such evidence is provided by the stereochemical test, since the rule for single enzymatic displacements is that they proceed with inversion of configuration, while double displacements proceed with net retention.

It is important to keep in mind that the stereochemical test has its limitations and cannot be the sole basis upon which to base mechanistic proposals. It can tell whether an even or odd number of inversions has occurred in the overall mechanism so that one could, in principle, observe inversion in the admittedly unlikely event of a triple or pentuple displacement. Moreover, the fact that pseudorotation leading to configurational retention has not been observed does not rule it out in future studies. The reactions so far studied are sufficiently numerous and varied in type to justify the formulation of the rule that single enzymatic displacements proceed with inversion, but exceptions to the rule may yet be found in special cases. Such exceptions would reflect special mechanistic aspects of those reactions without seriously undermining the rule.

2. RIBONUCLEASE

The first stereochemical study of an enzymatic P–O bond cleavage was that of Eckstein and Usher and their associates on pancreatic ribonuclease A. They showed that each of the two P–O bond cleaving steps catalyzed by this enzyme proceeds with inversion of configuration.

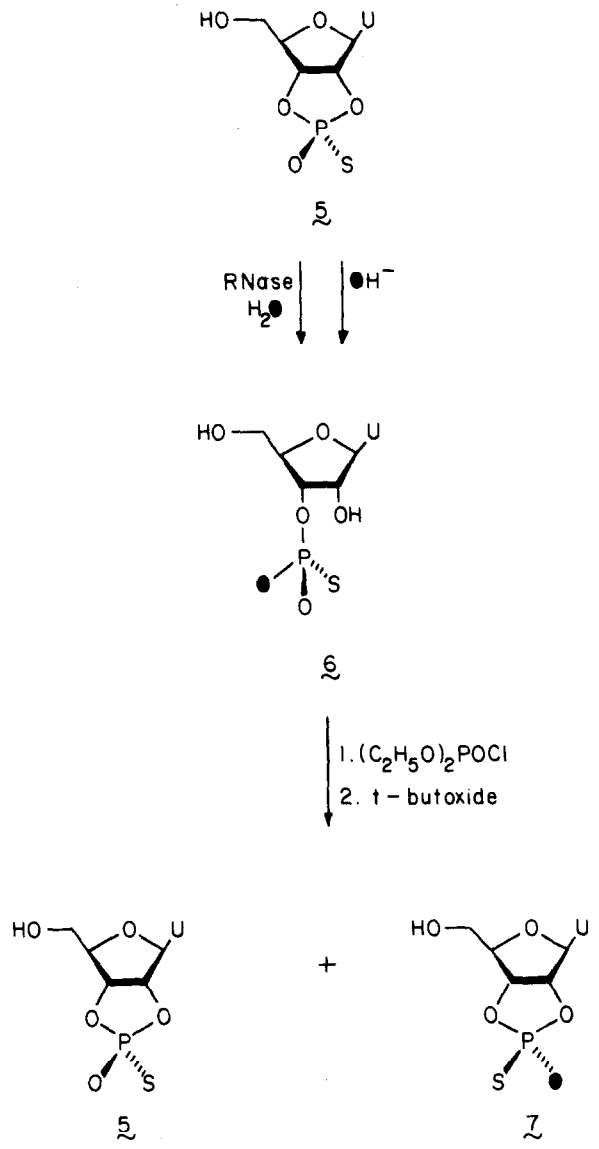


A = adenine , U = uracil , C = cytidine , G = guanine

Scheme 1.

Ribonuclease catalyzes the degradation of RNA by hydrolytic cleavage of the bond linking the 3′-ribosyl oxygen to its phosphodiester linkage specifically at pyrimidine sites of the polymer. The purine sites are not hydrolyzed. Scheme 1 shows the two steps in the overall hydrolysis. In the first step the enzyme catalyzes the attack of the 2′-OH of a uridyl or cytidyl residue on the neighboring phosphodiester, cleaving the polymer at that point and forming the 2′,3′-cyclic nucleotide at the pyrimidine end of the cleaved chain. In the second step the enzyme catalyzes the hydrolytic opening of the cyclic phosphodiester ring. The enzyme will cleave smaller pyrimidine substrates such as dinucleotides, and it will catalyze the hydrolysis of uridine or cytidine 2′,3′-cyclic phosphates to the corresponding 3′-phosphates.

Mechanisms involving pseudorotatory rearrangements such as that in equation 3 were first observed in reactions of cyclic phosphates. For that reason the ribonuclease reaction was an especially interesting and timely subject for stereochemical investigation. Eckstein and Usher and their associates addressed the issue of the possible involvement of a pseudorotatory step in the action of ribonuclease by performing a series of experiments designed to determine whether the formation and decomposition of the cyclic nucleotide intermediate proceeds with inversion or retention of configuration. Eckstein and Gindl has synthesized *endo*- uridine 2′, 3′-cyclic phosphorothioate **5** (in Scheme 2) by reaction of 5′-acetyl uridine with *tris* imidazolyl phosphine sulfide followed by aqueous workup and deblocking.[6] The mixture of *exo*- and *endo*- isomers produced was separable into its components by the fact that one isomer crystallized while the other remained an oil. The crystalline isomer was thereby purified and shown by



5 6 5 7

Scheme 2.

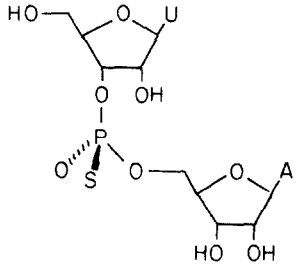
X-ray crystallographic analysis to be the *endo*- isomer **5** having the R_p configuration.[7] This isomer was shown to be hydrolyzed by the action of ribonuclease to uridine 3'-phosphorothioate.[8]

Usher *et al.* utilized **5** to show that the stereochemical course of its hydrolysis catalyzed by ribonuclease is the same as that of its hydroxide ion-catalyzed hydrolysis.[9] The strategy developed by Usher *et al.* is presented in Scheme 2. Compound **5** was subjected to both ribonuclease and hydroxide catalyzed hydrolysis in $H_2{}^{18}O$ to **6**, uridine 3'-[^{18}O]phosphorothioate. Recyclization of the two samples of **6** was carried out by first activating the [^{18}O]thiophosphoryl group with diethylphosphorochloridate and then generating the vicinal 2'-alkoxide ion with t-butoxide. The mixture of **5** and its *exo*-isomer **7** was obtained and separated into its components. Upon analysis for ^{18}O compound **5** was found to be essentially unenriched with ^{18}O while **7** contained essentially the same enrichment as that of **6**, regardless of whether ribonuclease or OH^- was used to catalyze the hydrolysis. According to the rules for pseudorotation the hydroxide catalyzed hydrolysis should have proceeded by an in-line mechanism without pseudoratation of the pentacovalent intermediate, i.e. with inversion of configuration. Since both reactions followed the same stereochemical course, the ribonuclease reaction must also have proceeded with inversion of configuration.

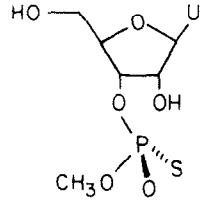
An internal confirmation of the stereochemical analysis was provided by the experimental findings for the ribonuclease reaction alone. Since the *endo*- isomer **5** resulting from the recyclization lacked ^{18}O, both the hydrolysis of **5**–**6** and its recyclization to **5** must have followed the same stereochemical course. According to the rules of pseudorotation the base catalyzed recyclization should have been an in-line reaction accompanied by configurational inversion,[1,3] so the hydrolysis must also have proceeded with inversion.

The foregoing method was subsequently applied to ribonucleases from two other sources and to a nonspecific phosphohydrolase.[10,12] Inversion was observed in these studies as well.

The first step of ribonuclease action in Scheme 1, the cleavage of the phosphodiester linkage to form the cyclic nucleotide intermediate, was also shown to proceed with inversion of configuration.[13] The dinucleoside phosphorothioate **8** was synthesized and assigned the R_p configuration on the basis of the fact that in its base catalyzed cyclization attack of the 2'-alkoxide at the chiral phosphorus led exclusively to **5**. By the rules for pseudorotation this cyclization should be an in-line displacement, so **5** could arise only from the R_p configuration in **8**. Compound **8** proved to be a substrate for ribonuclease, which also catalyzed its cyclization to **5** by an in-line mechanism with inversion of configuration.



8



9

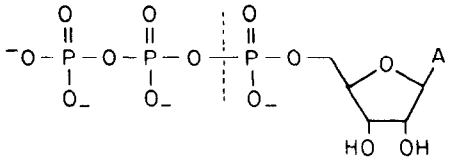
U = uracil

A = adenine

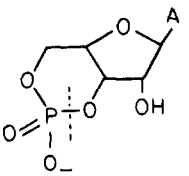
The conclusions in both of these studies were based on the rules for pseudorotation. They were confirmed directly in a third experiment in which **5** was subjected to ribonuclease catalyzed methanolysis in aqueous methanol to produce **9**, a methyl ester of uridine 3'-phosphorothioate. This was crystallized and subjected to X-ray crystallographic analysis; its configuration at phosphorus was found to be R_p, corresponding to inversion from that of **5**.[14] Inasmuch as methanol could be considered to be an analog of water in the hydrolysis of **5** or of adenosyl 5'-OH in the microscopic reverse of the cleavage of **8**, this experiment served to confirm the stereochemical findings in both of the preceding experiments.

3. NUCLEOTIDYL TRANSFER

Many enzymatic reactions involve cleavage of bonds of the type indicated in **10** and **11**. The reacting phosphorus atoms in these molecules are prochiral centers; they can be made chiral by substituting



10



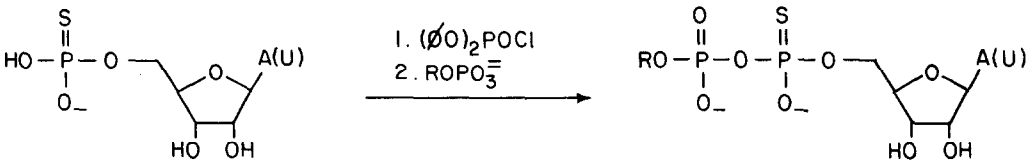
11

A = adenine

sulfur for one of the diastereotopic oxygens or by enriching one of them with ^{18}O or ^{17}O. Significant progress has been made in synthesising such molecules, which have been used as substrates to elucidate the stereochemical courses of nucleotidyl group transferring enzymes.

3.1 *Synthesis*

The α-thio analogs of ATP, ADP, UTP, UDP and UDP-glucose have been synthesized according to Scheme 3[15,16] starting with **12a** or **12b**, AMPS or UMPS, which were synthesized by thiophosphoryl-



A = adenine U = uracil

12a A = adenine
12b U = uracil

13a R = H
13b R = $HOPO_2$

14a R = H
14b R = $HOPO_2$
14c R = α-1-glucosephosphoryl

Scheme 3.

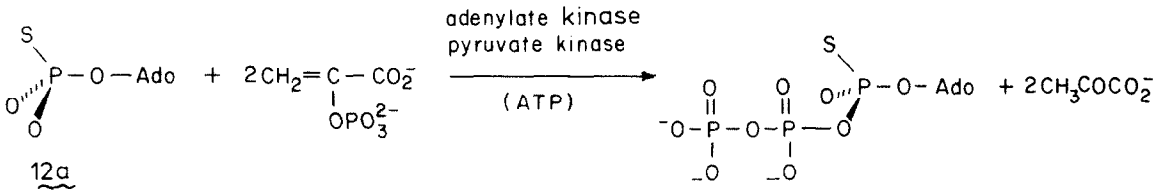
ation of adenosine or uridine with $PSCl_3$ in triethylphosphate followed by aqueous workup. α-Thionucleotides were synthesized by activating **12a** or **12b** by reaction with diphenylphosphorochloridate followed by condensation with phosphate to produce **13a** or **14a**, pyrophosphate to produce **13b** or **14b**, or α-D-glucose-1-phosphate to produce **14c**. The synthetic compounds consist of diastereomeric, actually epimeric, mixtures of isomers differing in configuration at the α-phosphorus atom.

The pure epimers can be obtained by enzymatic and chromatographic separation techniques, and they can be distinguished by their relative reactivities as substrates for enzymes.[15,16] They are also conveniently distinguished by their ^{31}P-NMR spectra.[19,20] The proton spin decoupled spectra of the epimers of **13a**, for example, consist of two doublets, one 7.00 ppm upfield from H_3PO_4 assigned to the β-phosphorus and a second downfield 40.65 or 40.95 ppm from H_3PO_4 assigned to the α-phosphorus. The two epimers exhibit identical P_β-chemical shifts but a 0.3 ppm difference in their P_α-chemical shifts. Similar differences in P_α-chemical shifts are exhibited by the other α-thionucleotides.

The absolute configurations at P_α of the epimers of **13a**, **13b**, **14a** and **14b** have been assigned by correlation with **8**,[21] which had been correlated as described above with **5**, whose crystal structure had been determined. Rabbit muscle pyruvate kinase catalyzes the phosphorylation by phosphoenolpyruvate of one isomer of **13a** to the corresponding isomer of **13b**.[15] The same enzyme also accepts one isomer of **14a**, converting it to a single isomer of **14b**.[16] These isomers of **13** and **14** presumably have the same configuration at P_α. This configuration was assigned S_p using snake venom phosphodiesterase as follows.[21] Venom phosphodiesterase catalyzes the hydrolysis of **8** (the R_p compound) to **12a** about 1800 times faster than the S_p isomer. The enzyme also catalyzes the hydrolysis of one epimer of **13b** to **12a** much faster than the other; this isomer was assigned the R_p configuration by analogy with **8**, and it was

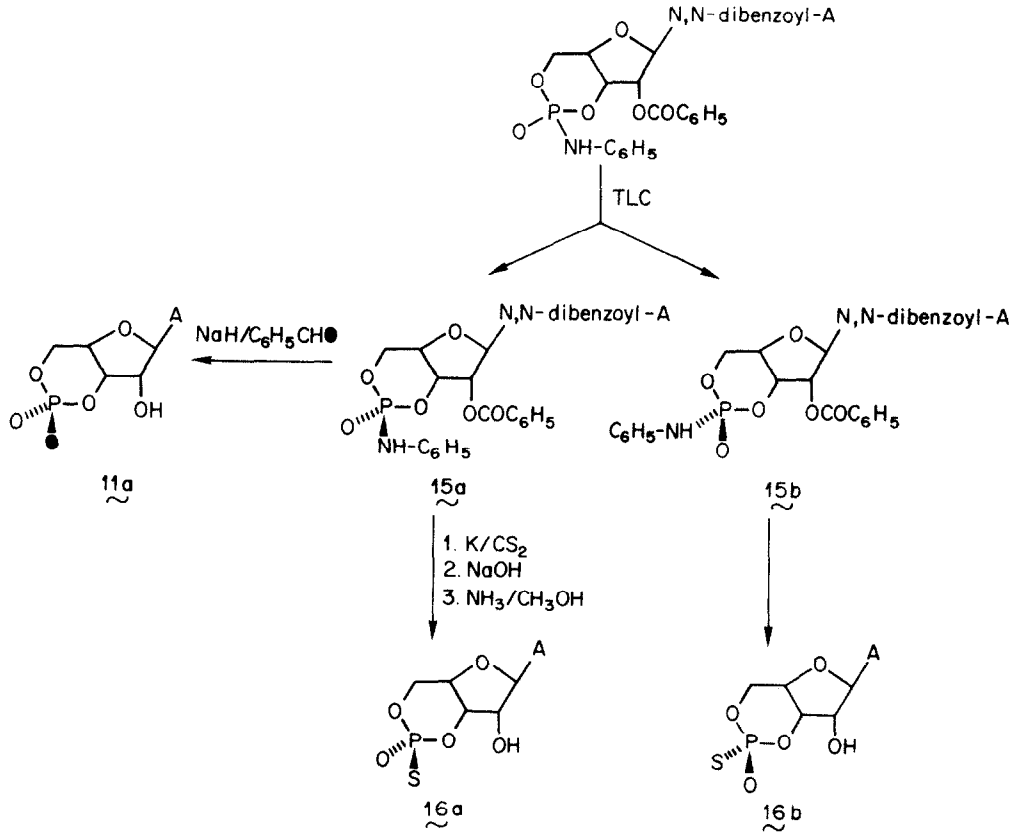
this isomer that was not a substrate for pyruvate kinase. The epimers of **13a** and **13b** which served as substrates for pyruvate kinase were therefore assigned the S_p configuration. Similar assignments were made for **14a** and **14b**. These assignments were confirmed by a stereospecific synthesis in which **12a** was synthesized with ^{18}O stereospecifically replacing the pro-R or pro-S ^{16}O of the phosphorothioate group. These compounds were used to show that upon phosphorylation to the diphosphate **13a**, the substrate for pyruvate kinase is the one in which the pro-R oxygen of **12a** is phosphorylated, i.e. the isomer of **13a** having the S_p configuration at P_α.[22]

The S_p epimer of **13b** is conveniently prepared from **12a** by stereospecific enzymatic phosphorylation according to eqn 7.[19] This reaction is a useful method for obtaining the S_p isomer in quantitative



(7)

yield from **12a**; however, highly stereoselective or stereospecific phosphorylation of nucleoside phosphorothioates has had a much broader impact in the field of phosphate stereochemistry because it has provided a means by which the configurations of chiral [^{18}O]phosphorothioates can be analyzed. The principle is general for enzymatic reactions. The thiophsophoryl group, as in **12a** and **12b**, is a prochiral center in contrast to the terminal phosphoryl group in **10**. A general property of enzymes is that they recognize the steric inquivalence of enantiotopic or diasterotopic groups; in catalyzing reactions involving such groups they generally act upon them with a high degree of stereoselectivity. Enzymatic phosphorylation of thiophosphoryl groups is a case in point; it has been widely used in the synthesis and configurational analysis of [^{18}O]phosphorothiotes.



Scheme 4.

Stereospecific syntheses of the epimeric thiophosphate analogs of cyclic nucleotides were devised by Stec and associates.[23-27] Epimeric adenosine-3′,5′-cyclic phosphoranilidates **15a** and **15b** were synthesized from the cyclic nucleotide and separated by tlc (Scheme 4).[23] Alternatively, epimeric nucleoside 3′- or 5′-O-arylphosphoranilidates were synthesized from blocked nucleosides and O-arylphosphoranilidochloridates, separated by chromatography, and cyclized stereospecifically by t-butoxide in DMF to chiral analogs of **15a** and **15b**.[27ab,28b] Treatment of phosphoranilidates with base followed by CS_2, CO_2, benzaldehyde or CSe_2 has been developed as a general method to replace the anilino group with S, O, or Se with *retention* of configuration (Scheme 4).[24-28] It has been used to prepare a variety of chiral cyclic nucleotides including the chiral ^{18}O-substituted cAMP, **11a**, and its 2′-deoxy analog.[26,28a]

3.2 *Nucleic acid polymerases*

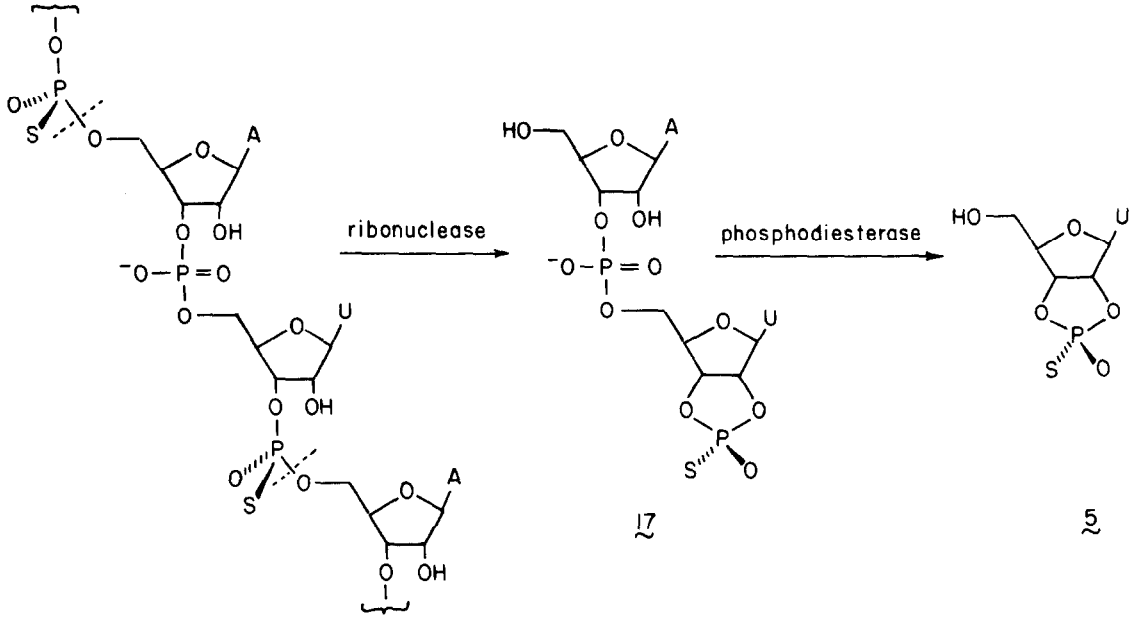
DNA-dependent RNA polymerase is a complex enzyme whose essential function is to transcribe the base sequence in a segment of DNA into a complementary base sequence of a messenger RNA molecule. The messenger RNA is transported to the sites of protein biosynthesis, and the information in its base sequence is translated into the amino acid sequence of a protein. Nucleoside triphosphates such as **10** are the substrates that serve as the nucleotide units in RNA, and the bond indicated in **10** is cleaved in the polymerization reaction. In the polymerization of triphosphates the enzyme requires a DNA segment that serves as a template for the base sequence in the newly synthesized RNA.

The stereochemical course of *Escherichia coli* RNA polymerase-catalyzed substitution at the α-phosphorus of nucleotides was determined by Eckstein and associates using **13b**, the α-thio analog of ATP, as a substrate.[21,29] The S_p epimer of **13b** was found to serve as a substrate and was used with uridine triphosphate (UTP) and a DNA template of poly d(AT), an alternating copolymer

$$\frac{n}{2}\,\mathbf{13b} + \frac{n}{2}\,\mathrm{UTP} \xrightarrow[\text{-dApdTpApdTp-}]{\text{RNA polymerase}} \text{+(Up(S)ApUp(S)Ap)+}_{n/4} + n(\mathrm{PPi}) \qquad (8)$$

of 2′-deoxyadenosine and 2′-deoxythymidine in 3′,5′-phosphodiester linkage (see eqn 8). The resulting RNA polymer was the complementary alternating copolymer of adenosine and uridine linked by alternating 3′,5′-phosphodiester and 3′,5′-thiophosphodiester bonds.

The configuration at the 3′,5′-*thio*phosphodiester linkages was shown to be R_p, resulting from inversion of the α-phosphorus in the S_p epimer of **13b**, by the procedure outlined in Scheme 5.[29] Treatment of the polymer with ribonuclease produced a dinucleotide containing adenine and uridine by cleavage at the pyrimidine sites. The specificity of ribonuclease, described above, required that the structure of the dinucleotide would be **17** (Scheme 5), resulting from cleavage of R_p thiophosphodiester

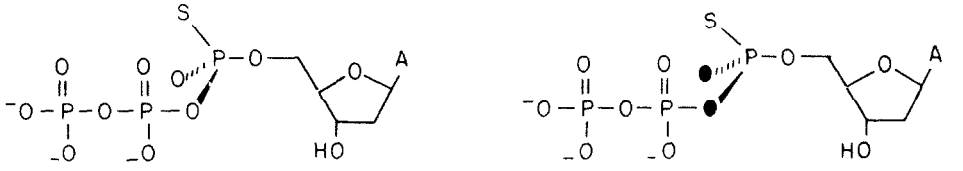


Scheme 5.

linkages by the known in-line mechanism. That this was indeed the case was verified by further degrading the product dimer with spleen phosphodiesterase to **5**, which was crystallized and characterized.

The same procedure as also used to show that the addition of the AMP residue at the 3′-end of transfer RNA catalyzed by tRNA nucleotidyltransferase proceeds with inversion of configuration.[30]

DNA polymerase I from *Escherichia coli* catalyzes the polymerization of deoxynucleoside triphosphates in the presence of a primer-template. This reaction was shown by two methods to proceed with inversion of configuration. The enzyme utilizes the α-thio analog of dATP **18a** (S_p) as efficiently as the



natural substrate. Burgers and Eckstein used DNA polymerase to polymerize this substrate with poly-dT as the primer template according to eqn (9).[31]

$$\text{n } \mathbf{18a} \xrightarrow[\substack{\text{-dTpdTpdTPDT-}\\\text{(primer-template)}}]{\text{DNA polymerase}} \{\text{dAp(S)dAp(S)dAp(S)dAp(S)}\}_{n/4} + \text{n(PPi)} \qquad (9)$$

The product was found to be susceptible to hydrolytic degradation by snake venom phosphodiesterase. Since venom phosphodiesterase had been shown to exhibit a strong preference for the R_p configuration in dinucleotide substrates such as **8**, it was concluded that the configurations of the 3′,5′-thiophosphodiester linkages in the polymerization product were also R_p, corresponding to inversion of the configuration in **18a**.

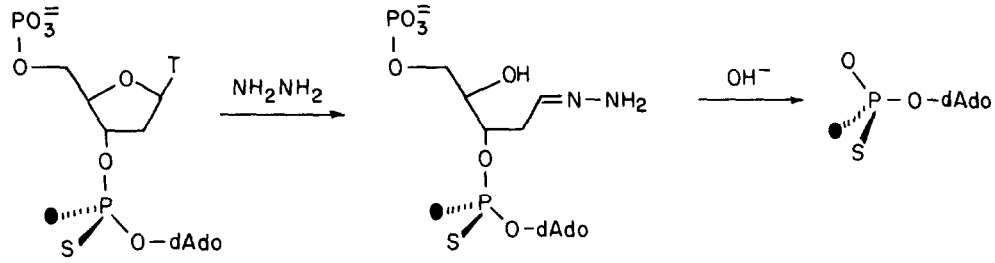
An ambiguity existed in the foregoing study in that venom phosphodiesterase is not absolutely specific and had not previously been used with polymeric thiophosphates. The enzyme had been shown to catalyze the hydrolysis of **8** 1770 times faster than its S_p epimer, and the assignment of the R_p configuration in the enzymatic polymer rested essentially on this rate difference. However, no such polymer known to have the S_p configuration at the thiophosphodiester linkages was available for kinetic comparison, so it could not be known at what rate the enzyme might act on such a polymer. The analysis, therefore, rested on the reasonable but unproven assumption that the relative rates observed for the R_p and S_p configurations in polymeric substrates would be similar to those for dimeric substrates.

That the DNA polymerase reaction does proceed with inversion of configuration was confirmed in a second study.[18] The chiral substrate was **18b**, which had been prepared by stereospecific enzymatic phosphorylation of 2′-deoxyadenosine 5′-[^{18}O]phosphorothioate according to eqn (7).[19] A polymerization was carried out with **18b** and dTTP as copolymerization substrates using poly d (AT) as the primer-template according to eqn (10). The pyrophosate produced was shown to contain one ^{18}O per molecule. The alternating copolymer was then degraded to the dimer **19** (Scheme 6) by the nucleolytic activity of DNA polymerase. This dimer was degraded to 20 by treatment with hydrazine to remove thymine followed by β-elimination in base. The configuration of **20** was established by again phosphorylating it enzymatically according to eqn (7) and again polymerizing it according to eqn (10). The pyrophosphate produced again contained one ^{18}O per molecule, and the polymer was free of ^{18}O. From this analysis, the ^{18}O in **20** was in the R position, but it must have been in the S position of **18b**. Inasmuch as the only bond to phosphorus cleaved in the transformation of **18b–20** was cleaved in the polymerization reaction, the configuration must have been inverted by the action of DNA polymerase.

$$\frac{n}{2}\,\mathbf{18b} + \frac{n}{2}\,\text{dTTP} \xrightarrow[\substack{\text{-dApdTpdApdT-}\\\text{(primer-template)}}]{\text{DNA polymerase}} -(\text{dTp(S, }^{18}\text{O)dApdTp(S, }^{18}\text{O)dAp})_{-n/4} + \text{n(PPi}^{18}\text{O)} \qquad (10)$$

3.3 *Uridylyltransferases*

The stereochemical courses of two uridylyltransferases were determined using α-thionucleotides as

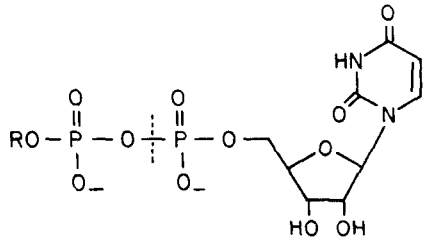


19 20

dAdo = 2′-deoxyadenosine
T = thymine

Scheme 6.

substrates and ^{31}P-NMR for configurational analysis.[16,32] Uridine diphosphate glucose pyrophosphorylase (UTP: α - D-glucose-1-phosphate uridylyltransferase) and galactose-1-phosphate uridylyltransferase (UDP-glucose: α - D-galactose-1-phosphate uridylyltransferase) catalyze the transfer of the uridylyl group in compounds **21** to phosphate acceptors with cleavage of the indicated bond. UDP-glucose **21b**



21a R = $PO_3^{=}$
21b R = α-D-glucosyl
21c R = α-D-galactosyl

arises in living cells mainly by the action of UDP-glucose pyrophosphorylase on UTP **21a** and α - D-glucose-1-phosphate, and the metabolism of galactose is dependent upon the action of galactose-1-phosphate uridylyltransferase in catalyzing the reaction of UDP-glucose **21b** with α-D-galactose-1-phosphate to produce UDP-galactose **21c** and α-D-glucose-1-P.

The α-thionucleotides **14b** and **14c** were synthesized and used as substrates for these enzymes. The stereochemical course of uridylyl transfer catalyzed by UDP-glucose pyrophosphorylase from yeast was determined by submitting the synthetic mixture of S_p and R_p epimers of **14b** along with the cosubstrate α - D-glusose-1-phosphate to the action of the enzyme. A maximum of approximately half of the **14b** reacted, indicating that one of the two isomers had been converted to **14c**. This was confirmed by separating the α-thionucleotides chromatographically and submitting each of them to configurational analysis by ^{31}P-NMR. The unreacted **14b** was found to have the S_p configuration, which meant that the R_p isomer had reacted. The **14c** produced was degraded to the corresponding isomer of **14a** by mild acid hydrolysis, which removed the glycosyl group without affecting the configuration at P_α. The configuration in the resulting **14a** was shown to be S_p, corresponding to inversion in the transferase reaction. This result was confirmed in the reverse direction by reacting the synthetic mixture of S_p and R_p epimers of **14c** with pyrophosphate. Approximately half of the **14c** reacted to form **14b**. The unreacted **14c** was found after hydrolysis to **14a** to have the R_p configuration, confirming that the S_p isomer had reacted. The isomer of **14b** produced was found by ^{31}P-NMR analysis to have the R_p configuration at P_α, confirming inversion of this S_p configuration in the precursor.

A similar analysis was applied to the galactose-1-phosphate uridylytransferase from *Escherichia coli*. The preceding study paved the way by providing pure samples of the R_p and S_p epimers of **14c**, the α-thio analog of UDP-glucose **21b**. The S_p epimer was unreactive; but the enzyme accepted the R_p epimer as a good substrate. This isomer was reacted with α - D-galactose-1-phosphate in the presence of

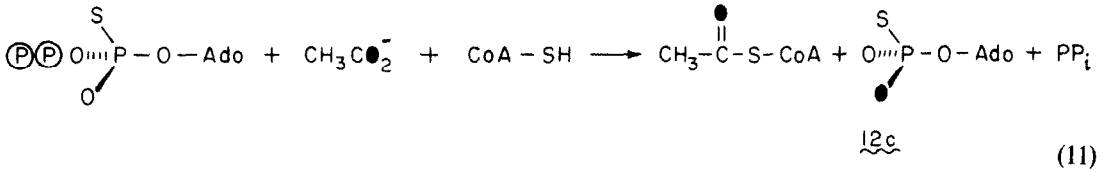
the enzyme, producing the galactosyl analog of **14c**, i.e. the α-thio analog of **21c**. This product was hydrolyzed in dilute acid to remove the galactosyl group, and the configuration at P_α in the resulting sample of **14a** was found by ^{31}P-NMR analysis to be R_p, the same as the configuration of the substrate from which it was derived. It was concluded that uridylyl transfer had in this case proceeded with retention of configuration.

The stereochemical findings for these ezymes, which catalyze chemically similar reactions, constituted unequivocal proof of a fundamental difference in their mechanisms of action. Inversion by UDP-glucose pyrophosphorylase was taken as positive evidence of a single displacement mechanism; and retention by galactose-1-phosphate uridylyltransferase confirmed the double displacement mechanism which had been assigned on the basis of the reaction kinetics and the isolation and characterization of the intermediate uridylylenzyme.[33-36]

3.4 *Acetyl-coenzyme A synthetase*

Acetyl-coenzyme A synthetase from yeast catalyzes the reaction of acetate with ATP and coenzyme A to produce acetyl coenzyme A, pyrophosphate and adenosine monophosphate, AMP. When the reaction is carried out with [^{18}O]acetate, the ^{18}O is equally partitioned between acetyl coenzyme A and AMP. Partitioning is understood to result from the mechanism by which acetate is activated for reaction with the thiol group of coenzyme A. The enzyme first catalyzes the reaction of [^{18}O]acetate with the α-phosphorus of ATP, cleaving the bond indicated in **10** by displacement of pyrophosphate concomitant with the formation of acetyl adenylate. The thiol group of coenzyme A then displaces AMP from the activated acetyl group.

Midelfort and Sarton-Miller used **13b**, the α-thio analog of ATP, to show that the displacement of pyrophosphate by acetate proceeds with inversion of configuration.[37] The R_p isomer of **13b** was used as the substrate in place of ATP and converted enzymatically by reaction with [^{18}O]acetate and coenzyme A to **12a**, the thio analog of AMP, which was shown to contain ^{18}O from [^{18}O]acetate. This product was found to have the R_p configuration of **12c** in eqn (11). In the configurational analysis the R

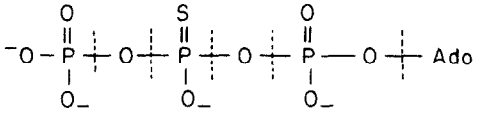


12c (11)

oxygen in **12c** was enzymatically phosphorylated as in eqn (7) and the product analyzed for bridging and nonbridging ^{18}O. The ^{18}O was found to be exclusively bridging, verifying the R_p configuration in **12c**.

The analysis for bridging and nonbridging ^{18}O developed and used in this study involved the systematic degradation of the S_p epimer of **13b**. The compound was cleaved enzymatically to pyrophosphate and **12a**, AMPS. These two compounds were further degraded to trimethylphosphate which was analyzed for ^{18}O by the gas chromatographic-mass spectroscopic (GC–MS) technique. Since all of the ^{18}O was found to be associated with pyrophosphate, it must have been bridging in the sample of **13b** produced by enzymatic phosphorylation of **12c**. This method has been used in a number of other studies to determine the configuration of ^{18}O-enriched AMPS.

An alternative and more general GC–MS method was developed to determine whether ^{18}O is bridging or nonbridging in the thio analogs of ATP, **22**.[38] The method is applicable to cases such as the above as



22

well as to determining whether ^{18}O bonded to P_β of the β-thio analog of ATP is bridging or non-bridging. In the procedure **22** (or **13b**) is chemically degraded to α- or β-thio tripolyphosphate, methylated with diazomethane, and solvolyzed in methanol to a mixture of trimethylphosphate and trimethylphosphorothioate. These compounds are subjected to GC–MS analysis. The degradation involves the cleavages indicated in **22**. Note that bridging oxygen is partitioned between trimethylphosphate and

trimethylphosphorothioate whereas nonbridging oxygen is associated exclusively with trimethylphosphorothioate.

The GC–MS methods are effective for small samples; however, two ^{31}P-NMR techniques are available for larger samples. One utilizes the effect of ^{18}O on the ^{31}P chemical shift of phosphorus to which it is bonded. The ^{18}O isotope induces a small upfield shift in the NMR signal of any ^{31}P atom to which it is bounded.[39,40] The magnitude of the isotope shift is dependent upon the order of the ^{18}O–^{31}P bond, 0.02–0.03 ppm for double bonded ^{18}O and < 0.02 ppm for single bonded (bridging) ^{18}O in aqueous solution. These isotope shifts can be detected with high field instruments.

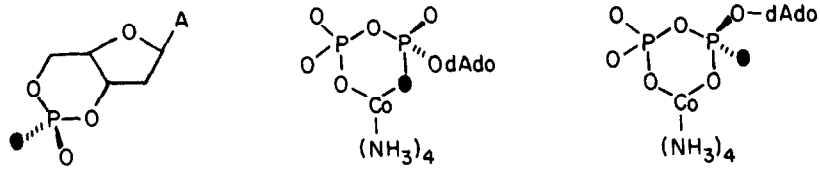
Another NMR technique was developed by M. D. Tsai in his study confirming the stereochemical course of the acetyl coenzyme A synthetase reaction. His method takes advantage of the interaction of the electric quadrupole of the ^{17}O nucleus interacting with the electric dipole of ^{31}P when the two are spin–spin coupled.[41] The quadrupole of ^{17}O rapidly relaxes any ^{31}P nucleus to which it is bonded and thereby broadens the ^{31}P signal to such degree that it is effectively silent in the ^{31}P spectrum.[37] When ^{17}O is bridging two phosphorus atoms, it broadens both signals; and when it is nonbridging, it broadens only the one signal. Tsai repeated the experiment of eqn (11) using [^{17}O]acetate in place of [^{18}O]acetate and confirmed the result of Midelfort and Sarton-Miller by showing that ^{17}O in the enzymatic phosphorylation product of **12c** broadened both the P_α and P_β NMR signals.

3.5 *Adenylyl cyclase*

Adenylyl cyclases catalyze the internal displacement of pyrophosphate in ATP, **10**, by the 3′-OH group, producing 3′,5′-cyclic AMP, **11**. Many adenylyl cyclases are membrane-bound proteins which are under hormonal control. The hormonal messages are relayed to regulated enzymes of metabolism by **11**, which stimulates or depresses their activities and thereby controls the flow of metabolism. This role of **11** has led to its recognition as a "second messenger" of hormonal regulation.

The stereochemical course of a bacterial adenylyl cyclase which is not under hormonal control has been shown to involve inversion of configuration at the α-phosphorus in **10**.[42] The S_p epimer of **13b**, the α-thio analog of ATP, was prepared and shown to be a good substrate for the adenyl cyclase from *Brevibacterium liquefaciens*. The ^{31}P-NMR spectrum of the cyclization product was compared with those of the thio analogs of **11**, which had been synthesized by the procedure in Scheme 4, and shown to have the R_p configuration of **16b**. This result showed that the cyclization had proceeded with inversion of configuration.

The foregoing was confirmed using **23**, the 2′-deoxy analog of **11a**, as the chiral substrate.[43] **23** was synthesized by the general procedure given in Scheme 4 for **11a** and used as the substrate for adenylyl cyclase. With pyrophosphate as the cosubstrate it was converted to ^{18}O-enriched 2′-deoxy ATP and then by enzymatic dephosphorylation to [α-^{18}O]ADP. To determine the configuration at P_α the [α-^{18}O]ADP was converted to the coordination exchange-inert Co(III) tetrammine complexes **24a** and **24b**. The



23 24a 24b

dAdo = 2′-deoxyadenosine

configurations and ^{31}P-NMR spectra of these complexes had been assigned,[44–46] so the isotope shift effect of ^{18}O could be used to determine in which of them ^{18}O was bridging and in which it was nonbridging. It was found to be bridging in **24a** and non-bridging in **24b**, corresponding to the S_p configuration in [α-^{18}O]2′-deoxy ATP, and inversion in the enzyme-catalyzed ring-opening of **23**.

3.6 *RNA ligase*

RNA and DNA ligases catalyze the formation of 5′,3′-phosphodiester linkages between oligonucleotides. The ligases play essential roles in DNA replication, recombination, and repair and in RNA processing. The mechanism by which these enzymes catalyze the joining of nucleic acid fragments can

be represented by eqns (12)–(14), in which ligase represents the enzyme, $p(Np)_n$ represents an oligonucleotide with the 5′-OH end phosphorylated, and $(Np)_mN$

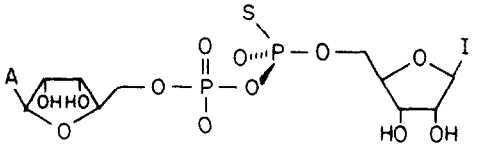
$$\text{ATP} + \text{ligase} \rightleftharpoons \text{AMP-ligase} + \text{PPi} \tag{12}$$

$$\text{AMP-ligase} + p(NP)_n \rightleftharpoons App(Np)_n + \text{ligase} \tag{13}$$

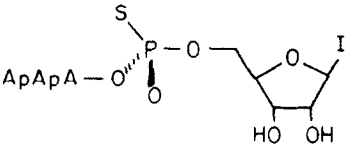
$$App(Np)_n + (Np)_mN \rightleftharpoons (Np)_mNp(Np)_n + \text{AMP} \tag{14}$$

represents an oligonucleotide with both the 5′- and 3′-OH ends unphosphorylated. In the first step a nucleophilic group of the enzyme displaces pyrophosphate from ATP, forming a covalent bond to the α-phosphorus of the nucleotide. The intermediate AMP-ligase then reacts with the 5′-phosphate end of $p(Np)_n$, forming the phosphoanhydride in $App(Np)_n$ and displacing the ligase. The enzyme then catalyzes the reaction of the 3′-OH group in $(Np)_mN$ with the phosphoanhydride to produce the new phosphodiester linkage and release AMP.

The third step of the bacteriophage T7 RNA ligase reaction has been shown to proceed with inversion of configuration.[47] The compound **25** was synthesized as a model for $App(Np)_n$ and used as the donor substrate with the trinucleotide ApApA serving as the acceptor $(Np)_mN$ in eqn (14). The product **26** was purified, characterized, and shown to have the R_p configuration by the fact that snake venom



25



26

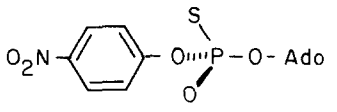
A = adenine I = inosine

ApApA = adenine trinucleotide

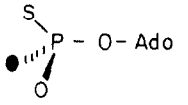
phosphodiesterase catalyzed its hydrolysis. Venom phosphodiesterase was known to exhibit a strong preference for the R_p configuration in dinucleoside phosphorothioates. Moreover, under conditions in which **26** was hydrolyzed by the enzyme the S_p diastereomer of Ap(S)A was not attacked.

3.7 *Nucleotide phosphodiesterases*

Snake venom phosphodiesterase catalyzes the cleavage of nucleotide phosphodiesters with overall retention of configuration at phosphorus. Bryant and Benkovic used this enzyme to catalyze the hydrolysis of the R_p diastereomer of **13b**, the α-thio analog of ATP, in $H_2{}^{18}O$, producing a sample of AMPS, ^{18}O which they subjected to enzymatic stereospecific phosphorylation according to eqn (7). Analysis of the phosphorylation product for bridging and nonbridging ^{18}O showed that the hydrolysis product had the S_p configuration, i.e. its structure was **12d**.[48] Burgers *et al.* obtained the same result in an analogous study in which they used **27** as the substrate in $H_2{}^{18}O$. The product was again shown to be **12d**.[49]



27

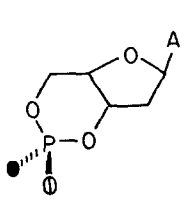


12d

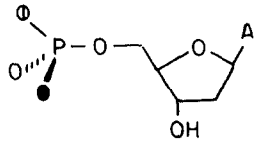
3′,5′-Cyclic AMP phosphodiesterase catalyzes the hydrolysis of **11** to AMP with inversion of configuration at phosphorus. This stereochemistry was established by using both the phosphorothioate analog of cyclic AMP and the chirally ^{18}O-enriched 3′,5′-cyclic 2′-deoxy AMP as substrates. In the first study the sulfur analog **16a** in Scheme 4 was subjected to cAMP phosphodiesterase-catalyzed hydrolysis in $H_2{}^{18}O$[50]. The resulting ^{18}O AMPS was subjected to configurational analysis as described in Section 3.5 and shown to be **12d**, the same isomer produced above by venom phosphodiesterase. Both **16a** and **12d**

are S_p diastereomers by the rules for assigning R, S symbols, but they have the opposite configurations at phosphorus.

Inversion by cAMP phosphodiesterase has recently been confirmed using **28**, the 2'-deoxy analog of **11a** as the substrate.[51] **28** was synthesized by the phosphoroanilidate method shown in Scheme 4 for **11a** and subjected to enzymatic hydrolysis in $H_2{}^{16}O$. the product was shown to be **29**, the R_p isomer of

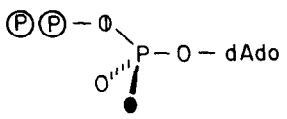


28

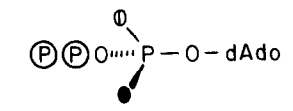


29

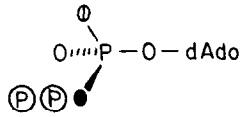
[^{16}O, ^{17}O, ^{18}O]AMP. The stereochemical analysis exploited the ^{18}O-induced isotope shift and the ^{17}O-induced broadening of the phosphorus NMR signal. These effects had been elegantly combined by Webb and Trentham in their configurational analysis of **4** and by Buchwald and Knowles in their configurational analysis of chiral [^{16}O, ^{17}O, ^{18}O]phosphate esters (see Sections 4.3 and 4.4). **29** was phosphorylated enzymatically to the mixture of **30a**, **30b** and **30c**. This mixture was cyclized with inversion of configuration to the mixture of **31a**, **31b** and **31c** by the action of adenylyl cyclase. **31b** and **31c** were silent in the phosphorus NMR spectrum because of the presence of ^{17}O; **31a** was the major species visible in the spectrum.† The configuration of **31a** could be assigned by the same method by which the configuration of **11a** had originally been assigned. This involved alkylation to the mixture of axially and equatorially alkylated triesters, in this case with diazomethane to **32a** and **32b**. The phosphorus NMR signals of axial and equatorial



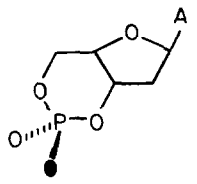
30a



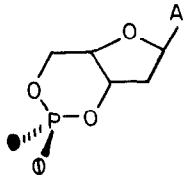
30b



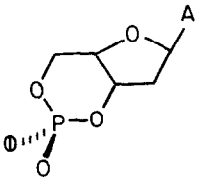
30c



31a

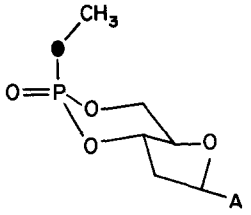


31b

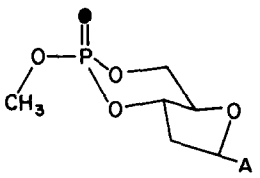


31c

alkylated triesters are well separated, and the magnitudes of the ^{18}O isotope shifts in **32a** and **32b** clearly showed that ^{18}O was axial in **32a** and in **32b**. This corresponded to the R_p configuration in **31a**, the same as that in **28**. Since cyclization by adenylate kinase had inverted the configurations in **30**, and this was the only step



32a



32b

†Other species were also present in lesser amounts because the position represented as ^{17}O in **28** was in reality enriched with *both* ^{17}O and ^{18}O. This did not undermine the analysis because the percent ^{17}O and ^{18}O enrichment were known and taken into account.

of the procedure other than cAMP phosphodiesterase that involved bond cleavage at phosphorus, identical configurations in **28** and **31a** could only have resulted from inversion by cAMP phosphodiesterase.

4. PHOSPHORYL TRANSFER

The steric courses of phosphotransferase reactions have been unmasked using chiral [^{18}O]phosphorothioates and [^{16}O, ^{17}O, ^{18}O]phosphates as phosphoryl donor substrates. The methodologies were developed concurrently and led quickly to the elucidation of the stereochemistries of a number of phosphoryl group transfer reactions which are discussed in the following sections.

4.1 *Glycerokinase, hexokinase and pyruvate kinase*

Glycerokinase, hexokinase and pyruvate kinase catalyze eqns (15), (16) and (17), respectively. Knowles and associates showed in a two-stage study that

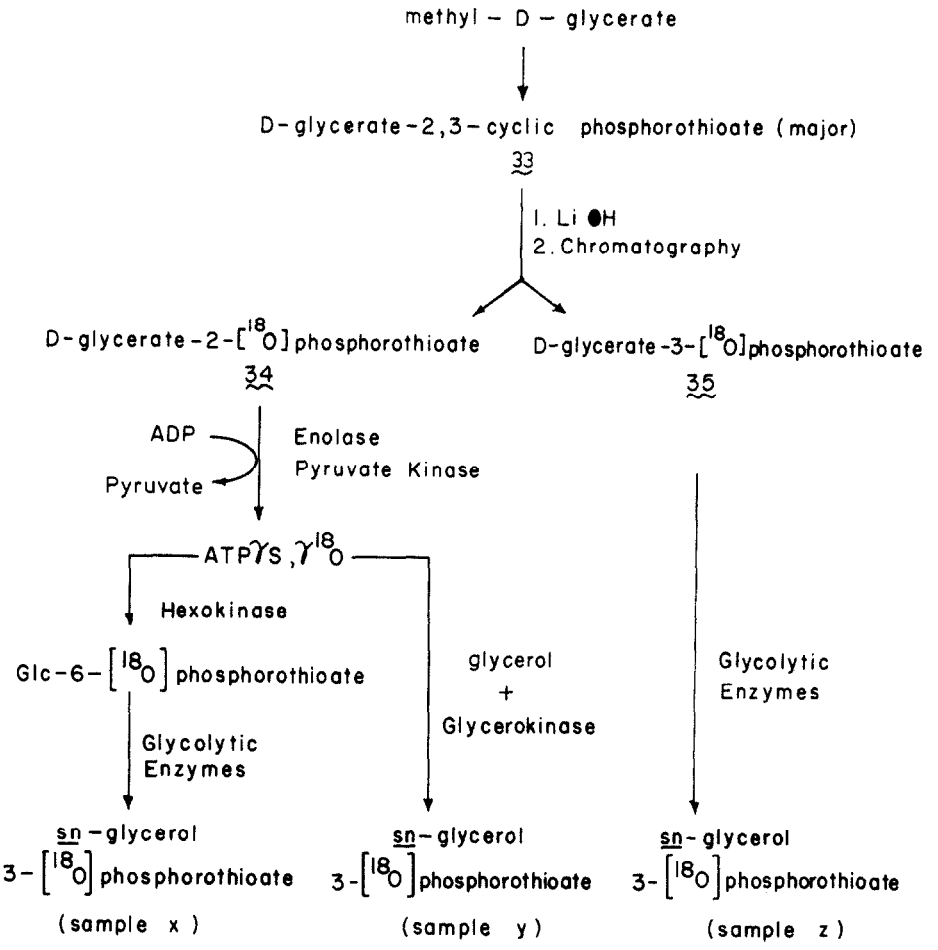
$$\text{Glycerol} + \text{ATP} \rightleftharpoons \text{Glycerophosphate} + \text{ADP} \quad (15)$$

$$\text{Glucose} + \text{ATP} \rightleftharpoons \text{Glucose-6-phosphate} + \text{ADP} \quad (16)$$

$$\text{2-Phosphoenolpyruvate} + \text{ADP} \rightleftharpoons \text{Pyruvate} + \text{ATP} \quad (17)$$

these reactions proceed with inversion of configuration at chiral phosphorus centers.

In their first experiment they showed that the three enzymes catalyze the stereospecific transfer of the [^{18}O]thiophosphoryl group with the same stereochemical consequences, either inversion or retention.[52] Following the strategy outlined in Scheme 7 they reacted methyl-D-glycerate with PCl_3 and sulfur

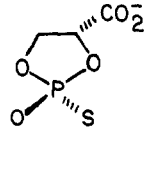


Scheme 7.

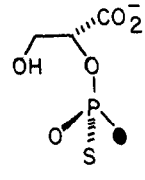
followed by aqueous work up and deesterification, affording a mixture of epimeric D-glycerate-2,3-cyclic phosphorothioates differing in configuration at phosphorus. These were separated by chromatography. The major isomer **33** was hydrolyzed with $Li^{18}OH$ to a mixture of D-glycerate-2-[^{18}O]phosphorothioate **34** and D-glycerate-3-[^{18}O]phosphorothioate **35**. By the rules of pseudorotation this hydrolysis was known to follow an in-line steric course so that, although their absolute configurations were unknown, **34** and **35** had opposite relative configurations at phosphorus. Compounds **34** and **35** were converted by three different enzymatic routes to three samples x, y and z of sn-glycerol-3-[^{18}O]phosphorothioate. **35** was transformed directly into sample z by the use of glycolytic enzymes which did not affect the configuration of the [^{18}O]phosphorothioate group. The [^{18}O]phosphorothioate group in **34** was transferred by the coupled actions of enolase and pyruvate kinase to ADP, forming ATPγS, γ^{18}O which was used to prepare samples x and y by two different routes. For sample y, glycerokinase catalyzed the transfer of the [^{18}O]thiophosphoryl group to glycerol according to eqn (15). Hexokinase catalyzed the transfer to glucose according to eqn (16), and glucose-6-[^{18}O]phosphorothioate was further transformed to sample x of sn-glycerol-3-[^{18}O]phosphorothioate by the actions of glycolytic enzymes which cleaved and reduced the carbon skeleton without affecting the configuration of the [^{18}O]phosphorothioate group.

The configurations about phosphorus in samples x, y and z were compared by a procedure analogous to that of Scheme 2 employed by Usher *et al.* in their study of ribonuclease. Each sample was activated with diethylphosphorochloridate and cyclized in base to the mixture of *syn*- and *anti*-glycerol-2,3-cyclic phosphorothioates. The isomers were separated by chromatography and analyzed for their ^{18}O contents. Samples x and y were found to have the same configuration while sample z had the opposite configuration. Inasmuch as **34** and **35** had opposite configurations at phosphorus and the transformation of **35** to sample z could not have altered its phosphorus configuration, the [^{18}O]phosphorothioate in **34** must have been transferred to samples x and y with net retention of configuration. The latter transformations each involved two transfers of the [^{18}O]thiophosphoryl group in **34**, both enzymatic and both involving pyruvate kinase. They differed in that the transfer from ATPγS, γ^{18}O involved glycerokinase for sample y and hexokinase for sample x. Since net retention was observed by both routes, the two transfers in each route must have followed the same steric course; and since pyruvate kinase was common to the two routes, all three transfers followed the same steric course, either inversion or retention.

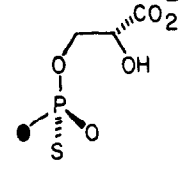
In the second stage the glycerokinase reaction was shown to proceed with inversion of the configurations of chiral [^{16}O, ^{17}O, ^{18}O]phosphate and [^{18}O] thiophosphate groups.[53,54] The method developed by Knowles and associates for the [^{16}O, ^{17}O, ^{18}O]phosphate experiment is described in section 4.3. For the [^{18}O]phosphorothioate study Scheme 7 was completed with two additional bits of information.[54] First, the absolute configuration of the major isomer of **33** was determined by crystallographic analysis to be **33a**. According to the rules of pseudorotation, the alkaline hydrolysis of **33** to **34**



33a



34a



35a

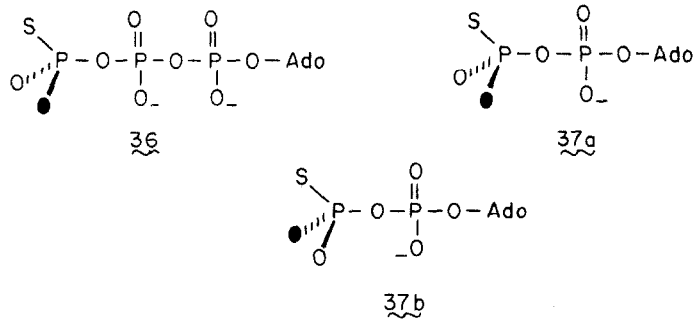
and **35** proceeds by an in-line mechanism so that the structure of **33a** dictated the phosphorus configurations of the $Li^{18}OH$ hydrolysis products to be those of **34a** (S_p) and **35a** (R_p). Therefore, the configuration in sample z of sn-glycerol-3-[^{18}O]phosphorothioate derived from **35a** was also R_p and that in samples x and y was S_p.

The second bit of information required to complete Scheme 8 was the phosphorus configuration in ATPγS, γ^{18}O. This proved to be R_p by the following analysis. An authentic sample of R_p ATPγS, γ^{18}O synthesized as described in section 4.2, structure **36**, when used as a substrate for glycerokinase, produced a sample of sn-glycerol-3-[^{18}O]phosphorothioate whose phosphorus configuration proved to be S_p by correlation with **35a**, the same as that of samples x and y in Scheme 7. This meant that the glycerokinase reaction, and therefore the pyruvate and hexokinase reactions as well, proceed with inversion of configuration at phosphorus.

4.2 *Nucleotide and nucleoside kinases*

The steric courses of [^{18}O]thiophosphoryl group transfers catalyzed by adenylate kinase, nucleoside diphosphate kinase, nucleoside phosphotransferase, and adenosine kinase were uncovered in this laboratory by an alternative method. The strategy followed with these enzymes involved the synthesis of substrates with chiral [^{18}O]phosphorothioate groups. The experimental design was such that knowledge of their absolute configurations was never required, since in each case the relative configurations in substrates and products could always be correlated; however, by the time of the completion of the syntheses the absolute configuration of the synthetic precursor of the chiral [^{18}O]phosphorothioates had been determined so that the configurations were, in fact, known to be as shown in the structures given below.

Adenylate kinase catalyzes eqn (18), the transfer of the terminal phosphoryl group in ATP to the phosphate in AMP to form two molecules of ADP. The enzyme plays an important role in the functions of muscle and other tissues by maintaining balance in the adenine nucleotide pool. The following strategy was devised for uncovering the steric course of this reaction.[55] The γ-thio, γ-^{18}O analog of ATP **36** and the β-thio, β-^{18}O analogs of ADP **37A** and **37b**, all chirally enriched with ^{18}O and having known



Ado = Adenosine

relative configurations, would be synthesized. **36** would then be used as the phosphoryl donor substrate

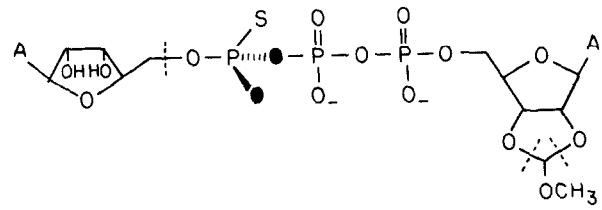
$$\text{ATP} + \text{AMP} \rightleftharpoons \text{ADP} + \text{ADP} \tag{18}$$

$$\mathbf{36} + \text{AMP} \rightleftharpoons \text{ADP} + \text{ADP}\beta\text{S}, \beta^{18}\text{O} \tag{19}$$

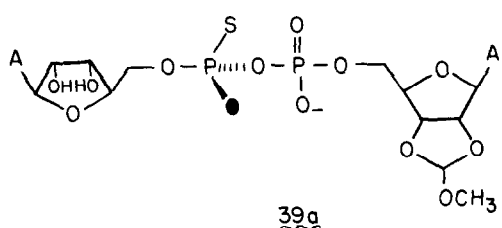
for adenylate kinase with AMP as acceptor according to eqn (19). The phosphorus configuration of the resulting sample of ADPβS, β^{18}O would be compared with those of **37a** and **37b** by stereoselective enzymatic phosphorylation followed by analysis for bridging and nonbridging ^{18}O in the corresponding triphosphates.

The synthesis of **36** began with stereospecific enzymatic phosphorylation of ^{18}O-enriched AMPS according to eqn (7) followed by hexokinase catalyzed dephosphorylation of the resulting ^{18}O-enriched S_p epimer of **13b** to the ^{18}O-enriched S_p epimer of **13a**. Activation of 2',3'-methoxymethylidene-AMP with diphenylphosphorochloridate followed by coupling with the ^{18}O-enriched S_p epimer of **13a** produced **38**, the half blocked diadenosyl-1-thio-1-[$^{18}O_2$]triphosphate, which was chemically degraded to **36**. The degradataion began with periodate cleavage of the unblocked ribose ring in **38** to the dialdehyde, followed by destruction of IO_3^- and unreacted IO_4^- with $HPSO_3^-$ or β-mercaptoethanol. After removing the methoxymethylidene blocking group in dilute acid, the periodate-cleaved nucleoside 2',3'-dialdehyde was removed by β-elimination at pH 10.5.[55]

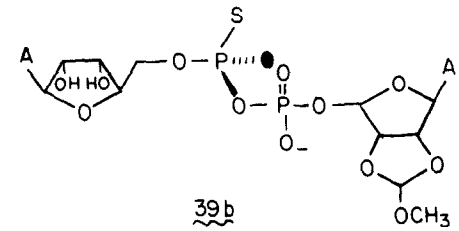
Samples of ADPβS, β^{18}O **37a** and **37b** were synthesized from ^{18}O-enriched **12a** and 2',3'-methoxymethylidene-AMP.[38] [^{18}O] **12A** was activated with diphenylphosphorochloridate and coupled with the blocked nucleotide, affording a mixture of epimers **39a** and **39b**. These isomers were separated by chromatography and their configurations assigned by chemical degradation to known compounds whose configurations could be assigned by ^{31}P-NMR analysis. A single isomer **39a** was deblocked with dilute acid to **40a** which was further degraded by reaction with one equivalent of periodate followed by alkaline β-elimination. Since periodate cleavage was almost random, all possible cleavage-elimination products were produced and isolated. Two formed in almost equal amounts were ADPαS, α^{18}O **13a** and ADPβS, β^{18}O **37a** or **b**. The ^{31}P-NMR chemical shift for the α-phosphorus in **13a** established its configuration as S_p, and this configuration was also assigned to **39a**. This meant that the ADPβS, β^{18}O obtained in the



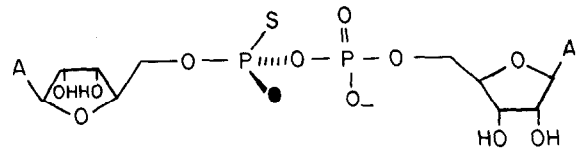
38



39a



39b



40a

A = Adenine

degradation was **37a**. The same analysis were applied to **39b**. Once their configurations had been assigned, **39a** and **39b** were converted to **37a** and **37b** by the same method used to degrade **38** to **36**.

The availability of **37a** and **37b** provided a means by which the orientations of stereoselective phosphorylations of ADPβS catalyzed by pyruvate and acetate kinases could be established.[38] Phosphorylation of **37a** by phosphoenolpyruvate catalyzed by pyruvate kinase produced ATPβS with largely nonbridging ^{18}O, and acetate kinase-catalyzed phosphorylation by acetyl phosphate led to bridging ^{18}O in the ATPβS. Therefore, pyruvate and acetate kinases catalyze stereoselective phosphorylation of the R and S oxygens, respectively, in **37a** and **37b**.

The adenylate kinase-catalyzed reaction of **36** with AMP produced a sample of ADPβS, $\beta^{18}O$ which by the above configurational analysis was identical to **37b**.[35] Therefore, the configuration of the ^{18}O phosphorothioate group in **36** must have been inverted by adenylate kinase. A similar procedure showed that nucleoside diphosphate kinase catalyzes the transfer of the [^{18}O]thiophosphoryl group in **36** to [^{14}C]ADP with retention of configuration.[16]

Nucleoside phosphotransferase catalyzes eqn (20), in which N_1 and N_2

$$N_1MP + N_2 \rightleftharpoons N_1 + N_2MP \tag{20}$$

signify nucleosides such as adenosine, uridine, guanosine, cytidine and their 2'-deoxy analogs. Although the enzyme exhibits broad specificity for nucleosides, it is more active toward 2'-deoxy nucleosides and their monophosphates than it is toward the ribonucleosides.

The synthesis of the chiral sample of AMPS, ^{18}O to investigate this stereochemistry began with the synthesis of **40a** and its epimer **40b** (not shown) from AMPS, $^{18}O_2$, [^{18}O]**12a** and AMP.[56] Activation of [^{18}O]**12a** with diphenylphosphorochloridate followed by coupling to AMP produced **40a** and its epimer **40b** (not shown). The epimers were separated by chromatography and their configurations assigned as described above. Nucleotide pyrophosphatase from *Crotalus adamanteus* catalyzed the hydrolysis of **40a** to **12d** and of **40b** to **12c**. This hydrolysis proceeded with retention of configuration at the chiral

12d 12c

Ado = Adenosine

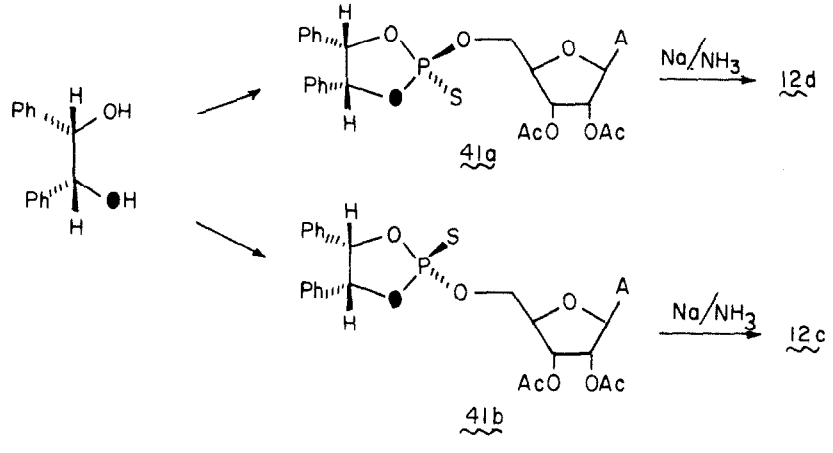
phosphorus centers, as shown by the fact that enzymatic phosphorylation of **12d** (eqn 7) produced [^{18}O]ATPαS, **13b**, with ^{18}O exclusively nonbridging at P_α.

Nucleoside phosphotransferase catalyzed eqn (21), in which the [^{18}O] thiophosphoryl donor was **12d** and the acceptor was [^{14}C]adenosine. The appearance of ^{14}C in AMPS, ^{18}O verified the transfer of the [^{18}O]thiophosphoryl group to [^{14}C]adenosine. Since eqn (21) represents an exchange at chemical equilibrium, the species present as [^{14}C]AMPS, ^{18}O could have been **12d** + [^{14}C] **12c** corresponding to inversion, or **12d** + [^{14}C]**12d** corresponding to retention of configuration. Stereochemical analysis of AMPS, ^{18}O as described above revealed that the sample was indistinguishable from **12d**.[56] It must therefore, have consisted of **12d** + [^{14}C]**12d** the products of retention of configuration. In a related study adenosine kinase from bovine liver catalyzed the transfer of the [^{18}O]thiophosphoryl group in **36** to adenosine.[57] The AMPS, ^{18}O produced was shown to be **12d** which corresponded to inversion of configuration.

$$\textbf{12d} + [^{14}\text{C}]\text{adenosine} \rightleftharpoons [^{14}\text{C}]\text{AMPS}, {}^{18}\text{O} + \text{adenosine} \tag{21}$$

12d and **12c** have been synthesized by two additional procedures. The epimers of **13a** were synthesized as a mixture with ^{18}O enrichment in the nonbridging position at P_α The mixture was separated by pyruvate kinase catalyzed phosphorylation of the S_p diastereomer with phosphoenol pyruvate. Chromatographic separation of S_p[^{18}O]**13b** from R_p[^{18}O]**13a** followed by alkaline phosphatase-catalyzed hydrolysis of both compounds produced **12d** and **12c**, respectively.[58] The hydrolysis was conveniently arrested at the AMPS, ^{18}O stage because of the resistance of phosphorothioate esters to the action of alkaline phosphatase.

Jarvest and Lowe synthesized **12d** and **12c** by the transformations in Scheme 8.[59] Reaction of

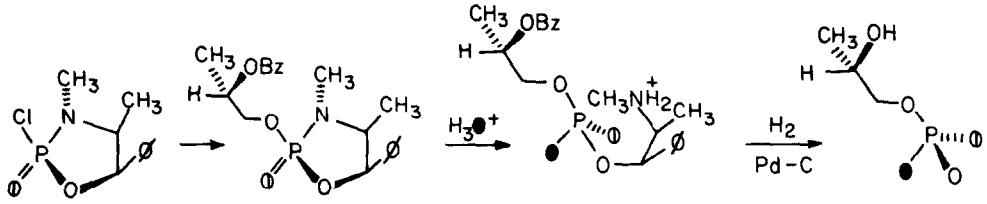


Scheme 8.

(1R,2S)-1,2-[1-^{18}O]dihydroxy-1,2-diphenylethane with thiophosphoryl bromide under thermodynamic or kinetic control followed by reaction with 2',3'-diacetyladenosine gave **41a** or **41b**, respectively. These were converted to **12d** or **12c** by reduction with sodium in liquid ammonia.

4.3 *Chiral* [^{16}O, ^{17}O, ^{18}O]*phosphates*

Chiral [^{16}O, ^{17}O, ^{18}O]phosphates have been synthesized by two general procedures. Knowles and associates devised the route outlined in Scheme 9,[60] which has been used to prepare the phenol[61] and glycerate[62] esters as well as the propylene glycol ester **42a** shown. The procedure has also been adapted

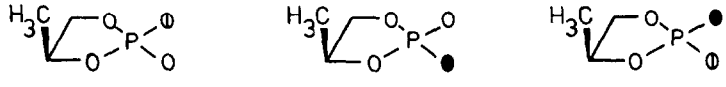


42a

Scheme 9.

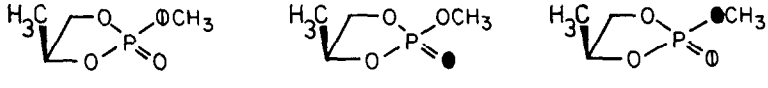
to the synthesis of [γ-^{16}O, ^{17}O, ^{18}O]ATP.[49] The starting cylic adduct was prepared by reaction of (−)ephedrine with $P^{17}OCl_3$, giving a separable mixture of 2-chloro-1,3,2-oxazaphospholidin-2-ones whose chemistry had been described.[63] The major isomer was converted to S-1,2-propanediol-R_p-1-[^{16}O, ^{17}O, ^{18}O]phosphate.

Although the stereochemistry at each step in Scheme 9 was well precedented, the configuration was verified by the following two methods.[64,65] Both methods involved cyclization of S-1,2-propanediol-1-R_p or S_p-[^{16}O, ^{17}O, ^{18}O]phosphate by random activation of the peripheral phosphorus oxygens and internal displacement of activated oxygen by the vicinal hydroxyl group. Cyclization of a given isomer leads to mixture of three cyclic phosphodiesters which differ in their isotopic compositions. The cyclic products from S-1,2-propanediol-1-(R_p)-[^{16}O, ^{17}O, ^{18}O]phosphate (**42a**) are **43a**, **43b** and **43c**. Those derived from

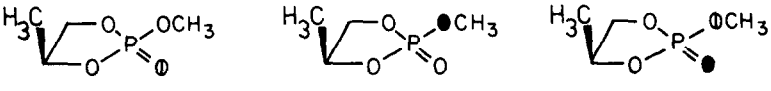


43a 43b 43c

the S_p epimer would have the opposite configurations at phosphorus; for example, in the species **43c** containing both ^{17}O and ^{18}O, the ^{17}O would be *syn-* to the methyl group rather than *anti-* as in **43c**. Since the *syn-* and *anti-* oxygens are chemically equivalent they are not easily distinguished, yet they must be distinguished if the species **43a**, **43b** and **43c** are to be distinguished from the epimeric cyclic phosphodiesters derived from the S_p epimer. Methylation of compounds **43** makes these oxygens chemically inequivalent and thereby more easily distinguishable. Six methylation products are obtained, three *syn-* and three *anti-* methyl esters. The *syn-* and *anti-*esters are diasteromeric and, therefore, separable by physical methods such as chromatography; and this separation is fundamental to the present analysis. The *syn-* isomers **44a**, **44b** and **44c** are used here to exemplify the configurational analysis but the *anti-* isomers contain equivalent information and can also be used. The *syn-* isomers **44** derived from the R_p epimer **42a** differ from the *syn-* isomers **45** derived from the S_p epimer. Comparing **44a** and **45a**, the former contains an ^{17}O-methoxyl while the latter contains as ^{16}O-methoxyl; however, this is alone not sufficient to distinguish the R_p and S_p configuration in the parent **42** because **45c** also



44a 44b 44c

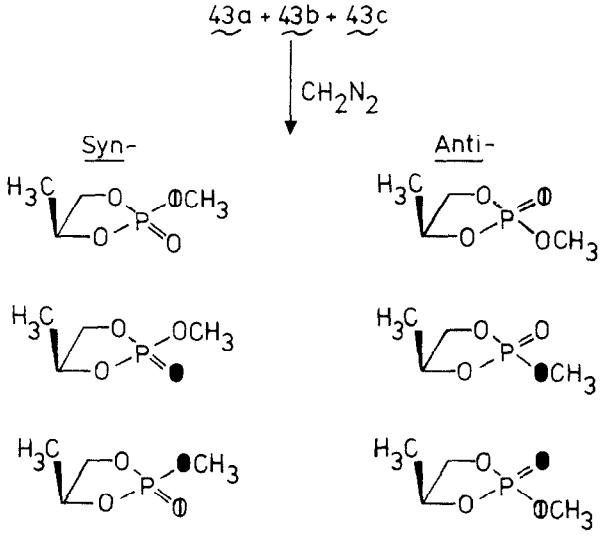


45a 45b 45c

contains an ^{17}O-methoxyl group. To make the configurational assignment it is necessary to show that the ^{17}O-methoxyl resides in the m + 1 species **44a**, distinct from the ^{17}O-methoxyl group in the m + 3 species **45c**.

Loss of formaldehyde from the methoxyl group is a common fragmentation mode for methyl phosphates. If it is shown that for one set of *syn*- isomers, **44a**, **44b** and **44c**, the m + 1 parent (**44a**) loses $CH_2{}^{17}O$ to form the m' daughter while the m + 2 ion (**44b**) loses $CH_2{}^{16}O$ to form the m' + 2 daughter and the m + 3 parent (**44c**) loses $CH_2{}^{18}O$ to form the m' + 2 daughter, the R_p configuration may be assigned to the precursor of that set. Metastable ion mass spectrometry can, in principle, be used to establish such parent-daughter relationships. The methyl cyclic phosphates **44** and **45** do not exhibit the necessary metastable transitions; however, their methanolysis products do exhibit them and these products contain all the stereochemical information. For example, the species of dimethyl-1,2-propanediol-1-[^{16}O, ^{17}O, ^{18}O]phosphate obtained by methanolysis of **44a** contains the ^{17}O-methoxyl group while that from **45a** does not, and similar relationships extend to **44b** vs **45b** and **44c** vs **45c**. Accordingly, metastable ion mass spectrometry of these derivatives has led to unambiguous assignment of configuration to the [^{16}O, ^{17}O, ^{18}]phosphate group in **42a**.

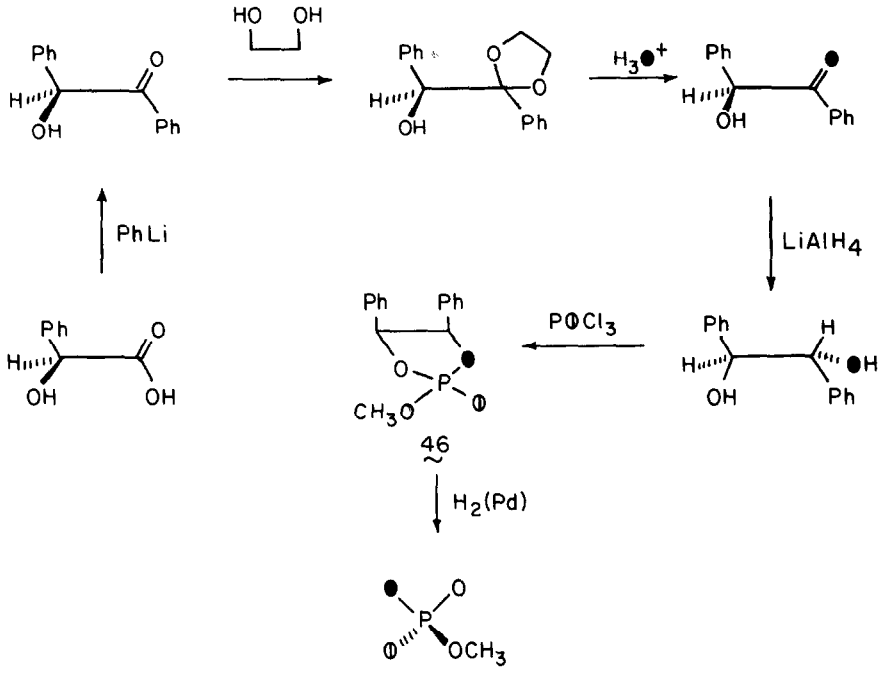
The second method of configurational assignment is simpler experimentally in that the *syn*- and *anti*-isomers of methyl-1,2-propanediol cyclic phosphate do not have to be separated or methanolyzed.[65] Returning to structures **44a**, **b** and **c** and **45a**, **b** and **c**, note that only one species in each set is devoid of ^{17}O. Recalling that phosphorus is silent in ^{31}P-NMR spectra when it is bonded to ^{17}O, it is immediately clear and significant that the ^{31}P-NMR spectra will reflect mainly species **44b** and **45b**. The reader is reminded that all samples of ^{17}O-enriched material are also enriched with ^{18}O; therefore, all atomic positions designated as ^{17}O in the structures are also enriched with ^{18}O. While this must and can be taken into account, it is not considered here in order to simplify the presentation of the principles upon which the configurational analysis is based. The ^{31}P-NMR spectra of **44b** and **45b** differ since ^{18}O is double bonded in **44b** (nonbridged) and single bonded (bridging) in **45b**. Therefore, the differential ^{18}O-isotope shift effects on the phosphorus chemical shift can be used to determine which species predominates and, therefore, the phosphorus configuration in the precursor 1,2-propanediol-1-[^{16}O, ^{17}O, ^{18}O]phosphate. In practice the *syn*- and *anti*- isomers are not separated in this analysis because their phosphorus chemical shifts are well separated and the ^{18}O-isotope shift effect on each of them can be measured in the mixture. This provides a necessary internal check. Since the nonbridging ^{18}O in **44b** will be bridging in its *anti*- isomer (Scheme 10), both shifts can be detected in the spectrum of the



Scheme 10.

mixture. It is important to measure both isotope shifts in the same sample because thay are small and their difference is even smaller; so they should be directly compared.

An alternative synthetic method for chiral [^{16}O, ^{17}O, ^{18}O]phosphate esters was devised by Lowe *et al.*, who began with S-mandelic acid according to Scheme 11.[66] They obtained (1R,2S)-[1-^{18}O]-1,2-dihydroxydiphenylethane by asymmetric synthesis and reacted it first with $POCl_3$ and then methanol to give the crystalline cyclic triester **46**, which they cleaved by hydrogenolysis to S_p methyl-[^{16}O, ^{17}O, ^{18}O]phosphate. The configuration was originally assigned as R_p because the configuration of **46**



Scheme 11.

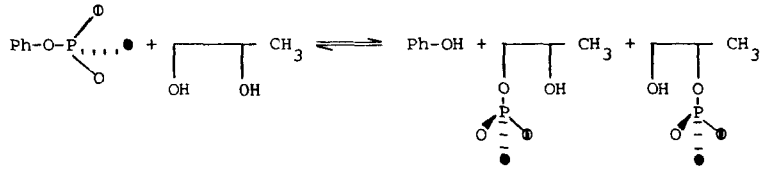
was thought to be S_p. The latter assignment was later revised to that of **46**, which meant that the configuration of chiral methyl phosphate was S_p as shown.[67]

Chiral [^{16}O, ^{17}O, ^{18}O]phosphate esters have been used to determine the stereochemical courses of phosphoryl transfer catalyzed by alkaline phosphatase, several kinases and phosphoglycerate mutase. Alkaline phosphatase from *Escherichia coli* catalyzes the hydrolysis of phosphate monoesters by a mechanism involving the transformations of eqns (22) and (23), in which E–OH

$$RO\textcircled{P} + E-OH \rightleftharpoons E-O\textcircled{P} + ROH \tag{22}$$

$$E-O\textcircled{P} + H_2O \rightleftharpoons E-OH + HO\textcircled{P} \tag{23}$$

represents the enzyme and the β-OH group of a serine residue in the active site, RO$\textcircled{P}$ a monoalkyl or aryl phosphate, and HO$\textcircled{P}$ inorganic phosphate. The enzyme also catalyzes transphosphorylation reactions in which the phosphoryl group of a phosphomonoester is transferred to an alcohol to generate a second phosphomonoester and a second alcohol. As such, it is understood to be an exchange of the phosphoryl group between two alcohols resulting from the reversibility of eqn (22), and for this reason it should proceed with overall retention of configuration at phosphorus when a chiral [^{16}O, ^{17}O, ^{18}O]phosphomonoester is used as the substrate. That this is the case was verified by Jones *et al.* who synthesized R_p phenyl-[^{16}O, ^{17}O, ^{18}O]phosphate and used alkaline phosphatase to catalyze the transfer of the [^{16}O, ^{17}O, ^{18}O]phosphoryl group to 1,2-propanediol according to eqn (24).[61] The 1,2-propanediol[^{16}O, ^{17}O, ^{18}O]phosphate produced had the same phosphorus configuration as phenyl[^{16}O, ^{17}O, ^{18}O]phosphate.



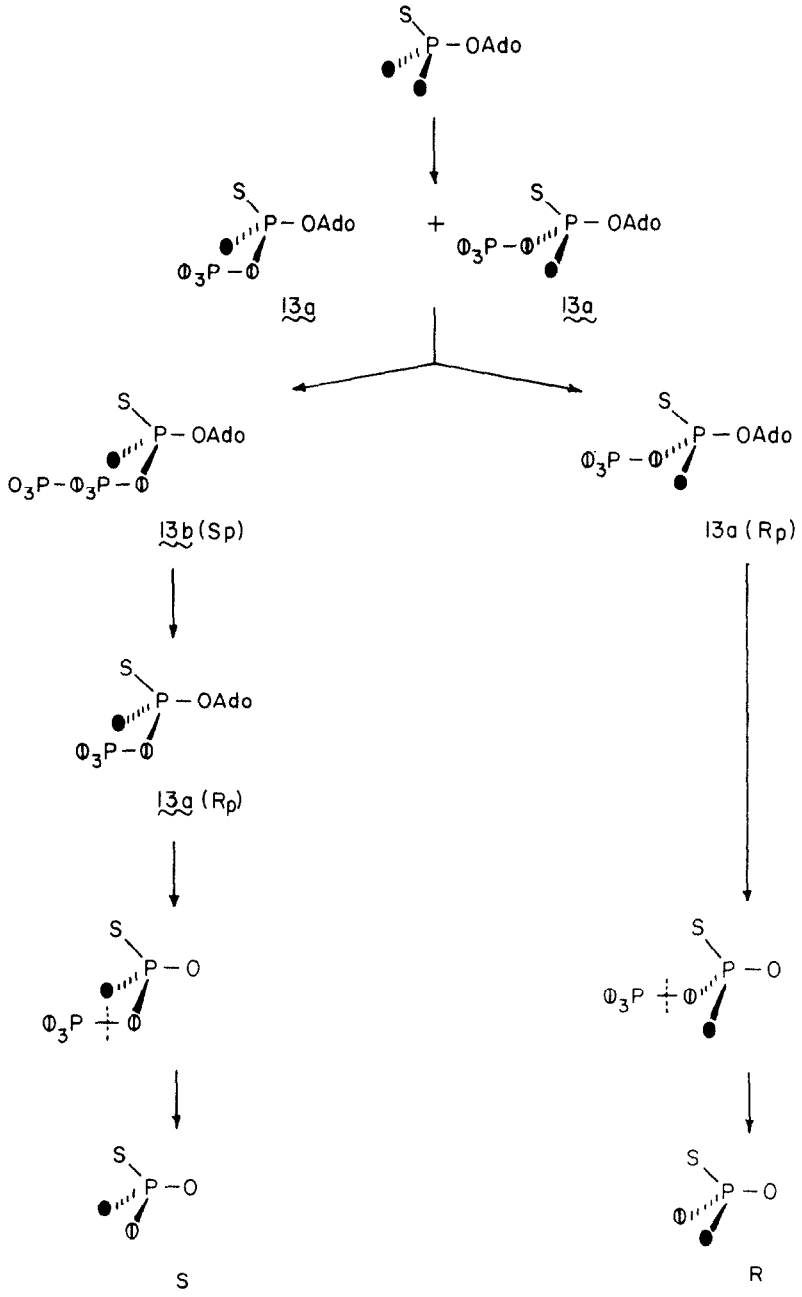
(24)

In addition to confirming expectations regarding its stereochemistry, the alkaline phosphatase study provided the basis for the configurational analysis of chiral [^{16}O, ^{17}O, ^{18}O]phosphate esters resulting from phosphotransferase reactions. In kinase reactions [γ-^{16}O, ^{17}O, ^{18}O]ATP is used with the appropriate enzyme to phosphorylate an alcohol. The resulting [^{16}O, ^{17}O, ^{18}O] phosphomonoester is substituted for phenyl-[^{16}O, ^{17}O, ^{18}O]phosphate in eqn (24) to generate a sample of 1,2-propanediol[^{16}O, ^{17}O, ^{18}O]phosphate having the

same configuration; and this sample is finally subjected to configurational analysis as described above. The method has been used to show that the acetate kinase,[68] glycerokinase,[53] and creatine kinase reactions proceed with inversion; and an analogous method was used to show that the interconversion of 2-phosphoglycerate and 3-phosphoglycerate catalyzed by phosphoglycerate mutase proceeds with retention of configuration.[62]

4.4 *Chiral* [^{16}O, ^{17}O, ^{18}O]*thiophosphate*

The synthetic method used by Webb and Trentham to synthesize the R_p and S_p enantiomers of [^{16}O, ^{17}O, ^{18}O]thiophosphate is outlined in Scheme 12.[69] Activation of AMPS, $^{18}O_2$ with diphenylphosphorochloridate

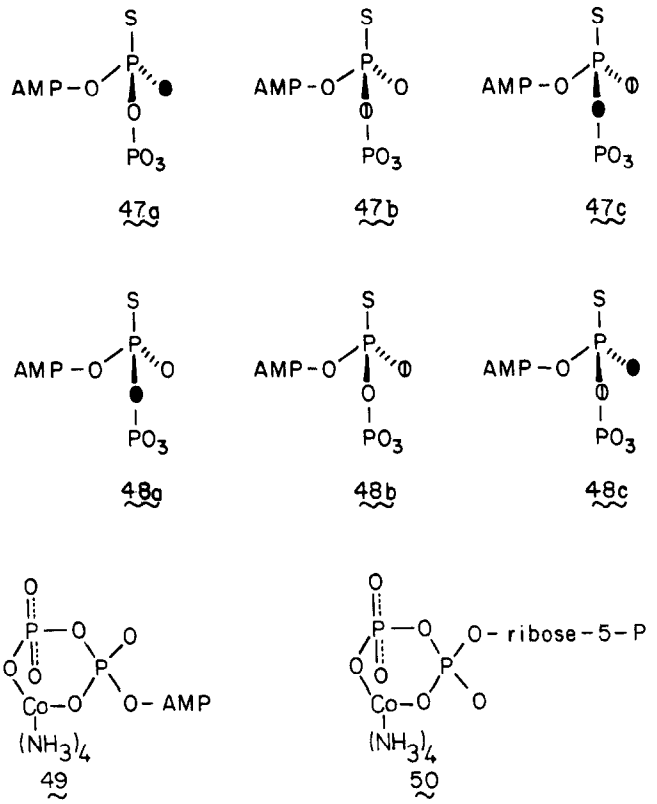


Scheme 12.

followed by coupling with $H_2P^{17}O_4^-$ led to the mixture of ^{17}O and ^{18}O-enriched diastereomers of **13a**. Pyruvate kinase catalyzed phosphorylation of this mixture was specific for the S_p diastereomer (Secion 3.1)

and the resulting S_p **13b** was separated by chromatography from unreacted R_p **13a**. S_p **13b** was dephosphorylated by the action of hexokinase with glucose to S_p **13a**. The separated isomers of **13a** were degraded to the corresponding isomers of ^{17}O, ^{18}O-enriched pyrophosphorothioate by periodate-cleavage and alkaline elimination, analogous to the syntheses of **36**, **37a** and **37b**; and the action of the enzyme inorganic pyrophosphatase cleaved the indicated bonds, producing the enantiomers of [^{16}O, ^{17}O, ^{18}O]thiophosphate.

The coupled actions of glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, and adenylate kinase together with their associated substrates and coenzymes incorporate inorganic phosphate into ATP in the β-position. This process also incorporates thiophosphate into the β-position of ATPβS. The stereochemical course of the adenylate kinase reaction is known to be inversion. The only other reaction in the process that involved cleavage of bonds to this phosphorus was the phosphoglycerate kinase reaction, which was also shown in the same study to proceed with inversion of configuration. The *known* stereochemistries of the enzymes involved provide a means by which the configuration of a sample of [^{16}O, ^{17}O, ^{18}O]thiophosphate can be determined. The structures of the major species of ^{17}O, ^{18}O-enriched ATPβS, produced by this process from the S enantiomer of [^{16}O, ^{17}O, ^{18}O]thiophosphate are **47a, b** and **c** and from the R enantiomer they are **48a, b** and **c**. The isomers **47** differ from **48** in the positions of ^{17}O and ^{18}O enrichment.



With reference to the configurational analysis, the essential aspects of these differences are the fact that the β-phosphorus signals for **47b** and **c** and **48b** and **c** are silenced by the presence of ^{17}O; and ^{18}O is nonbridging in **47a** and bridging in **48a**. Since **47a** and **48a** are the major β-phosphorus signals observed and they differ in the isotope shifts they exhibit, the enantiomers of [^{16}O, ^{17}O, ^{18}O] thiophosphate can be identified. The use of the combined effects of ^{17}O and ^{18}O on phosphorus NMR signals was originally introduced in this analysis.[69]

Webb and Trentham and M. D. Tsai have applied these methods to the stereochemical analyses of ATPases and 5′-nucleotidase. Myosin ATPase catalyzes the hydrolysis of ATP to ADP and inorganic phosphate. Webb and Trentham used myosin to catalyze the hydrolysis of **36** in $H_2{}^{17}O$, producing a sample of [^{16}O, ^{17}O, ^{18}O]thiophosphate. The configuration of [^{16}O, ^{17}O, ^{18}O]thiophosphate was R, the product corresponding to inversion of configuration at phosphorus.[70] 5′-Nucleotidase catalyzes the hydrolysis of AMP to adenosine and phosphate. Tsai and Chang used this enzyme to catalyze the hydrolysis of **12c** and **12d** in $H_2{}^{17}O$, producing two samples of [^{16}O, ^{17}O, ^{18}O]thiophosphate.[71] Applying the foregoing method, they

assigned the configuration S to the sample obtained from **12d** and the configuration R to the sample derived from **12c**. These assignments corresponded to inversion of configuration in the 5'-nucleotidase-catalyzed hydrolysis.

4.5 *Pyrophosphoryl transfer*

An early stereochemical study involved the transfer of the pyrophosphoryl group of ATP to ribose-5-phosphate catalyzed by 5-phosphoribosyl-1-pyrophosphate synthetase (eqn 25). Li, Mildvan and Switzer devised a novel

$$\text{ATP} + \text{ribose-5-P} \rightleftharpoons \text{AMP} + \text{5-phosphoribose-1-PP} \tag{25}$$

method to show that this transfer involves inversion of configuration at the β-phosphorus of ATP.[72] They synthesized **49**, a coordination exchange-inert cobalt(III) tetrammine-ATP complex, and used it as a substrate in place of ATP and Mg^{2+} to produce a sample of the corresponding cobalt(III) complex of 5-phosphoribosyl-1-pyrophosphate **50**. Note that the β-phosphorus in **49** is chiral and that this becomes the chiral center in **50**. That the configuration had been inverted in the reaction was concluded by the fact that the long wavelength CD bands corresponding to the Co(III) chromophores were nearly mirror images of each other.

5. CONCLUSION

The results of over thirty stereochemical studies of enzymatic substitution at phosphorus confirm the value of this approach in characterizing the catalytic mechanisms by which these enzymes function. There is as yet no indication of the involvement of pseudorotatory rearrangements in pentavalent intermediates of enzymatic substitutions at phosphorus. All reactions studied to date are stereospecific, and the available data strongly support the hypothesis that each enzymatic substitution at phosphorus proceeds by an in-line mechanism and inversion of configuration. Double displacements proceed with net retention of configuration. The stereochemical test is presently accepted as a highly reliable indicator of whether a given reaction follows a single displacement or a double displacement mechanism.[4,5,16,68]

Many of the studies reported have involved the use of chiral phosphorothioates as substrates and an increasing number have involved chiral phosphates. The phosphorothioates are not naturally occurring substrates and, in many cases, react at much slower rates than phosphates. This raises the question whether the stereochemical data obtained with phosphorothioates reflect the stereochemistries of the reactions of phosphates in general. Inasmuch as the stereochemical courses of six enzymatic phosphate substitutions have been shown to be the same with chiral thiophosphates as with chiral phosphates, it appears that chiral thiophosphates are valid substrates for stereochemical analysis. This is not an unexpected finding since the thiophosphates judged to be acceptable substrates generally react at rates of about 1–50% of the rates of phosphates. These are modest to substantial rate differences in terms of their experimental manifestations, but they are not large relative to the 10^8 or more rate accelerations achieved by enzymic catalysis. The thiophosphates and phosphates do not, therefore, appear to react by such grossly different mechanisms as to result in different stereochemical consequences. The chiral thiophosphates offer some advantages in that smaller amounts of material are needed for configurational analysis, but the chiral phosphates are essential for certain types of applications, especially for studies of a few enzymes such as alkaline phosphatase and phosphoglycerate mutase, which will not utilize thiophosphates as substrates.

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