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Epimerization in the Synthesis of Thiophosphoanhydrides

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Reaction of (R_p)-[β-^{17}O]ADPβs or (S_p)-[β-^{17}O]ADPβs (adenosine 5′,2-thio[2-^{17}O]diphosphate with *R* and *S* configurations, respectively, at P^2) with diphenylchlorophosphate for 15 min in hexamethylphosphoramide followed by aqueous workup produces (R_p)- and (S_p)-P^1-5′-ado-P^3-phenyl-2-thio[2-^{17}O]triphosphate as an epimeric mixture. Separation of the epimeric coupling products and analysis for bridging and nonbridging ^{17}O reveals that the labeling pattern is similar for products derived from either (R_p)- or (S_p)-[β-^{17}O]ADPβS, with ^{17}O distributed between bridging and nonbridging positions in both epimers. Similar labeling patterns in the coupling products must have arisen from epimerization at chiral P in (R_p)-[β-^{17}O]ADPβS and (S_p)-[β-^{17}O]ADPβS during the initial coupling reaction. Otherwise, one of the coupling products from each stereoisomer would have contained ^{17}O in the P^2-nonbridging position and the other in the P^2–P^3-bridging position. Epimerization at P^2 of [β-^{17}O]ADPβS could in principle involve either reversible formation of [^{17}O]thiometaphosphate or reversible transfer of the [^{17}O]thiophosphoryl group to the solvent, hexamethylphosphoramide. Consideration of the rate of epimerization observed in these experiments and the rate of thiometaphosphate formation in solvolysis reactions suggests that epimerization involves solvent participation via the resonance-stabilized zwitterionic species *O*-[^{17}O]thiophosphoryl-hexamethylphosphoramide.

A recently published method for the synthesis of ATPβS[1] involves the reaction of 2′,3′-methoxymethylidene-ADPβS with adenosine 5′-phosphorodichloridate in hexamethylphosphoramide, a reaction which produces the dialkyl 2-thio-*cyclo*-triphosphate **1** as an intermediate (*1*). Aqueous workup results in cleavage of **1** to the P^1,P^3-dialkyl 2-thiotriphosphate **2** by addition of H_2O to either P^1 or P^3. Selective removal of the unblocked adenosyl followed by deprotection of the 2′,3′-methoxymethylidene-adenosyl moiety produces ATPβS in good yield.

In a simple formulation of the mechanism by which the dialkyl *cyclo*-triphosphate **1** is produced, one can visualize a two-step process beginning with the displacement of Cl^- from adenosine 5′-phosphorodichloridate by 2′,3′-methoxymethylidene-ADPβS, forming **3** as a transient species which quickly collapses to **1** by internal displacement of Cl^- from P^3 by a P^1-oxygen.

[1] Abbreviations used: ATPβS, adenosine 5′,2-thiotriphosphate; ADPβS, adenosine, 5′,2-thiodiphosphate: (R_p)- and (S_p)-[β-^{17}O]ADPβS, adenosine 5′,2-thio[2-^{17}O]diphosphate having the *R* and *S* configuration at P^2.

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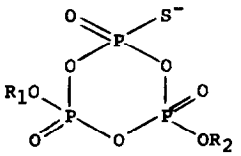

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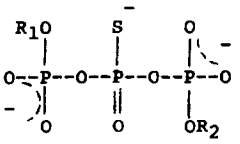

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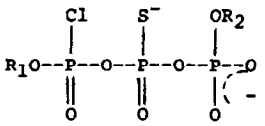

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R_1 = Ado
R_2 = 2',3'-methoxymethylidene-Ado

According to this formulation the reaction might be useful for preparing polyphosphates with exclusively bridging or nonbridging ^{17}O or ^{18}O by using (R_p)- or (S_p)-[β-^{17}O- or ^{18}O]ADPβS as the substrate. Since **2** is produced as a mixture of P^2-epimers, one epimer would have ^{17}O or ^{18}O bridging P^1 and P^2, which the other would have the heavy isotope nonbridging at P^2. The separated P^2-epimers could be used as precursors of ATP with bridging or nonbridging ^{18}O (or ^{17}O).

In this paper we show that reaction of (R_p)-[β-^{17}O]ADPβS or (S_p)-[β-^{17}O]ADPβS with phenyl phosphorodichloridate followed by aqueous workup produces similar mixtures of (R_p + S_p)-P^1-5'-adenosine-P^3-phenyl-2-thiotriphosphate, with ^{17}O in both bridging and nonbridging positions in both epimers. Therefore, (R_p)- and (S_p)-[β-^{17}O]ADPβS undergo epimerization at P^2 in hexamethylphosphoramide at a rate that exceeds the coupling rate. Epimerization most likely involves solvent participation, although reversible formation of [^{17}O]thiometaphosphate monoanion cannot be excluded by the results.

MATERIALS AND METHODS

Materials. Hexamethylphosphoramide, triethylphosphate, and phenyldichlorophosphate (Aldrich Chemical Co.) were dried over Linde 4-A molecular sieves and then distilled just before use. Triethylamine and tri-*n*-butylamine were distilled before use. Reagent grade salts were purchased from commercial suppliers and used without further purification. Both $H_2{}^{17}O$ (52.8% ^{17}O, 37.8% ^{18}O, and 9.4% ^{16}O, or 51.1% ^{17}O, 39.0% ^{18}O, and 9.9% ^{16}O) and $H_2{}^{18}O$ (95.2% ^{18}O, 2.6% ^{17}O, and 2.2% ^{16}O) were purchased from Monsanto Research Corp., Mound Laboratory. DEAE-Sephadex A-25 and Chelex 100 were purchased from Sigma and Bio-Rad, respectively.

General methods. Thin-layer chromatographic analysis of nucleotides was carried out using fluorescent-indicating Eastman 13181 silica gel plates, with 1-propanol–NH_3–H_2O in the ratio 6 : 3 : 1 as the mobile phase. Adenine nucleotides were visualized as fluorescence-quenched spots under an ultraviolet lamp. Nucleotides containing sulfur were identified by staining with the vapors of I_2.

Nucleotides were purified by anion-exchange chromatography through columns of DEAE Sephadex A-25, in the HCO_3^- form, eluted by linear gradients of triethylammonium bicarbonate at 4°C. Nucleotides in pooled fractions were desalted by rotary evaporation *in vacuo* using a Buchi apparatus with a bath temperature of 30°C. The dried nucleotides initially obtained were twice dissolved in small volumes of methanol and again evaporated, to remove residual traces of triethylammonium bicarbonate, and finally dissolved in H_2O and stored at −15°C.

Thiophosphate and nucleotides with terminal thiophosphate groups in column effluents were detected using 5,5′-dithiobis(2-nitrobenzoate) as described (*2*). Adenine nucleotide concentrations were calculated from measurements of A_{260} using the extinction coefficient 15×10^3 M^{-1} · cm^{-1}.

High-performance liquid chromatography was performed using a Waters Model 440 system equipped with ultraviolet detector and a Waters Novopak C_{18} reverse-phase column (3.9 × 15 cm) or a Waters Porasil C_{18} reverse-phase column (7.8 × 30 cm).

^{31}P NMR spectra were determined at 25°C using a Nicolet 200-MHz (proton) spectrometer, operating at 80.1 MHz for ^{31}P, with quadrature detection. The spectrometer was field frequency locked on the resonance of deuterium in the solvent. Chemical shifts were related to the signal for an external sample of H_3PO_4. Nucleotides were prepared for NMR analysis by dissolving in deionized water and percolating the solutions through small columns (Pasteur pipet) of Chelex 100 (Na^+). The columns were washed with 5 ml of deionized water and the effluents lyophilized. Residues were dissolved in 0.6 ml of 1 mM EGTA in redistilled ^2H_2O, pD 9.0, and filtered through 0.45-μm filters directly into NMR tubes. In calculations of percentage ^{18}O-containing species the areas under resolved signals were determined by computer-assisted integration.

Except as indicated below, the ^{16}O, ^{17}O, and ^{18}O contents of nucleotides were determined by chemical degradation to triethylphosphate and GC–MS analysis of this compound as described earlier (*3*).

Synthesis of [β-^{17}O]*ADPβS*. (R_p)-[β-^{17}O]ADPβS and (S_p)-[β-^{17}O]ADPβS were synthesized essentially as described for the ^{18}O-labeled compounds (*2*) except that the immediate precursors, the P-epimers of P^1-5′-ado-P^2-5′-(2′,3′-methoxymethylidene-ado) 1-thio[1-^{17}O]diphosphate, were separated by reverse-phase HPLC using 10 mM K-phosphate at pH 6 containing 12% methanol as the mobile phase and a flow rate of 1.5 ml/min. Under these conditions the S_p-epimer was eluted first and completely separated from the R_p-epimer, which emerged later. Multiple injections of 8 μmol of the mixture were carried out. Pooled fractions were separately converted to [β-^{17}O]ADPβS using the method reported by Ho and Frey (*1*).

Synthesis of P^1-5′-ado-P^3-phenyl 2-thio[2-^{17}O]*triphosphate*. This compound was prepared by coupling the R_p- and S_p-epimers of [β-^{17}O]ADPβS with phenyldichlorophosphate. The triethylammonium salt of (S_p)- or (R_p)-[β-^{17}O]ADPβS (46 μmol) in aqueous solution was dried by rotary evaporation. The nucleotide was dissolved in 1 ml of methanol, 92 μmol of tri-*n*-butylamine was added, and the methanol was removed by rotary evaporation. The compound was dissolved in 2 ml of water, frozen as a thin film on the inside wall of a flask, lyophilized to

dryness (16 h), and then desiccated in vacuo over P_2O_5 for 24 h. Separately, freshly distilled phenyldichlorophosphate (92 μmol) was dissolved in 0.5 ml of triethylphosphate. The lyophilized tri-*n*-butylammonium salt of [β-^{17}O]ADPβS was dissolved with vigorous swirling in 0.5 ml of hexamethylphosphoramide (*caution, hood, carcinogenic solvent*) and transferred to the flask containing the phenyldichlorophosphate together with a 0.2-ml wash of hexamethylphosphoramide. The reaction flask was sealed and the solution stirred at room temperature for 15 min. Cold diethyl ether (8 ml) was added and the resulting suspension centrifuged. After the supernatant fluid was decanted, the precipitate was dissolved with 2 ml of 1 M triethylammonium bicarbonate, diluted to 50 ml with water, and applied to a 1.5 × 30-cm column of DEAE-Sephadex A-25 resin equilibrated with 0.2 M triethylammonium bicarbonate. The column was eluted with a linear gradient of triethylammonium bicarbonate increasing in concentration from 0.2 to 0.4 M, total volume 2 liters. The compound emerged as the mixture of P^2-epimers at 0.32 M salt in a yield of 17 μmol (37%). ^{31}P NMR (^{16}O-species): (chemical shift, multiplicity, assignment) −11.701 ppm, doublet, P^1; 30.506 ppm, multiplet, P^2; −16.382 ppm, doublet, P^3; coupling constants, $J_{1,2}$ = 24.8 Hz and $J_{2,3}$ = 22.3 Hz.

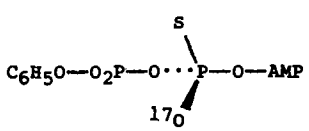
The P^2-epimers were separated by HPLC using a C_{18} reverse-phase column with 100 mM K-phosphate at pH 6.0 containing 0.5% methanol as the mobile phase at a flow rate of 1.5 ml/min. Under these conditions three peaks emerged from the column, an impurity with a retention time of 154 min and the two product epimers with retention times of 180 and 200 min, respectively. Multiple injections of 0.86 μmol were carried out until all of the impurity was removed and the two epimers were separated. To remove K-phosphate pooled epimer fractions were concentrated on the rotary evaporator until the salt began to crystallize, and 2 vol of methanol was then added to precipitate most of the salt. The last traces of K-phosphate were removed by rechromatography through a small column of DEAE-Sephadex (HCO_3^-) eluted with triethylammonium bicarbonate, which was in turn removed by rotary evaporation. Each epimer was obtained in a yield of 6 μmol.

GC–MS analysis of P^1-5′-ado-P^3-phenyl-2-thio[2-^{17}O]triphosphate. A sample of the HPLC-purified epimers prepared from (R_p)- or (S_p)-[β-^{17}O]ADPβS (four samples in all, 0.7 μmol each) was dried by rotary evaporation, dissolved in 0.5 ml of water, and adjusted to pH 8 using 0.1 M NaOH. Sodium periodate (1.1 μmol) was added with stirring and the pH checked and adjusted immediately. After 15 min at 25°C, 28 μmol of 2-mercaptoethanol was added and the pH readjusted to 11.0 by the addition of 5 M KOH. The solution was stirred at 55°C for 35 min, diluted with 1 ml of 0.2 M triethylammonium bicarbonate, and applied to a 1 × 10-cm column of DEAE-Sephadex A-25 equilibrated with the same buffer. After the column was washed with 50 ml of 0.3 M buffer, phenyl 2-thio[2-^{17}O]triphosphate was eluted with 50 ml of 0.7 M triethylammonium bicarbonate. The compound was detected in effluent fractions by phosphate analysis of ashed aliquots. Pooled fractions were dried by rotary evaporation and the last traces of buffer removed by repeated evaporation after additions of methanol. The residue was dissolved in 0.2 ml of ethanol containing 0.02 ml of water and 0.005 ml of 6 M

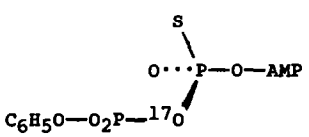
HCl and then ethylated using diazoethane (*3*). The solution was concentrated to 0.1 ml, heated at 100°C for 60 min, cooled to 25°C, and again ethylated. The resulting mixtures of triethylphosphate, triethylthiophosphate, and phenyldiethylphosphate were analyzed by GC–MS, using for the separation a 10-m All Tech RSL-100 column and a hyperbolic temperature program increasing from 40 to 80°C over 3 min, followed by a 10°/min increase to 170°C. Masses of parent peaks were analyzed.

RESULTS AND DISCUSSION

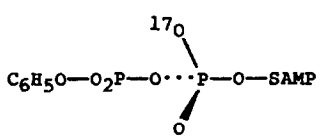
Coupling of ADPβS with phenyldichlorophosphate. Based on the known reaction of 2′,3′-methoxymethylidene-ADPβS with adenosine 5′-phosphorodichloridate, which proceeds via **3** and the 2-thio-*cyclo*-triphosphate diester **1** to produce **2** upon aqueous workup, it appeared reasonable to expect that this reaction could be used to produce (R_p)-[β-^{17}O]ATP and (S_p)-[β-^{17}O]ATP by using (R_p)- and (S_p)-[β-^{17}O]ADPβS as starting materials. For example, reaction of (R_p)-[β-^{17}O]ADPβS with phenyldichlorophosphate in hexamethylphosphoramidate followed by aqueous workup might produce **4a** and **4b** as an epimeric mixture. The separated epimers could be desulfurized by known general methods to **5a** and **5b**, which by catalytic hydrogenation are immediate precursors of (S_p)-[β-^{17}O]ATP and [βγ-^{17}O]ATP (bridging ^{17}O). Indeed, coupling of ADPβS with phenyldichlorophosphate, desulfurization of the product by reaction with BrCN in water (*4*), and catalytic hydrogenation to ATP[2] proceeded smoothly as expected.



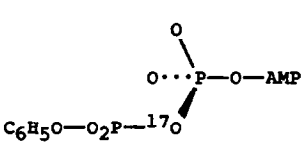
4a



4b



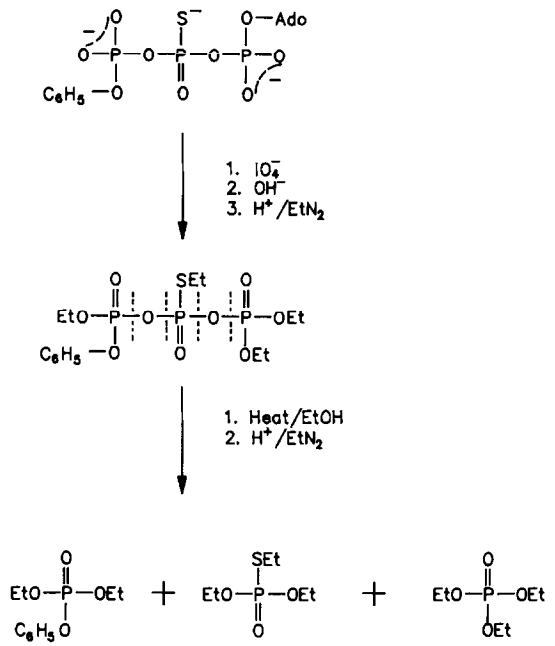
5a



5b

Coupling of (R_p)-[β-^{17}O]ADPβS produces an epimer mixture that can be separated by HPLC. However, ^{17}O in the separated epimers is not specifically in the bridging or nonbridging positions as in **4a** or **4b**. This is shown by the results of chemical degradation according to Scheme 1 followed by GC–MS analysis. The chemical degradation of p^1-5′-Ado-P^3-phenyl-2-thiotriphosphate leads to triethyl-

[2] P^1-5′-Adenosine-P^3-phenyl triphosphate was converted to ATP in 60% purified yield by catalytic hydrogenation over platinum oxide at 14 psi for 16 h.



SCHEME 1

phosphate derived from P^1, triethylphosphorothiolate from P^2, and phenyldiethylphosphate from P^3. The solvolysis step proceeds with partitioning of bridging oxygen between P^1 and P^2 and between P^2 and P^3. Therefore, ^{17}O in **4a** should be found in triethylphosphorothiolate and not in phenyldiethylphosphate. A smaller amount will appear in triethylphosphate owing to desulfurization during chemical degradation. Similar degradation of **4b** will lead to ^{17}O in both triethylphosphorothiolate and phenyldiethylphosphate in amounts reflecting the partitioning of bridging oxygen.

Table 1 presents the results of chemical degradation and GC–MS analysis of the separated epimeric products resulting from the coupling of (R_p)-[β-^{17}O]ADPβS with phenyldichlorophosphate, showing that both products contain ^{17}O in both bridging and nonbridging positions. Similar coupling of (S_p)-[$\beta^{17}O$]ADPβS with phenyldichlorophosphate leads to the same pair of epimers which, upon chemical degradation and GC–MS analysis, give similar ^{17}O-labeling patterns.

The ^{17}O enrichments in Table 1 reveal substantial scrambling of ^{17}O to both bridging and nonbridging positions of both epimers. However, ^{17}O (and ^{18}O) is not randomly distributed in either set of epimeric products. Considering the ^{17}O (and ^{18}O) enrichments in triethylphosphorothiolate and phenyldiethylphosphate obtained by degradation of epimers 1 and 2 derived from (R_p)-[β-^{17}O]ADPβS, it is clear that epimer 1 contains less bridging and more nonbridging ^{17}O (and ^{18}O) than epimer 2. Complementing this result, the labeling patterns for the products derived from (S_p)-[β-^{17}O]ADPβS are the reverse, i.e., higher ^{17}O (and ^{18}O)-enrichment in the bridging position and less in the nonbridging position in epimer 1 than in epimer 2. On the basis of these differential enrichments, we can assign the

TABLE 1

Mass Spectral Analysis of Coupling Products from (R_p)- and (S_p)-[β-^{17}O]ADPβS

	(R_p)-[β-^{17}O]ADPβS[a]		(S_p)-[β-^{17}O]ADPβS[a]	
	Epimer 1	Epimer 2	Epimer 1	Epimer 2
$(C_2H_5O)_2POSC_2H_5$				
M[b]	48.68	42.09	43.08	47.60
M + 1	29.75	32.99	32.65	30.55
M + 2	21.57	24.91	24.35	21.85
$(C_6H_5O)PO(OC_2H_5)_2$				
M[b]	60.38	69.02	66.52	63.56
M + 1	24.43	19.25	17.57	21.91
M + 2	15.19	11.73	15.91	14.53
$(C_2H_5O)_3PO$				
M[b]	83.53	75.83	84.74	83.83
M + 1	8.79	12.82	8.15	8.86
M + 2	7.68	11.35	7.11	7.31

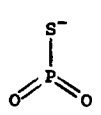
[a] Listed are percentage enrichments of the indicated species in the three chemical degradation products shown in Scheme 1 derived from epimers 1 and 2 of P^1-5′-adenosine-P^3-phenyl 2-thiotriphosphate prepared from (R_p)- and (S_p)-[β-^{17}O]ADPβS. Epimers 1 and 2 are eluted first and second, respectively, from the HPLC column (see Materials and Methods).

[b] M^+ is 138 for $(C_2H_5O)_2POSC_2H_5$, 202 for $(C_6H_5O)PO(OC_2H_5)_2$, and 99 for $(C_2H_5O)_3PO$.

configuration R_p to epimer 1 and S_p to epimer 2 of P^1-5′-adenosine-P^3-phenyl 2-thiotriphosphate.

The partial randomization of ^{17}O shown in Table 1 reveals the intervention, during the coupling process, of some chemical reaction leading to epimerization at P of (R_p)- or (S_p)-[β-^{17}O]ADPβS at a rate that is competitive with the rate of coupling. The coupling reaction itself proceeds rapidly and is terminated within 15 min. Based on the data in Table 1 epimerization must have been nearly complete (83%) within this time, since the labeling patterns in the products derived from the R_p- and S_p-epimers of [β-^{17}O]ADPβS are nearly the same.

The epimerization mechanism must involve reversible cleavage of the thiophosphoryl group from ADPβS to form an intermediate that either lacks asymmetry at P or itself undergoes rapid epimerization. Such an intermediate could in principle be thiometaphosphate **6**, which is planar. Another attractive candidate is *O*-thiophosphoryl hexamethylphosphoramidate **7** resulting from reaction of the solvent with ADPβS.



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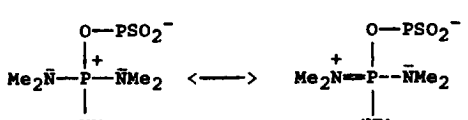

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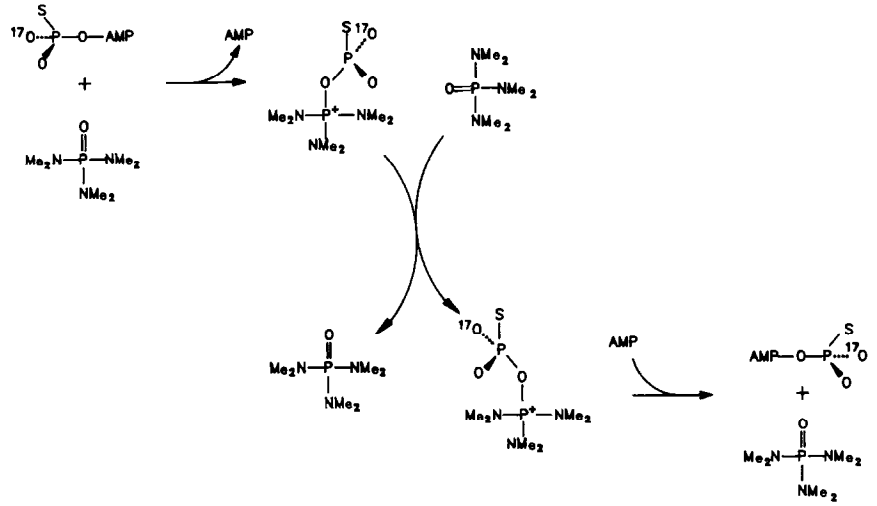
Thiometaphosphate is postulated to be a discrete intermediate in solvolysis reactions of *p*-nitrophenyl phosphorothioate (*5, 6*). These solvolyses, in which the leaving group is *p*-nitrophenolate, proceed at rates two orders of magnitude slower than the rate of epimerization in our experiments. In the case of ADPβS the leaving group is AMP dianion, which on the basis of the pK_a of its conjugate acid should be approximately as stable as *p*-nitrophenolate. We suggest, therefore, that the species **7** is more likely to be involved. This species is stabilized by delocalization of the positive charge over all three nitrogens and the phosphorus. The asymmetry of the $[^{17}O]PSO_2^-$ group would probably be initially retained in the formation of the intermediate from (R_p)- or (S_p)-[β-^{17}O]ADPβS, but this would be lost upon relay of the $[^{17}O]PSO_2^-$ group among other solvent molecules prior to being returned to AMP, as illustrated in Scheme 2.

Solvent participation by pyridine in alkylphosphoryl group transfer has previously been reported, also based on stereochemical evidence. Coupling of (R_p)- or (S_p)-P^1-5′-adenosine-P^2-diphenyl 1-thio[1-^{18}O]diphosphate with AMP in pyridine was found to proceed with epimerization at P^1 (*7*). This was attributed to nucleophilic catalysis of coupling by pyridine in a reaction pathway in which the adenosine 5′-thio[^{18}O]phosphoryl group was displaced from the substrate by pyridine, with release of diphenylphosphate, and epimerized by multiple solvent transfers prior to being transferred to AMP.

We have also considered whether the randomization of ^{17}O in our coupling products might have occurred through a rearrangement of the initial coupling intermediate **3**. A rearrangement leading to transfer of ^{17}O from P^2 to P^3 could involve the cyclization of **3** to a 1-thio-*cyclo*-diphosphate rather than the expected 2-thio-*cyclo*-triphosphate **1**. *Cyclo*-diphosphates have been proposed as rearrangement intermediates in BrCN-activated desulfurizations of adenosine 5′,1-thiodiphosphate and adenosine 5′,2-thiotriphosphate (*3*). These rearrangements were detected by the observation of positionally randomized ^{18}O and/or ^{17}O in reaction products.

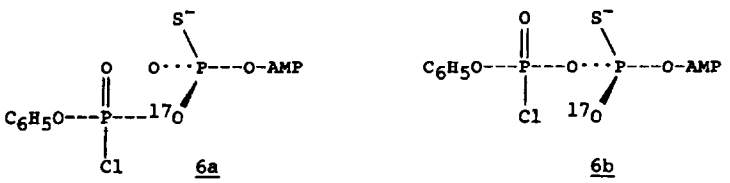
Rearrangements attributed to the intermediacy of *cyclo*-diphosphates were



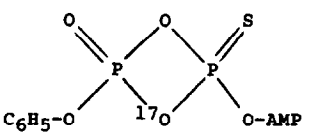
SCHEME 2

observed under conditions in which *cyclo*-triphosphate formation could not have been involved, and the pattern of H_2O addition to P^1 and P^2 and *cyclo*-diphosphomonoesters suggested that they are higher energy species than *cyclo*-triphosphomonoesters (*3*). Therefore, although the present work involves diesters, *cyclo*-diphosphates were not expected to be produced in competition with *cyclo*-triphosphates.

Regardless of expectations, our results cannot be explained by postulating the intermediate formation of 1-thio-*cyclo*-diphosphates. Consider either coupling product **6a** or **6b** resulting from reaction of diphenylchlorophosphate with (R_p)-[β-^{17}O]ADPβS. Cyclization of either isomer to a 1-thio-*cyclo*-diphosphate by internal displacement of Cl^- leads to the same compound **7**. If hydrolysis of **7** should proceed by addition of H_2O exclusively to P^3, **7** would be converted to **4a**



6a 6b



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and **4b** with no randomization of ^{17}O. Our observation of randomization, therefore, excludes this pathway from further consideration. Addition of H_2O exclusively to P^2 or randomly to P^2 and P^3 would lead to randomization of ^{17}O. These pathways involve incorporation of O from H_2O at P^2 in the coupling products. However, reaction of ADPβS with adenosine 5′-phosphorodichloridate (*1*) or diphenylchlorophosphate[3] in hexamethylphosphoramide followed by hydrolysis in $H_2{}^{18}O$ results in the incorporation of ^{18}O exclusively at P^1 and P^3 of the coupling product, and not at P^2. Therefore, these latter pathways are also excluded, and 1-thio-*cyclo*-diphosphates play no role in the randomization of ^{17}O.

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