cyclo-Diphosphates as Intermediates in Reactions of Nucleoside Phosphorothioates with Cyanogen Bromide

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Abstract: Adenosine 5'-*O*-(1-thiodiphosphate) (ADPαS) and adenosine 5'-*O*-(2-thiotriphosphate) (ATPβS) react with BrCN in neutral to alkaline aqueous solutions to produce adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP) in good yields. Reaction of ADPαS in 95% $H_2{}^{18}O$ produces [α-^{18}O,β-^{18}O]ADP with approximately 45% ^{18}O enrichment at each phosphoryl group. Similar reaction of ATPβS in 97% $H_2{}^{18}O$ produces [β-^{18}O,γ-^{18}O]ATP with equal ^{18}O enrichment at P_β and P_γ. Reaction of [α-$^{18}O_2$]ADPαS, with ^{18}O in both bridging and nonbridging positions on P_α, leads to [^{18}O]ADP in which a fraction of the bridging ^{18}O has migrated to a nonbridging P_α position and been replaced by ^{16}O. Similar reactions of [β,γ-^{18}O]ATPβS, with exclusively bridging ^{18}O, lead to [^{18}O]ATP in which part of the ^{18}O is nonbridging at P_β and replaced by ^{16}O in the P_β–P_γ bridge. Desulfurization of a mixture of (S_P)-adenosine 5'-*O*-(1-thio[1-$^{17}O_2$]diphosphate) plus (S_P)-adenosine 5'-*O*-(1-thio[2-$^{18}O_3$]diphosphate) by reaction with cyanogen bromide at pH 10.2 produces [^{17}O,^{18}O]ADP in which the distribution of ^{17}O and ^{18}O at P_β and P_α is consistent with intramolecular transfer of oxygen from P_β to P_α and incompatible with intermolecular transfer. Mass spectral analysis of the triethyl phosphates derived from P_α and P_β of the [^{17}O,^{18}O]ADP indicates that the cyanated intermediate is partitioned between two reaction pathways, displacement by water (33%) to produce ADP directly and internal displacement of the thiocyanate by the β phosphate group (67%) leading to the formation of the intermediate adenosine *cyclo*-diphosphate. Spontaneous hydrolysis of the latter occurs by attack at P_α (36%) and P_β (64%). At pH 7.0 mass spectral studies using (S_p)-adenosine 5'-*O*-(1-thio[1-$^{17}O_2$,2-$^{18}O_3$]diphosphate) provide supporting evidence that the desulfurization proceeds by an intramolecular pathway via a *cyclo*-diphosphate intermediate. All of the results are consistent with a mechanism involving the intermediate formation of *cyclo*-diphosphates. *cyclo*-Diphosphates have heretofore not been described nor postulated as reaction intermediates.

The elucidation of the stereochemical course of phosphoryl and nucleotidyl group transfer catalyzed by enzymes depends upon the availability of substrates that have chiral phosphate groups at the reacting centers. Many chiral phosphates can be synthesized by procedures that have been developed in recent years.[1a-f] These include chiral phosphorothioates such as the R_P and S_P epimers of ATPαS,[2] ADPαS, ATPβS, [γ-^{18}O]ATPγS, [β-^{18}O]ADPβS, [α-^{18}O]AMPS, 3',5'-cAMPS, 2',3'-cUMPS, D-glycerate 2- and 3-[^{18}O]phosphorothioates and others. Chiral phosphates can also be synthesized, and these include chiral [^{16}O,^{17}O,^{18}O]phosphoric esters, [γ-^{16}O,^{17}O,^{18}O]ATP and 3',5'-c[α-^{18}O]AMP.[3a-f]

Optically pure P_α epimers of [α-^{18}O]ATP and [α-^{18}O]ADP have until very recently not been accessible by chemical synthesis. The availability of chiral nucleoside [^{18}O]phosphorothioates by high yield, convenient synthetic routes made them logical precursors for these compounds. A method was needed whereby sulfur in a chiral phosphorothioate could be replaced by an oxygen, preferably from $H_2{}^{18}O$ or $H_2{}^{17}O$, in a stereochemically specific reaction preserving optical purity in the product.

We observed that reaction of ADPαS or ATPβS with BrCN in aqueous solutions produced high yields of ADP or ATP, suggesting that BrCN may be useful for facilitating the displacement of sulfur by H_2O. Experiments in $H_2{}^{18}O$ revealed, however, that the reaction follows a complex course culminating in the incorporation of ^{18}O into at least two positions. In addition, the desulfurization of (S_P)-[α-$^{18}O_2$]ADPαS at pH 10.6 with cyanogen bromide resulted in the transfer of bridging ^{18}O to a nonbridging position. These findings could be accommodated by a mechanism involving intramolecular neighboring group participation by the terminal phosphoryl group via a *cyclo*-diphosphate intermediate. However, the observations can also be rationalized by a mechanism involving intermolecular participation by the terminal phosphoryl group involving an eight-membered ring *cyclo*-tetraphosphate as an intermediate.

We present here the results of our experiments which demonstrate that the reactions of ADPαS and ATPβS with cyanogen bromide in aqueous solutions proceed by complex mechanisms involving intramolecular transfer of oxygen from the terminal

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(2) Abbreviations: AMP, adenosine 5'-phosphate; AMPS, adenosine 5'-*O*-phosphorothioate; [α-$^{18}O_2$]AMPS, adenosine 5'-[$^{18}O_2$]phosphorothioate; (R_P)- and (S_P)-[α-^{18}O]AMPS, [α-^{18}O]AMPS having the *R* and *S* configurations about phosphorus; [^{17}O,^{18}O]AMP, adenosine 5'-[^{17}O,^{18}O]phosphate; [α-$^{17}O_2$]AMPS, adenosine 5'-*O*-[$^{17}O_2$]phosphorothioate; ADP, adenosine 5'-diphosphate; (S_P)-[α-$^{17}O_2$]ADPαS, adenosine 5'-*O*-(1-thio[1-$^{17}O_2$]diphosphate) with the *S* configuration about P_α; (S_P)-[β-$^{18}O_3$]ADPαS, adenosine 5'-*O*-(1-thio[2-$^{18}O_3$]diphosphate) with the *S* configuration about P_α; (S_P)-[α-$^{17}O_2$,β-$^{18}O_3$]ADPαS, adenosine 5'-*O*-(1-thio[1-$^{17}O_2$,2-$^{18}O_3$]diphosphate) with the *S* configuration about P_α; (S_P)- and (R_P)-β-(cyanoethyl)-ADPαS, adenosine 5'-*O*-[1-thio-2-(cyanoethyl)diphosphate] with the *S* and *R* configuration about P_α, respectively; [β-^{18}O]ADPβS, adenosine 5'-*O*-(2-thio[2-^{18}O]diphosphate); (R_P)- and (S_P)-[β-^{18}O]ADPβS, [β-^{18}O]ADPβS having the *R* and *S* configurations about the β phosphorus; (S_P)-[α-$^{18}O_2$]ADPαS, adenosine 5'-*O*-(1-thio[1-$^{18}O_2$]diphosphate) with the *S* configuration about P_α; ATP, adenosine 5'-triphosphate; [γ-$^{18}O_3$]ATP, adenosine 5'-[3-$^{18}O_3$]triphosphate; [β-^{18}O,γ-^{18}O]ATP, adenosine 5'-[2-^{18}O,3-^{18}O]triphosphate; ATPαS, adenosine 5'-*O*-(1-thiotriphosphate); [β-$^{18}O_3$,γ-$^{18}O_3$]ATPαS, adenosine 5'-*O*-(1-thio[2-$^{18}O_3$,3-$^{18}O_3$]triphosphate); ATPβS, adenosine 5'-*O*-(2-thiotriphosphate); (S_P)-[β-^{18}O]ATPβS, adenosine 5'-*O*-(2-thio[2-^{18}O]triphosphate) with nonbridging ^{18}O and the *S* configuration about the β phosphorus; (S_P)-[β,γ-bridging-^{18}O]ATPβS, ATPβS with ^{18}O bridging P_β and P_γ and the *S* configuration about P_β; ATPγS, adenosine 5'-*O*-(3-thiotriphosphate); [γ-^{18}O]ATPγS, adenosine 5'-*O*-(3-thio[3-^{18}O]triphosphate); 3',5'-cAMPS, adenosine 3',5'-cyclic phosphorothioate; 2',3'-cUMPS, uridine 2',3'-cyclic phosphorothioate; [γ-^{16}O,^{17}O,^{18}O]ATP, adenosine 5'-[3-^{17}O,3-^{18}O]triphosphate; 3',5'-c[α-^{18}O]AMP, adenosine 3',5'-cyclic [^{18}O]phosphate; NADP, nicotinamide adenine dinucleotide phosphate; NAD, nicotinamide adenine dinucleotide; NADPH, reduced NADP; NADH, reduced NAD; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; PEP, phosphoenolpyruvate; [$^{18}O_3$]PEP, [$^{18}O_3$]phosphoenolpyruvate; TCA, trichloroacetic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoate); NMR, nuclear magnetic resonance.

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phosphoryl group to the neighboring phosphorothioate center. Our results are consistent with a mechanism involving the intermediate formation of *cyclo*-diphosphates as intermediates. A preliminary report of a portion of this work has appeared.[4]

Experimental Section

Materials. Enzymes, coenzymes, and other biochemicals were purchased from the Sigma Chemical Co. or Boehringer-Mannheim and used without further purification. DEAE-Sephadex A-25 was purchased from Sigma Chemical and Dowex 1-X8, chloride form, 200–400 mesh, from Bio-Rad Laboratories. Cyanogen bromide and triethylamine wer purchased from Aldrich Chemical Co. Triethylamine was redistilled before use. Dimethylformamide was purchased from Fisher Scientific and redistilled in vacuo at 25 °C from BaO before use. Reagent grade salts were purchased from commercial suppliers and used without further purification. Both 52% $H_2{}^{17}O$ (containing 52.4% ^{17}O, 35.1% ^{18}O, 12.5% ^{16}O) and 97% $H_2{}^{18}O$ (containing 97.5% ^{18}O, 2.0% ^{16}O, 0.5% ^{17}O) were purchased from Monsanto Research Corporation, Mound Laboratory.

$[^{18}O_4]KH_2PO_4$ was prepared by the method of Hackney et al.,[5] except that the product was purified by column chromatography on Dowex 1-X8 (chloride form) by elution with 20 mM HCl. [γ-$^{18}O_3$]ATP was prepared by published procedures[5] and purified by column chromatography on DEAE-Sephadex A-25. [$^{18}O_3$]PEP was synthesized by exchange of an acidic solution in $H_2{}^{18}O$.[6] AMPS and [α-$^{17}O_2$]AMPS were synthesized as described previously.[7] *N*-Nitroso-*N*-ethylurea was prepared by the method of Ardnt.[8]

Triethylammonium bicarbonate was prepared by passing CO_2 gas generated from vaporizing dry ice through a fine fritted-glass filter immersed in a well-stirred 1 M solution of triethylamine maintained at 4 °C until the pH reached the desired value (7.6–8.0). The solution was stored in a sealed bottle.

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

Nucleotides were purified by anion-exchange chromatography through columns of DEAE Sephadex A-25 in the HCO_3^- form. The columns were eluted with linear gradients of triethylammonium bicarbonate at 4 °C. Nucleotides in pooled column fractions were desalted by rotary evaporation in vacuo using a Büchi apparatus with a bath temperature no higher than 30 °C. The dried residues initially obtained were twice dissolved in small volumes of 95% ethanol and again evaporated to remove final traces of triethylammonium bicarbonate. The nucleotides were finally dissolved in a minimum volume of water, adjusted to pH 10 by addition of triethylamine, and stored at −15 °C.

AMP, ADP, and ATP in reaction mixtures and column fractions were identified and quantified by enzymatic assays. AMP and ADP were measured in the presence of AMPS and ADPαS using a coupled enzymatic assay with alkaline phosphatase and adenosine deaminase. The assay was based on the phosphatase-catalyzed conversion of AMP and ADP, but not AMPS or ADPαS, to adenosine followed by deamination to inosine. The decrease in ϵ_{265} due to this process was 7.08×10^3 M^{-1} cm^{-1}. AMP was assayed in the absence of AMPS by the coupled actions of adenylate kinase, pyruvate kinase, and lactate dehydrogenase with excess cosubstrates phosphoenolpyruvate, NADH, and ATP. The overall reaction produced ATP and lactate and consumed NADH and AMP in equivalent amounts. The decrease in A_{340} due to NADH consumption (ϵ_{260} 6.22×10^3 M^{-1} cm^{-1}) was used to calculate AMP concentration. ADP was measured in the same way as AMP but in the absence of adenylate kinase and ATP. ATP was measured by the coupled actions of hexokinase and glucose-6-phosphate dehydrogenase in the presence of excess cosubstrates D-glucose and NADP. NADPH was produced in amounts equivalent to ATP consumed and measured by the increase in A_{340} (ϵ_{340} 6.22×10^3 M^{-1} cm^{-1}) for NADPH. The phosphorothioate groups of AMPS, ADPβS, and thiophosphate were measured by reaction with excess 5,5′-dithiobis(2-nitrobenzoate). The concentration was calculated from the increase in A_{412} and the extinction coefficient for 3-carboxy-4-nitrothiophenolate anion at pH 8 (13.6×10^3 M^{-1} cm^{-1}).[9] Inorganic phosphate was detected with malachite green and acid molybdate.[10,11] Organic phosphates were ashed and then assayed for inorganic phosphate.[12] To avoid contamination by inorganic phosphate all glassware was acid washed.

^{31}P NMR Spectral Measurements. ^{31}P NMR spectra of nucleotides were obtained in the Fourier transfer mode using Bruker 200-, 300-, and 360-MHz spectrometers and a Nicolet 200-MHz instrument. Samples contained 1–5 mM nucleotide, 30–50 mM EDTA and 40% D_2O in 2.0–2.5-mL volumes at pHs between 9 and 10. The spectrometers were field-frequency locked on the resonance of deuterium in the solvent. The external reference was 1 N (0.33 M) $^2H_3PO_4$ in ^2H_2O. Most spectra were collected with broad-band proton spin decoupling at 2-W power (300 MHz) or 1.5 W (200 MHz). These power levels provided adequate decoupling without excessive heating of the samples. For observing effects of ^{18}O on the P_β or P_γ signals of ATP or ATPβS, the decoupler was not used. This improved temperature control and optimized resolution.

Mass Spectral Analysis of Triethyl Phosphate. The isotopic enrichments of ADP and AMPS samples were determined by mass spectral analysis of their volatile triethyl phosphate derivatives. The nucleotides were enzymatically degraded to two inorganic phosphate samples derived from P_α and P_β. Samples of inorganic phosphate, 1.0–2.0 μmol, were dissolved in 200 μL of ethanol containing 20 μL of water. The phosphates were ethylated by the addition of an ether solution of diazoethane that was generated from *N*-nitroso-*N*-ethylurea by the addition of 40% KOH.[13] Diazoethane was made in small quantities and distilled just prior to use. The ether solution of diazoethane at 0 °C was added to the phosphate solution until the solution turned yellow. After 15 min at ambient temperature the diazoethane and ether were removed by evaporation in a stream of nitrogen, and the sample was concentrated to 20 μL. Samples (~10 μL) were injected into a Packard Model 427 gas chromatograph equipped with a thermal conductivity detector. The column was a 0.6 mm × 6 ft glass column packed with 10% SE-30 on chromosorb W and was purchased from Supelco. At a column temperature of 105 °C, detector temperature of 260 °C, and a 10 mL/min nitrogen carrier gas flow rate, the triethyl phosphates had a retention time of 8 min. Samples were recovered by condensation onto a liquid nitrogen cooled 0.6 mm × 1.3 ft Pyrex glass capillary tube of 0.2-mm internal diameter. The purified product was immediately dissolved in ether by rinsing the tube with ~6 mL of ether. The etheral solution was concentrated to ~50 μL under a stream of nitrogen. The recoveries of the triethyl phosphate as determined by the ashing procedure of Ames[12] were typically 50%.

The mass spectra of the triethyl phosphates were recorded on an AEI MS9 equipped with a ES-50 data system. The ionization electron energy was 70 mev and source temperature 90–100 °C. Samples (5 μL) were volatilized using a direct-inlet probe. Multiple scans were made of m/e 28–830, and the intensities of the base peak (m/e 99) corresponding to $H_4PO_4^+$ was used to determine the isotopic enrichments. At least four scans were used for the calculation of the enrichments.

The M + 1 peak (m/e 100) of the triethyl phosphate derived from P_β of the product ADP was used as a measure of the ring opening of the *cyclo*-diphosphate intermediate by attack of water at P_α. The M + 6 peak (m/e 105) was used to calculate the amount of desulfurization that occurred by direct displacement of the thiocyanate by water. When the M + 6 peak was corrected for the transfer of ^{18}O from P_α to the P_β moiety by ring opening of the *cyclo*-diphosphate intermediate by attack of water at P_α, the values obtained did not differ significantly from those reported in Tables I–IV. In all the calculations it was assumed that ring opening of the cyclic intermediates occurred by cleavage of the two P–O bonds with equal probability.

Synthesis of ADPαS and ADPβS Specifically Enriched with ^{17}O or ^{18}O. (R_P)-[β-^{18}O]ADPβS and (S_P)-[β-^{18}O]ADPβS were synthesized as described by Richard and Frey.[1d] ADPβS was synthesized by a simplified modification of the same procedure. (S_P)-ATPβS was prepared by enzymatic phosphorylation of ADPβS using phosphoenolpyruvate and pyruvate kinase as described by Eckstein and Goody.[1b] [α-$^{18}O_2$]AMPS and (S_P)-[α-$^{18}O_2$]ADPαS were synthesized as described by Richard and Frey,[1d] and the same procedures were used for (S_P)-[α-$^{17}O_2$]ADPαS and [α-$^{17}O_2$]AMPS.

To prepare (S_P)-[β-$^{18}O_3$]ADPαS, AMPS was converted to (S_P)-[β-$^{18}O_3$,γ-$^{18}O_3$]ATPαS by the coupled reaction of adenylate kinase and

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pyruvate kinase using [γ-$^{18}O_3$]ATP and [$^{18}O_3$]PEP.[14,15] The reaction mixture consisted of 14.2 mM AMPS, 0.41 mM [γ-$^{18}O_3$]ATP, 48.2 mM [$^{18}O_3$]PEP, 30 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 100 mM triethanolamine buffer (pH 8.2), adenylate kinase (300 units), and pyruvate kinase (160 units) in a total volume of 5 mL. After 20 h at ambient temperature, the reaction mixture was applied to a 2.5 × 30 cm column of DEAE Sephadex A-25 (bicarbonate form) and eluted at 4 °C with a 4-L linear gradient of 0.2–0.8 M triethylammonium bicarbonate. The fractions containing [β-$^{18}O_3$,γ-$^{18}O_3$]ATPαS, which came off the column at ~0.6 M buffer, were pooled and desalted. The ATPαS (57 μmol) was converted to [β-$^{18}O_3$]ADPαS (95% yield) by enzymatic degradation with hexokinase.[16] (S_P)-[α-$^{17}O_2$,β-$^{18}O_3$]ADPαS was prepared by the same procedure starting with [α-$^{17}O_2$]AMPS.

Preparation of (S_P)-[β-^{18}O]ATPβS and (S_P)-[β,γ-bridging-^{18}O]ATPβS. (R_P)-[β-^{18}O]ADPβS (20 μmol) and (S_P)-[β-^{18}O]ADPβS (25 μmol) were converted to (S_P)-[β-^{18}O]ATPβS and (S_P)-[β,γ-bridging-^{18}O]ATPβS, respectively, by stereoselective phosphorylation with phosphoenolpyruvate catalyzed by pyruvate kinase.[1b] Pyruvate kinase catalyzes selective phosphorylation of the S oxygen at the β-position of these substrates so that the product resulting from (R_P)-[β-^{18}O]ADPβS has mainly nonbridging ^{18}O at P_β while that from (S_P)-[β-^{18}O]ADPβS has mainly β,γ-bridging ^{18}O.[17a,b] The 10-mL phosphorylation reaction mixtures contained 40 mM Tris-HCl buffer at pH 8.0, 4 mM $MgCl_2$, 0.38 M KCl, 0.86 mM dithiotreitol, 2.5 mM phosphoenolpyruvate, 2 mM (R_P)- or (S_P)-[β-^{18}O]ADPβS, and 40 units mL^{-1} of pyruvate kinase. The reaction proceeded for 6 h at 25 °C, production of ATPβS being monitored by thin-layer chromatography of aliquots. ATPβS was purified by chromatography through a 1.5 × 25 cm column of DEAE Sephadex A-25 eluted with a gradient of triethylammonium bicarbonate increasing in concentration from 0.2 to 0.55 M and generated from 0.4 L of each component. Fractions 7.5 mL in volume were collected at a flow rate of 0.5 mL min^{-1}. A_{260} measurements on selected fractions revealed a prominent band of nucleotide eluting between fractions 85 and 95. Peak fractions were pooled and desalted as described above. ATPβS was identified by comparison of its ^{31}P NMR spectrum and thin-layer chromatographic properties with those of authentic material. The yield of chromatographically pure material was 85%. The position of ^{18}O as bridging in (S_P)-[β,γ-^{18}O]ATPβS and nonbridging in (S_P)-[β-^{18}O]-ATPβS was confirmed by ^{31}P NMR.

Reaction of (S_P)-ATPβS with BrCN in $H_2{}^{18}O$. To conserve $H_2{}^{18}O$ the reaction was carried out on a small scale in three repetitions, $H_2{}^{18}O$ being recovered after each reaction and reused in the next. The triethylammonium salt of (S_P)-ATPβS (10 μmol) was dried by rotary evaporation. The residue in a 10-mL pear-shaped flask was dissolved in 0.5 mL of 0.1 M sodium phosphate buffer at pH 7.0, frozen as a thin film on the wall of the flask and lyophilized. $H_2{}^{18}O$ (0.5 mL, 95.2% ^{18}O) and BrCN (20 μmol) were added to the dried residue. After 30 min at 25 °C, 12 μmol of cysteine crystals were added to the solution to consume unreacted BrCN. $H_2{}^{18}O$ was recovered by connecting the flask through a three-way connecting tube to a water aspirator and a second flask cooled in liquid nitrogen. The temperature of the reaction flask was maintained with a luke warm bath and the $H_2{}^{18}O$ transferred and trapped in the cold flask (0.4 mL). ATPβS (8 μmol) lyophilized with sodium phosphate as described above was dissolved in the 0.4 mL of $H_2{}^{18}O$ recovered from the first reaction. BrCN (16 μmol) was added and the procedure repeated. The second recovery of $H_2{}^{18}O$ (0.37 mL) was again used in the reaction of 7 μmol of ATPβS with 14 μmol of BrCN. The final recovery of $H_2{}^{18}O$ was 0.35 mL and its ^{18}O enrichment by mass spectroscopy found to be 83%.

The products of the three reactions were dissolved in distilled water, combined, and absorbed on a 1 × 20 cm column of DEAE Sephadex A-25. The column was eluted with a gradient of triethylammonium bicarbonate increasing in concentration from 0.2 to 0.45 M, total volume 1.0 L. The major band and the last one eluted was identified as ATP by enzymatic and ^{31}P NMR analysis after pooling fractions and desalting. The yield was 12.7 μmol (51%). The band (3 μmol) that appeared just ahead of ATP gave a positive reaction with 5,5′-dithiobis(2-nitrobenzoate) and may have been ADPβS.

Reaction of (S_P)-ADPαS with BrCN in $H_2{}^{18}O$. (S_P)-ADPαS (50 μmol) as the triethylammonium salt was dissolved in 0.5 mL of 0.5 M potassium tetraborate at pH 10.6 and the solvent removed by rotary evaporation. The residue was dried in vacuo over P_2O_5 for 8 h before adding 0.5 mL of $H_2{}^{18}O$ enriched with 0.97 mol ^{18}O/mol. To this solution was added 12.1 mg of solid BrCN (114 μmol). The solid slowly dissolved as the flask was swirled by hand for 10 min. The pH was readjusted to 10.6 by adding 2.5 M KOH in $H_2{}^{18}O$. Another 12.1 mg of solid BrCN was added and dissolved. The reaction was stopped after 10 additional min by the addition of 31.5 mg of anhydrous cysteine. $H_2{}^{18}O$ was recovered by vacuum distillation into a liquid N_2 trap.

The product dissolved in 10 mL of 0.1 M triethylammonium bicarbonate was applied to a 1.5 × 30 cm column of DEAE Sephadex. The column was eluted with a gradient of triethylammonium bicarbonate increasing in concentration from 0.27 to 0.67 M, total volume 1.6 L. Fractions 20 mL in volume were collected. A_{260} measurements showed that two major bands were eluted, [^{18}O]ADP at 0.4 M salt in 20% yield and AMP at 0.35 M salt in 50% yield. These compounds were identified by enzymatic and ^{31}P NMR analyses after pooling the fractions and desalting as described under **General Methods**.

Reaction of (S_P)-[α-$^{18}O_2$]ADPαS with BrCN. (S_P)-[α-$^{18}O_2$]ADPαS (70 μmol) as its triethylammonium salt was dissolved in 0.8 mL of 1.0 M potassium tetraborate at pH 10.4, transferred to a 10-mm NMR tube, and combined with 0.1 mL of 0.1 M EDTA and ^2H_2O to a total volume of 1.8 mL. The ^{31}P NMR spectrum was obtained, 7.43 mg of solid BrCN were added, and the spectrum was redetermined after 5 min. The procedure was repeated twice more at 5-min intervals, first with 10.66 mg of BrCN and again with 7.79 mg to effect complete reaction. Overall 25.88 mg or 3.5 equiv of BrCN were added. The reaction was quenched by addition of 25 mg of cysteine. The sample was diluted with water to 8 mL and applied to a 1.5 × 20 cm column of DEAE Sephadex. The column was eluted with a gradient of triethylammonium bicarbonate increasing in concentration from 0.2 to 0.5 M, total volume 1.0 L. [^{18}O]ADP appeared as a major A_{260}-absorbing band in the effluent at 0.3 M salt in a yield of 71%. Pooled fractions were desalted and prepared for ^{31}P NMR analysis as described under **General Methods**.

Reaction of (S_P)-[β-^{18}O]ATPβS and (S_P)-[β,γ-bridging-^{18}O]ATPβS with BrCN. (S_P)-ATPβS with ^{18}O enrichment either in the nonbridging P_β position or in the position bridging P_β and P_γ was converted to [^{18}O]ATP by reaction with BrCN in H_2O. The reactions were carried out under the conditions described above for that of (S_P)-ATPβS in [^{18}O]H_2O but in more dilute solution: 10 mM nucleotide, 20 mM BrCN, and 50 mM potassium phosphate buffer at pH 7.0. After quenching the reaction with cysteine, [^{18}O]ATP was purified from the solution by chromatography through DEAE Sephadex as described above. The yield of purified [^{18}O]ATP was 50%.

Isolation of P_α, P_β, and P_γ from [β-^{18}O,γ-^{18}O]ATP. The P_γ, P_β, and P_α phosphoryl groups of [β-^{18}O,γ-^{18}O]ATP were isolated as phosphate for analysis of ^{18}O content by a systematic enzymatic degradation. [β-^{18}O,γ-^{18}O]ATP from the desulfurization of ATPβS with cyanogen bromide in $H_2{}^{18}O$ was first converted to ADP and glucose 6-phosphate (Glc-6-P). The 10-mL reaction mixture contained 25 mM Tris-HCl buffer at pH 8.0, 1 mM [β-^{18}O,γ-^{18}O]ATP, 10 mM glucose, and 5 mM $MgCl_2$, as well as hexokinase (5 units). After reaction at 25 °C for 1 h, the amount of Glc-6-P generated was measured by withdrawing an aliquot of the reaction mixture and assaying enzymatically for Glc-6-P. This assay verified that the reation was complete, and the products were purified by DEAE Sephadex A-25 chromatography through a 0.7 × 18 cm column eluted with a triethylammonium bicarbonate gradient increasing from 0.05 to 0.3 M, total volume 250 mL. Fractions containing ADP were detected by A_{260} measurements and those containing Glc-6-P by enzymatic analysis. The Glc-6-P contained the γ-phosphoryl group.

The [β-^{18}O]ADP isolated above was desalted as described under Methods and its β-phosphoryl group transferred to glucose by the coupled actions of adenylate kinase and hexokinase, producing glucose 6-[^{18}O]-phosphate and AMP. The reaction mixture contained 25 mM Tris-HCl buffer at pH 8.0, 10 mM $MgCl_2$, 2 mM [β-^{18}O]ADP, 20 mM glucose, 1 mM DTT, adenylate kinase (5 units), and hexokinase (5 units) in a total volume of 5 mL. The glucose-6-phosphate dehydrogenase assay was again used to monitor the formation of Glu-6-P. The phosphoryl group transferred to glucose was exclusively from the β-phosphoryl group.

The reaction products were purified by chromatography through a DEAE Sephadex A-25 column (0.7 × 18 cm) eluted with a gradient of triethylammonium bicarbonate at pH 8.0 increasing in concentration from 0.08 to 0.2 M, total volume 200 mL. AMP-containing fractions were identified by their A_{260} values and those containing Glc-6-P by enzymatic analysis. Fractions containing AMP and glucose 6-[^{18}O]-phosphate from this column (representing P_α and P_β of [β-^{18}O,γ-^{18}O]-ATP) and those containing glucose 6-[^{18}O]phosphate (representing P_γ) from the preceding column were separately pooled and desalted as described in the methods.

The two glucose 6-[^{18}O]phosphate solutions and the AMP solution were each subjected to alkaline phosphatase digestion to generate inor-

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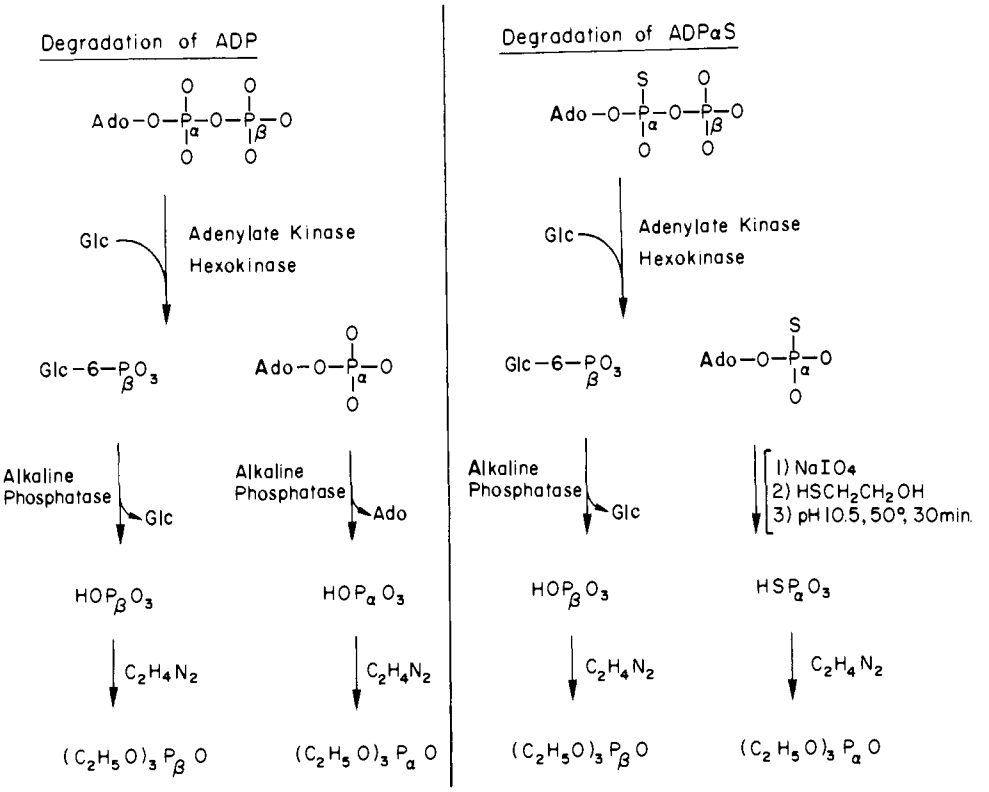
Scheme I. Degradation Sequence of ADP and ADPαS[a]



[a] The conditions for the degradation are described in the Experimental Section.

ganic phosphate. The reaction mixtures contained 0.1 M Tris-HCl buffer at pH 8.0, 0.1 M NaCl, 10 mM glucose 6-[^{18}O]phosphate (or AMP), and alkaline phosphatase (5 units) in a total volume of 1.0 mL. The solutions were incubated at 37 °C for 2 h and then applied to 0.7 × 18 cm columns of DEAE Sephadex A-25 to purify the inorganic phosphate.

The columns were eluted with triethylammonium bicarbonate gradients at pH 8.0 increasing in concentration from 0.05 to 0.14 M, total volume 150 mL. Phosphate-containing fractions were detected by colorimetric analysis, pooled, and desalted as described under **General Methods**. The purified [^{18}O]phosphates were subjected to ^{31}P NMR analysis to determine their ^{18}O enrichments.

Determination of Isotopic Enrichment of a Mixture of (S_P)-[α-$^{17}O_2$]-ADPαS (4) plus (S_P)-[β-$^{18}O_3$]ADPαS (5). To determine the isotopic enrichments at each of the phosphate atoms of a sample of (S_P)-[α-$^{17}O_2$]ADPαS (25.9 μmol) mixed with (S_P)-[β-$^{18}O_3$]ADPαS (28.5 μmol), a method for selectively degrading ADPαS to two inorganic phosphate samples derived from P_α and P_β was used (Scheme I). **4** plus **5** was first converted to [α-$^{17}O_2$]AMPS and Glc-6-[$^{18}O_3$]P_β by the combined actions of adenylate kinase and hexokinase. A 500-μL solution containing the triethylammonium salts of a mixture of **4** plus **5** (4-μmol total), 40 mM glucose, 40 mM $MgCl_2$, hexokinase (2 units), and adenylate kinase (5 units) in 0.1 M triethylammonium bicarbonate, pH 8.0, was incubated at ambient temperature. The extent of the reaction was followed by removing 5-μL aliquots and assaying for Glc-6-P. After the reaction was complete, the solution was diluted to 3 mL and applied to a 1 × 10 cm column of DEAE Sephadex A-25 (bicarbonate form). The column was first washed with 25 mL of water. Glc-6-[$^{18}O_3$]P was then eluted with 0.08 M triethylammonium bicarbonate and [α-$^{17}O_2$]AMPS with 0.2 M triethylammonium bicarbonate. The Glc-6-[$^{18}O_3$]P (3.24 μmol) and [α-$^{17}O_2$]AMPS (3.76 μmol) were separately pooled, desalted, and converted to inorganic phosphate and thiophosphate, respectively.

The triethylammonium salt of AMPS (2.02 μmol) recovered from the previous column was treated with 2 equiv of sodium periodate at neutral pH and ambient temperature for 15 min. 2-Mercaptoethanol (42.5 mmol) was added to reduce iodate and periodate to I^-. The solution was adjusted to pH 10.5 by using KOH and then placed at 50 °C for 30 min to effect the β-elimination of thiophosphate from the periodate cleaved nucleotide.[17] The solvent was removed on the rotary evaporator and the resulting residue dissolved in 5 mL of water and applied to a 1 × 10 cm column of DEAE Sephadex A-25 (bicarbonate form) equilibrated with water. The column was washed with water and then 0.1 M triethylammonium bicarbonate until the 260-nm absorbance was negligible. The [$^{17}O_2$]thiophosphate was eluted with 0.3 M triethylammonium bicarbonate. After removing the triethylammonium bicarbonate, the thiophosphate was dissolved in 0.2 mL of ethanol containing 0.02 mL of water, acidified with 2 μL of concentrated HCl, and then alkylated with diazoethane. During the ethylation procedure the thiophosphate was desulfurized without loss of the heavy oxygen isotopes. The resulting triethyl phosphate was purified by gas chromatography and analyzed by mass spectroscopy as described earlier. The mass spectral data are summarized in Table I.

Table I. Mass Spectral Analysis of Triethyl Phosphates Derived from P_α of **4** plus **5** after Desulfurization at pH 10.2[a]

m/e for base peak	rel peak intensities: [α-$^{17}O_2$]-ADPαS [β-$^{18}O_3$]-ADPαS	[^{17}O,^{18}O]-ADP	calcd for intramol mechanism	calcd for intermol mechanism
99 ^{16}O	57.74 ± 0.52	43.50 ± 0.73	44.22	49.46
100 $^{17}O_1$	5.02 ± 0.49	10.24 ± 0.66	8.47	4.30
101 $^{17}O_2$; $^{18}O_1$	18.79 ± 0.68	32.55 ± 0.28	32.33	24.43
102 $^{17}O_1{}^{18}O_1$	13.77 ± 0.41	10.69 ± 0.20	11.29	12.52
103 $^{18}O_2$; $^{17}O_2{}^{18}O_1$	4.49 ± 0.22	3.02 ± 0.26	3.68	6.57
104 $^{18}O_2{}^{17}O_1$				1.97
105 $^{18}O_3$				0.64

[a] The [^{17}O,^{18}O]ADP obtained by desulfurization of **4** plus **5** by cyanogen bromide at pH 10.2 was degraded to triethyl phosphate and analyzed by mass spectrography as described in the Experimental Section.

The ^{18}O-labeled Glc-6-P (1.62 μmol) isolated above was dissolved in 0.2 mL of 50 mM Tris-HCl, pH 8.0, and incubated with alkaline phosphatase (0.06 units) at ambient temperature. After ~80% reaction, the solution was acidified with TCA (0.2 N final concentration), readjusted to pH 8.0, diluted to 1 mL, and applied to a 0.5 × 5 cm column of Dowex 1-X8 (chloride form). The column was eluted with 20 mM HCl and the fractions containing phosphate were combined and ethylated with diazoethane. The mass spectral analysis of the GC-purified triethyl phosphate is presented in Table II.

Reaction of (S_P)-[α-$^{17}O_2$]ADPαS (4) plus (S_P)-[β-$^{18}O_3$]ADPαS (5) with Cyanogen Bromide at pH 10.2. A mixture of the triethylammonium salts of 4 plus **5** (20.62-μmol total) was dissolved in 0.5 mL of 0.08 M potassium tetraborate buffer, pH 10.2. Cyanogen bromide (42.45 μmol)

Table II. Mass Spectral Analysis of Triethyl Phosphates Derived from P_β of **4** plus **5** after Desulfurization at pH 10.2[a]

m/e for base peak	rel peak intensities: [α-$^{17}O_2$]-ADPαS [β-$^{18}O_3$]-ADPαS	[^{17}O,^{18}O]ADP	calcd for intramol mechanism	calcd for intermol mechanism
99 ^{16}O	59.33 ± 0.94	57.30 ± 0.49	57.53	64.26
100 $^{17}O_1$		4.31 ± 0.39	4.31	
101 $^{18}O_1$	21.74 ± 0.15	27.51 ± 0.25	25.59	23.17
103 $^{18}O_2$	14.06 ± 0.34	9.60 ± 0.25	10.99	10.99
105 $^{18}O_3$	4.80 ± 0.47	1.58 ± 0.26	1.58	1.58

[a] The [^{17}O,^{18}O]ADP obtained by desulfurization of **4** plus **5** by cyanogen bromide at 10.2 was degraded to triethyl phosphate and analyzed by mass spectroscopy as described in the Experimental Section.

was added and the solution stirred at ambient temperature for 10 min. Another 2 equiv of cyanogen bromide were added, and after 10 min cysteine (50 μmol) was added to consume the unreacted cyanogen bromide. The reaction mixture was diluted to 25 mL and applied to a 1.5 × 30 cm column of DEAE Sephadex A-25 that had been equilibrated with 0.1 M triethylammonium bicarbonate. The column was eluted at 4 °C with a 2-L linear gradient of triethylammonium bicarbonate increasing in concentration from 0.1 to 0.45 M. Fractions of 10-mL volume were collected. AMP appeared in fractions 51–58 (3.1 μmol) while ADP appeared in fractions 99–112 (13.72 μmol).

Degradation of [^{17}O,^{18}O]ADP Isolated from Cyanogen Bromide Reaction of 4 plus 5. The [^{17}O,^{18}O]ADP isolated from the preceding reaction was first degraded to [^{17}O,^{18}O]AMP and Glc-6-[^{17}O,^{18}O]P by the combined actions of adenylate kinase and hexokinase (Scheme I). The products were independently treated with alkaline phosphatase and the resulting samples of inorganic phosphate isolated and analyzed by mass spectroscopy of their triethyl esters. The [^{17}O,^{18}O]ADP (5.48 μmol) was dissolved in 0.5 mL of 0.1 M triethylammonium bicarbonate, pH 8.0, containing 40 mM glucose, 40 mM $MgCl_2$, hexokinase (2 units), and adenylate kinase (5 units). The reaction was followed by removing 5-μL aliquots and assaying for the formation of glucose 6-phosphate by using glucose-6-phosphate dehydrogenase. After the reaction was complete, the solution was diluted to 3 mL and applied to a 1 × 10 cm column of DEAE Sephadex A-25 (bicarbonate form) equilibrated with water, and the column was washed with 25 mL of water. Glc-6-[^{17}O,^{18}O]P (3.51 μmol) was eluted with 0.08 M triethylammonium bicarbonate and [^{17}O,^{18}O]AMP (3.75 μmol) with 0.2 M triethylammonium bicarbonate. The fractions containing Glc-6-P and AMP were separately pooled, desalted, and treated with alkaline phosphatase to produce inorganic phosphate. The phosphatase reaction mixture contained 50 mM Tris-HCl, pH 8.0, 10 mM Glc-6-P or AMP, and alkaline phosphatase (0.06 units) in 0.25 mL and was incubated at ambient temperature. After 80% reaction, TCA was added (0.2 N final concentration). The solution was reneutralized, diluted to 1 mL, and applied to a 0.5 × 5 cm column of Dowex 1-X8 (chloride form). The inorganic phosphate was eluted with 20 mM HCl. Phosphate-containing fractions were concentrated and then perethylated with diazoethane. The triethyl phosphates were purified by gas chromatography and analyzed by mass spectroscopy. The results are given in Tables I and II.

Determination of Isotopic Enrichment of (S_P)-[α-$^{17}O_2$,β-$^{18}O_3$]ADPαS (10). The procedure was analogous to that described for the determination of the isotopic enrichment for the mixture of **4** plus **5**. The mass spectral data for the triethyl phosphates derived from P_α and P_β of **10** are reported in Tables III and IV, respectively.

Reaction of 10 with Cyanogen Bromide in Potassium Phosphate Buffer, pH 7.0. The triethylammonium salt of (S_P)-[α-$^{17}O_2$,β-$^{18}O_3$]ADPαS (**10**) (31.2 μmol) was dissolved in 1.5 mL of 0.1 M potassium phosphate buffer, pH 7.0. Cyanogen bromide (32.12 μmol) was added, and after 15 min a second equivalent of cyanogen bromide was added. After 30 min the reaction was quenched by the addition of cysteine (34.85 μmol). The solution was diluted to 30 mL and applied to a 1.5 × 30 cm column of DEAE Sephadex A-25 that had been equilibrated with 0.1 triethylammonium bicarbonate. The column was eluted at 4 °C with a 2-L linear gradient of 0.1–0.45 M triethylammonium bicarbonate. Fractions of 10-mL volume were collected. AMP (4.81 μmol) was eluted in fractions 49–58, AMPS (14.53 μmol) in fractions 69–80, and ADP (5.16 μmol) in fractions 103–113. The nucleotides were separately pooled, concentrated, and desalted.

Determination of Isotopic Enrichment of [^{17}O,^{18}O]ADP Isolated from Reaction of 10 with Cyanogen Bromide at pH 7.0. The [^{17}O,^{18}O]ADP isolated from the previous reaction was converted to two triethyl phosphate samples derived from P_α and P_β. The procedure was the same as that described earlier, and the mass spectral data in Tables III and IV were obtained.

Table III. Mass Spectral Analysis of Triethyl Phosphate Derived from P_α of **10** after Desulfurization at pH 7.0[a]

m/e for base peak	rel peak intensities: [α-$^{17}O_2$,β-$^{18}O_3$]ADPαS	[^{17}O,^{18}O]-ADP	calcd for intramol mechanism	calcd for intermol mechanism
99 ^{16}O	3.21 ± 0.22	5.80 ± 0.35	4.61	2.59
100 $^{17}O_1$	12.76 ± 0.52	16.70 ± 0.55	17.38	10.27
101 $^{17}O_2$; $^{18}O_1$	35.72 ± 0.78	29.24 ± 0.80	29.43	29.39
102 $^{17}O_1{}^{18}O_1$	36.02 ± 0.52	25.34 ± 0.75	24.65	31.49
103 $^{18}O_2$; $^{17}O_2{}^{18}O_1$	12.29 ± 0.51	15.74 ± 0.53	14.52	16.87
104 $^{18}O_2{}^{17}O_1$		5.29 ± 0.52	7.02	7.02
105 $^{18}O_3$		1.89 ± 0.25	2.39	2.39

[a] The [^{17}O,^{18}O]ADP obtained by desulfurization of **10** by cyanogen bromide was degraded to triethyl phosphate and analyzed by mass spectroscopy as described in the Experimental Section.

Table IV. Mass Spectral Analysis of Triethyl Phosphate Derived from P_β of **10** at pH 7.0[a]

m/e for base peak	rel peak intensities: [α-$^{17}O_2$,β-$^{18}O_3$]ADPαS	[^{17}O,^{18}O]-ADP	calcd for intramol mechanism	calcd for intermol mechanism
99 ^{16}O	18.25 ± 0.49	23.38 ± 0.41	21.30	23.54
100 $^{17}O_1$		3.03 ± 0.45	2.24	
101 $^{17}O_2$; $^{18}O_1$	38.06 ± 0.75	37.38 ± 0.65	36.64	40.49
102 $^{17}O_1{}^{18}O_1$		3.73 ± 0.24	3.85	
103 $^{18}O_2$; $^{17}O_2{}^{18}O_1$	27.86 ± 0.34	20.38 ± 0.25	24.17	26.71
104 $^{18}O_2{}^{17}O_1$		2.84 ± 0.21	2.54	
105 $^{18}O_3$	15.81 ± 0.52	9.26 ± 0.47	9.26	9.26

[a] The [^{17}O,^{18}O]ADP obtained by desulfurization of **10** by cyanogen bromide at pH 7.0 was degraded to triethyl phosphate and analyzed by mass spectroscopy as described in the Experimental Section.

Table V. Mass Spectral Analysis of Triethyl Phosphate Obtained from [^{17}O]AMP by Desulfurization of **10** at pH 7.0[a]

m/e for base peak	rel peak intensities of P_α: [α-$^{17}O_2$,β-$^{18}O_3$]ADPαS	[^{17}O]AMP
99 ^{16}O	3.21 ± 0.22	3.41 ± 0.04
100 $^{17}O_1$	12.76 ± 0.52	15.09 ± 0.09
101 $^{17}O_2$; $^{18}O_1$	35.72 ± 0.79	35.03 ± 0.19
102 $^{17}O_1{}^{18}O_1$	36.02 ± 0.52	33.19 ± 0.11
103 $^{18}O_2$	12.29 ± 0.51	13.28 ± 0.19

[a] The [^{17}O]AMP obtained by desulfurization of **10** with cyanogen bromide at pH 7.0 was degraded to triethyl phosphate and analyzed by mass spectroscopy as described in the Experimental Section.

Determination of Isotopic Enrichment of [^{17}O]AMP Isolated from Reaction of 10 with Cyanogen Bromide at pH 7.0. The [^{17}O]AMP isolated from the reaction of **10** with cyanogen bromide at pH 7.0 was hydrolyzed by alkaline phosphatase to inorganic phosphate by the procedure described earlier. The mass spectral results of the triethyl phosphate derivative are given in Table V.

Results

Desulfurization of ATPβS and ADPαS. In preliminary experiments the production of ATP or ADP from ATPβS or ADPαS by reaction with BrCN in aqueous solutions could be demonstrated under a variety of conditions. Yields varied depending upon the pH and the ratios of BrCN to nucleotide. In sodium acetate, sodium citrate, and sodium phosphate buffers ranging in pH from 4.1 to 7.0, reaction of 10 mM ATPβS with 20–100 mM BrCN produced ATP in yields ranging from 18% to 64%. The best yield was observed at pH 7.0 with phosphate buffer and 20 mM BrCN. The same result was observed with 50 or 100 mM BrCN, but smaller amounts of BrCN reduced the yield. In most trials ATP

Table VI. Effect of pH and Cyanogen Bromide Concentration on Desulfurization of ADPαS[a]

buffer	pH	ADPαS, mM	BrCN, mM	% yield AMP	AMPS	ADP	ADPαS
0.1 M KPO_4	7.0	18.1	35.65	16.0	47.0	15.0	
0.1 M KPO_4	7.0	25.28	88.48	51.0	18.0	16.0	
0.1 M KPO_4	7.0	18.1	11.40	7.5	32.0	10.0	35.0
0.1 M $K_4B_2O_7$	10.2	20.22	113.23	28.0		65.0	
0.1 M $Na_2CO_3/NaHCO_3$	10.3	14.4	86.40	15.0		60.0	

[a] ADPαS was treated with cyanogen bromide at ambient temperature. After quenching the unreacted cyanogen bromide with cysteine, the products were separated by chromatography on a 1.1 × 30 cm column of DEAE Sephadex A-25 (bicarbonate form) at 4 °C. The column was eluted with a 1-L linear gradient of triethylammonium bicarbonate increasing in concentration from 0.1 to 0.45 M. Fractions of 9-mL volume were collected. AMP appeared in fractions 24–26, AMPS in fractions 32–38, ADP in fractions 47–52, and ADPαS in fractions 59–66.

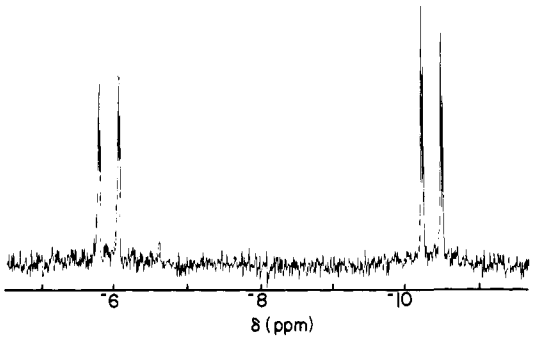


Figure 1. ^{31}P NMR spectrum of [α-^{18}O,β-^{18}O]ADP. The sample was prepared by reaction of ADPαS with BrCN in $H_2{}^{18}O$ as described in the Experimental Section. Shown is the 80.1-MHz ^{31}P NMR spectrum. Spectral parameters: δ_{P_β} −5.892, doublet; δ_{P_α} −10.453, doublet; $J_{P_\alpha P_\beta}$ = 22.3 Hz. Each doublet signal is accompanied by a second doublet 0.027 ppm upfield assigned to [β-^{18}O]ADP and [α-^{18}O]ADP with about 45% ^{18}O enrichment at each position.

was measured by enzymatic analysis, but in one experiment it was purified by ion-exchange column chromatography through DEAE Sephadex and characterized by thin-layer chromatography, enzymatic analysis, and ^{31}P NMR analysis.

Under identical reaction conditions with ADPαS, the conversion to ADP was only 15%, the other products isolated being AMP (16%) and AMPαS (47%) (Table VI). Additional cyanogen bromide resulted only in the conversion of AMPS to AMP. Inasmuch as the ADP formed was stable under the reaction conditions, these data suggested an initial partitioning of ADPαS between desulfurization to ADP and dephosphorylation to AMPS. Experiments using 0.6 equiv of cyanogen bromide indicated that the overall ratio of AMPS plus AMP to ADP was approximately 4:1. To determine the effect of pH on this partition, the reaction was studied at pH 10.2 (potassium borate buffer). In this case, using 5 equiv of cyanogen bromide, a 65% conversion to ADP was obtained; the only other product detected was AMP. To investigate if the improved yield at higher pH was due to complexation of the cis-vicinal 2′- and 3′-ribosyl hydroxy groups with borate buffer, the reaction was repeated using sodium carbonate/bicarbonate buffer with the same results, showing that the yield of ADP was affected only by the pH of the buffer.

Reaction of ADPαS and (S_P)-ATPβS with BrCN in $H_2{}^{18}O$. To determine the origin of oxygen replacing sulfur in the conversions of ADPαS and ATPβS to ADP and ATP, the reactions were carried out in $H_2{}^{18}O$. ^{31}P NMR analyses of the products verified that sulfur was replaced by oxygen from water.

The ^{31}P NMR spectrum of [^{18}O]ADP isolated from the reaction of ADPαS with BrCN in $H_2{}^{18}O$ is shown in Figure 1. The spectrum is typical of ADP with a doublet −5.9 ppm upfield from phosphoric acid assigned to P_β and a second at −10.5 ppm assigned to P_α. The presence of ^{18}O bonded to both P_α and P_β is clearly revealed by the presence of a second doublet 0.027 ppm upfield from the base signal for P_α and one 0.023 ppm upfield from that for P_β. The coupling constant is the same for these signals as for those assigned to ADP. The upfield shifts are caused by the presence of ^{18}O bonded to phosphorus.[18a-d]

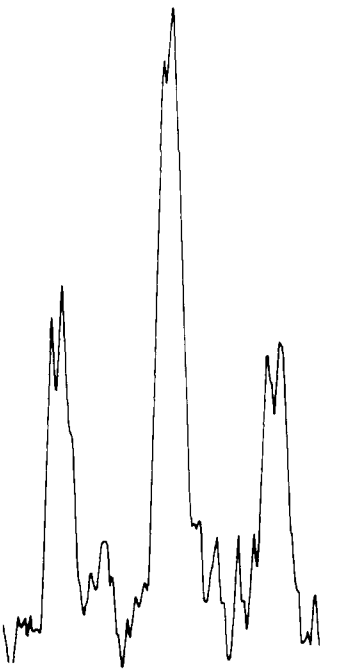


Figure 2. ^{31}P NMR spectrum of [β-^{18}O,γ-^{18}O]ATP. The sample was prepared by reaction of ATPβS with BrCN as described in the Experimental Section. Shown is the P_β region. Spectral parameters: δ_{P_β} −21.60, triplet (ATP); δ_{P_β} −21.63, triplet ([β-^{18}O]ATP). Other spectral parameters for signals not shown: δ_{P_α} −11.03, doublet; δ_{P_γ} −5.97, broadened doublet.

The ^{18}O-induced upfield shifts in Figure 1 demonstrate that ^{18}O is bonded to both P_α and P_β at nonbridging positions, with about 45 ± 5% enrichment at each position, 90% overall in [^{18}O]ADP. The slightly larger ^{18}O-induced upfield shift for P_α relative to P_β can be attributed to the slightly higher P–O bond order for nonbridging ^{18}O at P_α than at P_β. It is most unlikely that the spectrum could reflect bridging ^{18}O perturbing both P_α and P_β. This is because the P–O bond order with bridging oxygen is 1.0, which would result in a smaller ^{18}O-induced shift in the phosphorus resonance (0.02 ppm) than that actually measured for either P_α or P_β in Figure 1.

The reaction between (S_P)-ATPβS and BrCN in [^{18}O]H_2O also leads to incorporation of ^{18}O into ATP, the major product. Figure 2 shows the P_β region of the ^{31}P NMR spectrum obtained for a sample of [^{18}O]ATP prepared in this way. The P_β signal for ATP is a triplet centered at about −23.5 ppm upfield from phosphoric acid and appears in Figure 2 as two incompletely resolved triplets separated by about 0.027 ppm. The upfield triplet is assigned to species of [^{18}O]ATP with ^{18}O bonded to P_β and the downfield triplet to species of ATP devoid of ^{18}O at P_β. The signal intensities and integrations indicate about 50% incorporation of ^{18}O at P_β

(18) (a) Cohn, M.; Hu, A. *J. Am. Chem. Soc.* **1980**, *102*, 913–916. (b) Lowe, G.; Potter, B. V. L.; Sproat, B. S.; Hull, W. E. *J. Chem. Soc., Chem. Commun.* **1979**, 733–734. (c) Cohn, M.; Hu, A. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 200–202. (d) Lowe, G.; Sproat, B. S. *J. Chem. Soc., Chem. Commun.* **1978**, 565–566.

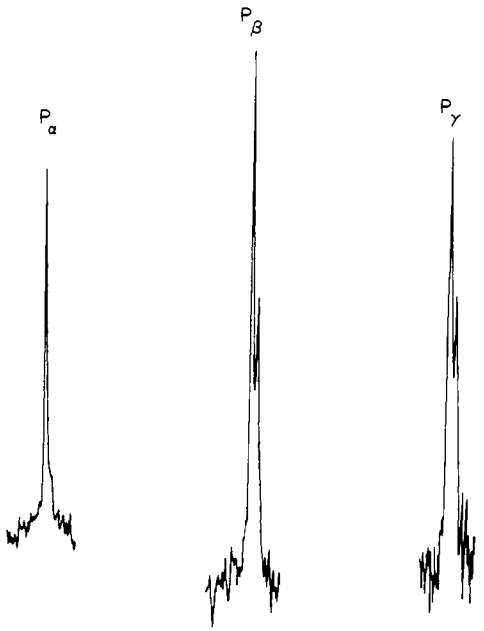
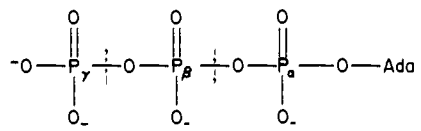


Figure 3. ^{18}O analysis of inorganic phosphate derived from P_α, P_β, and P_γ of [β-^{18}O,γ-^{18}O]ATP. The sample of [β-^{18}O,γ-^{18}O]ATP was enzymatically degraded to adenosine and inorganic phosphate as in the procedure described in the Experimental Section in such a way that P_α, P_β, and P_γ were separately isolated as inorganic phosphate. Shown are the 80.1-MHz ^{31}P NMR signals from the spectrum of each sample. Note the ^{18}O-isotope shifted components 0.02 ppm upfield from the signals for P_β and P_γ.

from $H_2{}^{18}O$, 95% enriched with ^{18}O.

The signal for P_γ in the above sample was broad relative to that for P_α but not well enough resolved to give a clearly defined ^{18}O-shifted signal. Given that ^{18}O incorporation at P_β was only 50%, it appeared likely that ^{18}O was also present at P_γ. To determine whether this was the case, P_α, P_β, and P_γ were isolated separately as inorganic phosphate and subjected to ^{31}P NMR analysis. These isolations involved the bond cleavages indicated below, so that P_γ retained only nonbridging oxygens while P_β and



P_α retained both bridging and nonbridging oxygens in the isolated phosphates. The ^{31}P NMR signals for these samples, shown in Figure 3, showed clearly that P_β and P_γ were equivalently labeled with ^{18}O, while P_α showed no sign of ^{18}O labeling.

The size of the ^{18}O-induced shift in the P_β signal for [^{18}O]ATP (Figure 2) and the presence of ^{18}O in phosphate derived from P_γ (Figure 3) showed that nonbridging ^{18}O was present at both positions. Owing to inadequate resolution in the ^{31}P NMR spectrum of [^{18}O]ATP, we could not conclude whether ^{18}O was present in the position bridging P_β and P_γ. However, results of experiments described in later sections did not indicate incorporation of oxygen from H_2O into this position.

The incorporation of ^{18}O into the P_β and P_γ positions of ADP and ATP, respectively, on reaction of ADPαS and ATPβS with BrCN in $H_2{}^{18}O$ and the correspondingly less than 100% incorporation at the P_α and P_β positions from which sulfur was displaced revealed the operation of an unexpectedly complex reaction mechanism. These results could not have arisen from a mechanism that involved the direct, nucleophilic displacement of thiocyanate exclusively by water.

Reaction of (S_P)-[α-$^{18}O_2$]ADPαS with BrCN. To obtain further information regarding the reaction mechanism, we traced the fate of ^{18}O in (S_P)-[α-$^{18}O_2$]ADPαS resulting from its conversion to [^{18}O]ADP by reaction with BrCN in H_2O. Monitoring the progress of the reaction by ^{31}P NMR during the addition of 3.5

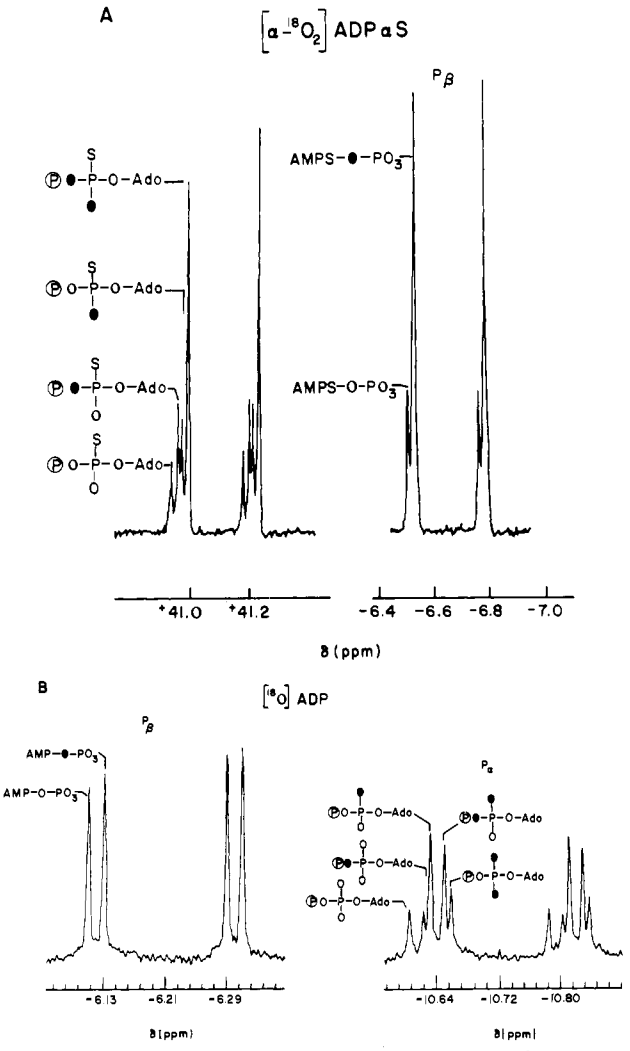
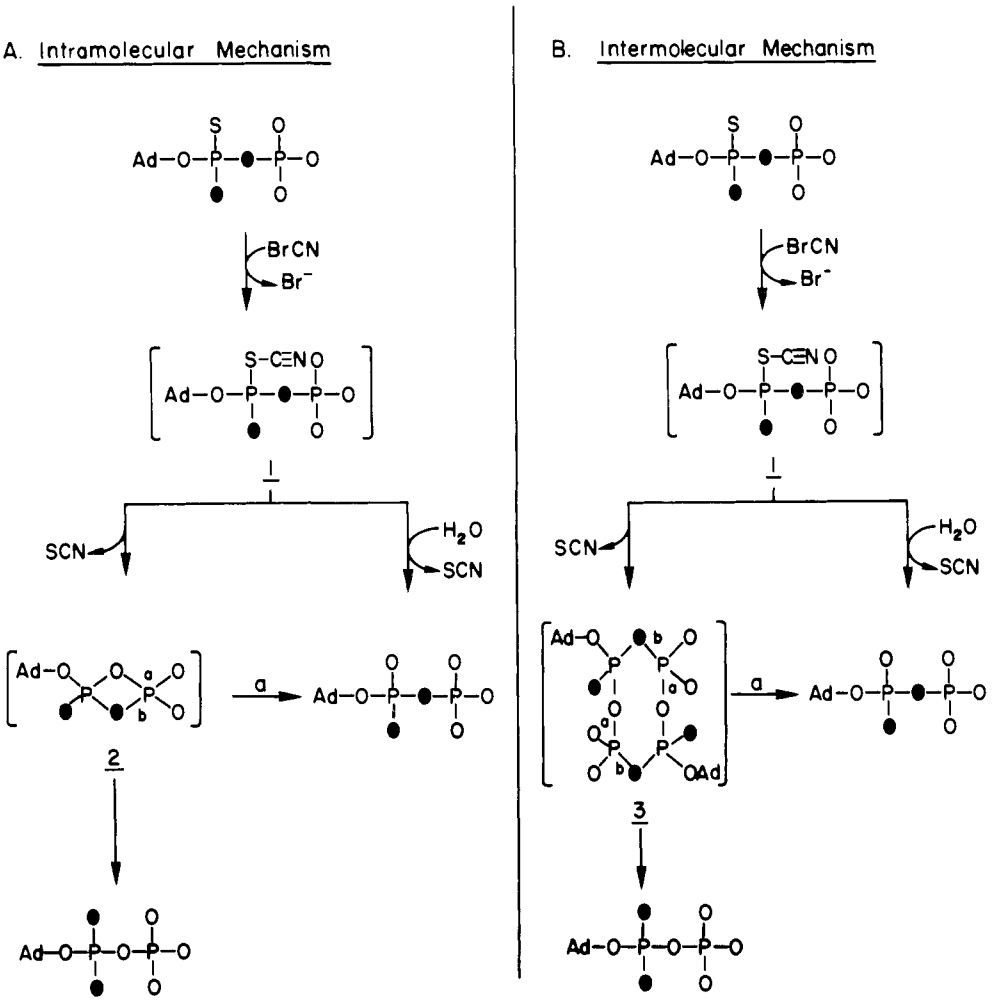


Figure 4. ^{18}O rearrangements in the reaction of (S_P)-[α-$^{18}O_2$]ADPαS with BrCN. The procedure for converting (S_P)-[α-$^{18}O_2$]ADPαS to [^{18}O]ADP by reaction with BrCN is described in the Experimental Section. Shown in part A is the 121.4-MHz ^{31}P NMR spectrum for (S_P)-[α-$^{18}O_2$]ADPαS. The assignments of spectral lines to ^{18}O-containing species are indicated in the figure, where ● designates ^{18}O. The signals for P_α and P_β are both doublets with $J_{P_\alpha P_\beta}$ = 31.3 Hz. Chemical shift values for the various species: δ_{P_α} 40.962, 40.936, 40.925, and 40.902; δ_{P_β} -6.620 and -6.642. Shown in part B is the ^{31}P NMR spectrum for [^{18}O]ADP. The assignments of spectral lines to ^{18}O-containing species are indicated in the figure. The signals for P_α and P_β are both doublets with $J_{P_\alpha P_\beta}$ = 22.6 Hz. Chemical shift values for the various species: δ_{P_α} -10.694, -10.713, -10.722, -10.739, -10.749; δ_{P_β} -6.199 and -6.220.

equiv of BrCN in three increments, we observed the complete transformation of ADPαS into three phosphorus-containing products, ADP in over 70% yield and AMP and inorganic phosphate in equivalent amounts and less than 30% yield. The ratios of products were the same throughout the course of the reaction; therefore, AMP and inorganic phosphate did not arise from hydrolysis of ADP.

The high-resolution ^{31}P NMR spectra of the reactant (S_P)-[α-$^{18}O_2$]ADPαS and [^{18}O]ADP purified from the reaction mixture are presented in Figure 4. The P_α region of the spectrum for (S_P)-[α-$^{18}O_2$]ADPαS in Figure 4A consists of four doublets, representing the four possible species that can be expected on the basis of the 72% overall ^{18}O enrichment at P_α. The species are identified in Figure 4A as one lacking ^{18}O, one with a single bridging ^{18}O, one with a single nonbridging ^{18}O, and one with two ^{18}O's, one bridging and one nonbridging. The latter is the strongest signal. The P_β region consists of two doublets, the downfield signal assigned to species containing ^{16}O in the P_α–P_β bridging position. The much larger signal 0.022 ppm further upfield is assigned to

Scheme II. Proposed Mechanism for the Desulfurization of [α-$^{18}O_2$]ADPαS at pH 10.6



species with ^{18}O in this position.

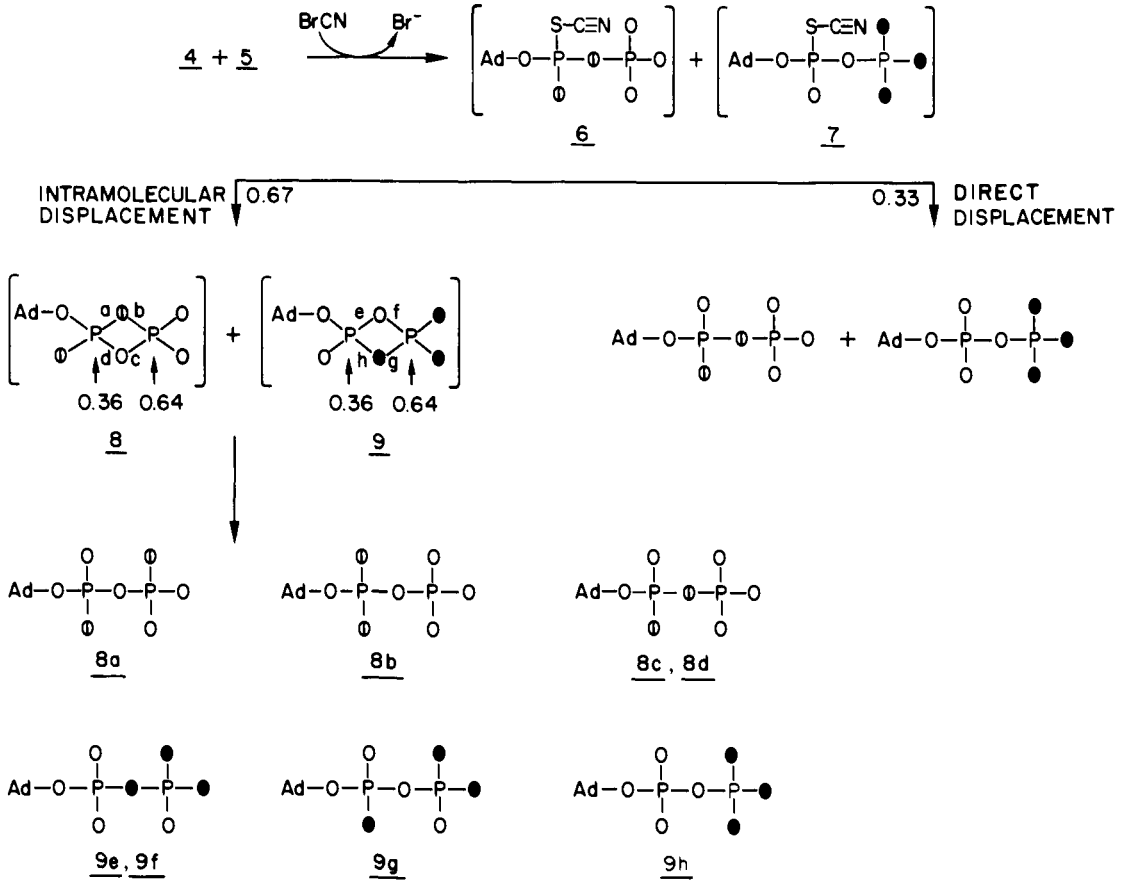
The ^{31}P NMR spectrum of [^{18}O]ADP isolated from the reaction mixture is quite revealing when compared with that of (S_P)-[α-$^{18}O_2$]ADPαS from which it was derived, with striking differences appearing in both the P_α and P_β regions (Figure 4B). In the P_β region for [^{18}O]ADP there are, as in the sulfur-containing precursor, two doublets; but the ratios of intensities are comparable in [^{18}O]ADP, showing that species with bridging ^{16}O are present in amounts comparable to those with bridging ^{18}O. Bridging ^{18}O in (S_P)-[α-$^{18}O_2$]ADPαS has clearly been replaced to a substantial extent with ^{16}O in the product. In the P_α region of the spectrum for [^{18}O]ADP there are *five* doublets compared with four in that for (S_P)-[α-$^{18}O_2$]ADPαS. The lowest field signal is assigned to species with no ^{18}O bonded to P_α. Three of the remaining four signals correspond to ^{18}O-containing species analogous to those in the reactant, ^{18}O bridging, ^{18}O nonbridging, and ^{18}O both bridging and nonbridging. The fifth signal represents the species with ^{16}O bridging and ^{18}O at *both* nonbridging positions. The relative magnitudes of the ^{18}O-induced shifts in the P_α resonance are consistent with these assignments. The most upfield signal in the P_α region, corresponding to product molecules with ^{18}O at both nonbridging P_α positions, and the markedly decreased ratio of ^{18}O to ^{16}O in the bridging position are especially significant for they show that sulfur at P_α in the reactant has been replaced by bridging oxygen in a substantial fraction of product molecules.

Reaction of (S_P)-[β,γ-bridging-^{18}O]ATPβS and (S_P)-[β-^{18}O]-ATPβS with BrCN. To further clarify the fates of bridging and nonbridging oxygen in this reaction, we prepared two samples of ^{18}O-enriched ATPβS, one with ^{18}O in the position bridging P_β and P_γ and the other with ^{18}O at the nonbridging P_β position. We submitted these compounds to reaction with BrCN in H_2O and traced the ^{18}O by ^{31}P NMR spectroscopy. The results fully confirmed and extended those from (S_P)-[α-$^{18}O_2$]ADPαS by showing that bridging ^{18}O in part migrates to a nonbridging P_β position and that nonbridging ^{18}O at P_β of ATPβS remains at that position in the product.

Migration of ^{18}O in the β,γ-bridge is demonstrated by the ^{31}P NMR spectra in Figure 5. The P_β and P_γ regions of the [β,γ-bridging-^{18}O]ATPβS spectrum presented in part A are typical of ATPβS, showing largely the ^{18}O-shifted signals due to the high enrichment in this sample. The small unshifted signals for the 10% of ATPβS unenriched with ^{18}O are poorly resolved in this sample. The P_β and P_γ regions in the spectrum of [^{18}O]ATP presented in part B show the enhanced ratio of ^{16}O to ^{18}O in the bridging position by the appearance of a second doublet in the P_γ region. This is mirrored in the P_β region, which reveals the appearance of a new triplet corresponding to species with nonbridging ^{18}O at P_β. This species can only have arisen by a mechanism that permits P_β–P_γ-bridging ^{18}O to migrate out of the bridge and into a nonbridging P_β position of ATP.

Intramolecular Oxygen Transfer. The transfer of bridging ^{18}O in (S_P)-[α-$^{18}O_2$]ADPαS and (S_P)-[β,γ-bridging-^{18}O]ATPβS to nonbridging positions can be rationalized on the basis of two mechanisms that involve transfer of oxygen from the terminal phosphoryl group to the neighboring phosphorothioate center. Scheme II illustrates an intramolecular transfer pathway (A) and an intermolecular pathway (B). Both mechanisms involve the initial reaction of BrCN with S in the nucleoside phosphorothioate, leading to the cyanated intermediate **1**. Thiocyanate is then displaced from **1** either by water or by the terminal phosphoryl group. In the intramolecular mechanism internal displacement of the thiocyanate group by the β-phosphoryl oxygen forms a *cyclo*-diphosphate intermediate **2**, which then undergoes spontaneous hydrolysis with cleavage of either P–O bond (pathway A

Scheme III. Intramolecular Mechanism for Desulfurization of (S_p)-[α-$^{17}O_2$]ADPαS (4) plus (S_p)-[β-$^{18}O_3$]ADPαS (5) at pH 10.2



and B). For the intermolecular pathway, mutual displacement of thiocyanate from P_α of intermediate **1** by P_β oxygen of a second molecule could lead to an eight-membered ring *cyclo*-tetraphosphate **3**, which would subsequently be decomposed by hydrolysis. To distinguish between these two mechanisms, the desulfurization was carried out using a mixture of [α-$^{17}O_2$]ADPαS (**4**) plus [β-$^{18}O_3$]ADPαS (**5**).

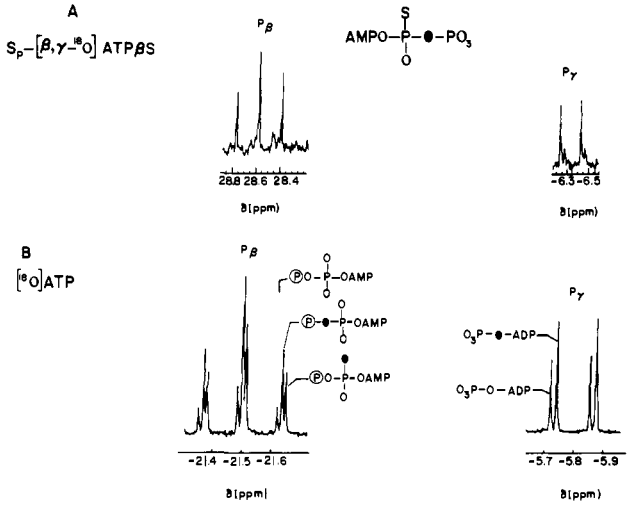


Figure 5. ^{18}O rearrangement in the reaction of (S_p)-[β,γ-^{18}O]ATPβS with BrCN. The procedure for converting (S_p)-[β,γ-^{18}O]ATPβS to [^{18}O]ATP is described in the Experimental Section. Shown in part A are the P_β and P_γ regions of the ^{31}P NMR spectrum of (S_p)-[β,γ-^{18}O]-ATPβS. Chemical shift values: δ_{P_β} 28.593, triplet; δ_{P_β} −6.330, doublet. Shown in part B are the P_β and P_γ regions of the ^{31}P NMR spectrum of [^{18}O]ATP obtained in the desulfurization of (S_p)-[β,γ-^{18}O]ATPβS. The assignments of spectral lines to ^{18}O-containing species are indicated in the figure. Chemical shift values: δ_{P_β} −21.487, triplet; −21.505, triplet; −21.515, triplet; δ_{P_γ} −5.791, doublet; −5.812, doublet.

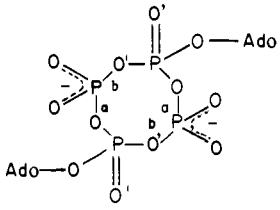
According to pathway A in Scheme II, reaction of **4** can lead only to ADP with ^{17}O and ^{16}O at P_α and reaction of **5** can lead only to ADP with ^{18}O and ^{16}O at P_α, even when **4** and **5** are mixed. According to pathway B, however, reaction of a mixture of **4** and **5** should lead to the formation of product species with both ^{17}O and ^{18}O at P_α. These species can be readily distinguished by mass spectroscopic analysis of triethyl phosphate derived from P_α and P_β of the ADP produced. In practice the analysis is slightly complicated by the presence of ^{18}O in the $H_2{}^{17}O$ used to synthesize **4**. However, the isotopic compositions are accurately known; and the appearance of ^{17}O-containing species with additional enrichment in ^{18}O can be easily detected.

Treatment of a mixture of **4** plus **5** with excess cyanogen bromide in 0.1 M tetraborate buffer at pH 10.2 gave [^{17}O,^{18}O]ADP in 66% yield. The P_α and P_β phosphoryl groups of the resulting ADP were isolated as inorganic phosphate by the enzymatic degradation outlined in Scheme I, in which the β-phosphoryl group was transferred to glucose by the coupled actions of adenylate kinase and hexokinase. The AMP and Glc-6-P were isolated and independently treated with alkaline phosphatase. The resulting samples of inorganic phosphate were ethylated using diazoethane, and triethyl phosphate was purified by GC and subjected to mass spectral analysis. The isotopic enrichment of the starting material **4** plus **5** was determined by a similar sequence of reactions (Scheme I). Since AMPS is not a substrate for alkaline phosphatase, it was converted to thiophosphate by alkaline β-elimination from the periodate-cleaved adenosyl moiety. During the ethylation of thiophosphate by diazoethane, desulfurization occurs without loss of the oxygen isotope.

The data obtained from the mass spectral analysis of the triethyl phosphates derived from P_α and P_β of the starting material **4** plus **5** and the product [^{17}O,^{18}O]ADP are presented in Tables I and II. The results support an intramolecular mechanism (Scheme III) and rule out the intermolecular pathway. In the first step the sulfur in **4** plus **5** displaces bromide from cyanogen bromide forming the cyanated intermediates **6** plus **7**. These intermediates

are partitioned between two pathways: (1) displacement of the thiocyanate by water (33%) and (2) internal displacement of the thiocyanate by the β-phosphoryl group (67%) leading to the adenosine *cyclo*-diphosphate intermediates **8** and **9**. Spontaneous hydrolysis of **8** and **9** occurs by attack of water at P_α (36%) and P_β (64%) with cleavage of either P–O bond (pathways a–h).

These conclusions arise from a straightforward rationalization of the mass spectral data in Tables I and II. Inspection of the second and third columns in Table I reveals that species of ADP containing either two ^{17}O and one ^{18}O or two ^{18}O at P_α, *m/e* 103, are decreased relative to the mixture of **4** plus **5**. In the intermolecular pathway the $^{17}O_2{}^{18}O_1$ intensity must increase due to transfer of ^{18}O from [β-$^{18}O_3$]ADPαS (**5**) to [α-$^{17}O_2$]ADPαS (**4**). In the intramolecular pathway this intensity can decrease but cannot increase. This point alone distinguishes the pathways. However, there are other features that support our conclusion. The second line in Table I shows that species with a single ^{17}O at P_α in ADP are markedly increased relative to P_α in **4** plus **5**. This should not occur in the intermolecular pathway, since the *cyclo*-tetraphosphate **3a** is expected to undergo hydrolysis to ADP



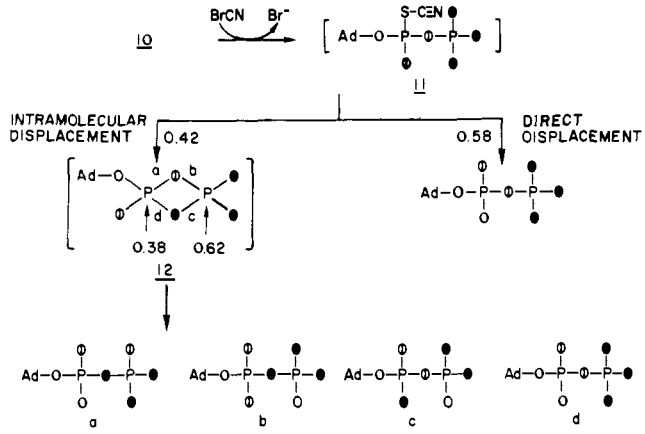
3a

by attack of water at the unesterified P^2 and P^4 with cleavage of the paired bonds a or b. **3a** is shown with symbols O′ for ^{17}O as if it were derived from reaction of two molecules of **4**, since the $^{17}O_1$ species could arise only from species of **4** with ^{17}O at P_α in both bridging and nonbridging positions (O′ is designated by O with a vertical line through it in Schemes III–V). Note that either cleavage route would lead to retention of both ^{17}O's at P_α in ADP, the only difference being that cleavage of bonds b would lead to exclusively nonbridging ^{17}O, whereas the other route would lead to [α-$^{17}O_2$]ADP with bridging and nonbridging ^{17}O. The isolation of P_α by the process of Scheme I would not distinguish between them.

This mode of hydrolytic cleavage for P^1,P^3-bis(alkyl) *cyclo*-tetraphosphates, though not directly proven here or elsewhere, follows logically from the known hydrolytic chemistry of alkyl *cyclo*-triphosphates, in which the orientation of ring opening is governed by the relative stabilities of the leaving groups.[19a-c] This rule predicts paired cleavage of bonds a or b in **3a**. We know of no reason for this rule to be violated in the hydrolysis of **3a**. There appears to be no serious bond angle strain or eclipsing interactions in a space-filling model; and the crystallographic O–P–O bond angles are 100° and 110° and the P–O–P angles 132° in tetrammonium *cyclo*-tetraphosphate.[20]

Contrasting with this the intramolecular pathway offers a straightforward rationale for the appearance of species with a single ^{17}O at P_α. Reaction of **4** by the intramolecular pathway would produce the *cyclo*-diphosphate **8** in Scheme III. If hydrolysis of **8** were governed solely by electronic factors, water would attack at P_β and ^{17}O would be partitioned between bridging and nonbridging positions at P_α in ADP. However, there must be significant bond angle strain in **8**; therefore, it can be expected to exhibit less selectivity in its reaction than monoalkyl *cyclo*-triphosphates. Consequent reaction of water at P_α would lead to the partitioning of bridging ^{17}O between P_α and P_β. Transfer of ^{17}O to a nonbridging P_β position would account for the increase in species containing a single nonbridging ^{17}O at P_α and also to the appearance of species containing a single nonbridging ^{17}O at P_β of ADP. The latter point is confirmed in Table II, line 2, by the presence of an $^{17}O_1$ species in triethyl phosphate derived from P_β. This was not present at P_β in the mixture of **4** and **5**.

Scheme IV. Intramolecular Mechanism for Desulfurization of (S_p)-[α-$^{17}O_2$,β-$^{18}O_3$] ADPαS (10) at pH 7.0



The mass spectral data, in addition to distinguishing the intramolecular and intermolecular pathways, also enable us to calculate partitioning ratios for the reaction intermediates in the intramolecular pathway. Two reasonable assumptions are (1) that bridging oxygens in *cyclo*-diphosphates are equally partitioned between bridging and nonbridging positions upon hydrolysis by reaction of water at either P_α or P_β and (2) that ^{17}O in a nonbridging position at P_β of ADP arises exclusively by attack of water at P_α of *cyclo*-diphosphates containing bridging ^{17}O (e.g., P_α of **8** in Scheme III). The partition ratios in Scheme III arise from the use of these assumptions in fitting the mass spectral data to pathway A in Scheme II. The calculated intensities for the intramolecular pathway based on these partitioning ratios are in reasonable agreement with the measured intensities.

The calculated intensities for the intermolecular pathway are based on the *same* assumptions used for the intramolecular pathway, including the assumption that hydrolysis can occur by nucleophilic attack of water at P_α. This assumption was used in order to achieve the best possible fit of the data despite the fact that for the reasons cited above it is very unlikely to be correct. Tables I and II show that the mass spectral results are incompatible with the intermolecular pathway. A more reasonable set of assumptions would lead to calculated intensities even more at variance with the measured intensities.

Effect of Phosphate Buffer. If desulfurization occurs via an intermolecular pathway, then, in phosphate buffer at 10 times the concentration of ADPαS, nucleophilic displacement of the thiocyanate group by phosphate should predominate. To investigate this possibility, a sample of [α-$^{17}O_2$,β-$^{18}O_3$]ADPαS **10** was desulfurized by treatment with cyanogen bromide in 0.1 M potassium phosphate buffer at pH 7.0, producing AMP (15%), AMPS (46%), and ADP (16%). The isotopic enrichments of the [^{17}O,^{18}O]ADP was determined by mass spectral analysis of the triethyl phosphates derived from P_α and P_β by the procedure described earlier (Scheme I). The results presented in Tables III and IV eliminate an intermolecular pathway and are consistent with an intramolecular mechanism (Scheme IV). The initially formed cyanated intermediate **11** is partitioned between direct displacement by water (58%) and the formation of the *cyclo*-diphosphate intermediate **12** (42%). Spontaneous hydrolysis of **12** occurs by attack at P_α (38%) and P_β (62%). Displacement of thiocyanate by phosphate would have led to the production of ADP with substantial dilution of ^{18}O at both P_α and P_β.

Origin of Monophosphates. To investigate the site of bond cleavage for the formation of AMP during the desulfurization of ADPαS at pH 7.0, the [^{17}O]AMP produced from the reaction of [α-$^{17}O_2$,β-$^{18}O_3$]ADPαS with cyanogen bromide at pH 7.0 was analyzed by mass spectroscopy. The data obtained from the mass spectral analysis of the triethyl phosphate samples derived from AMP and P_α of **10** are presented in Table V. The results show

(19) (a) Ho, H.-T.; Frey, P. A. *Biochemistry* **1984**, *23*, 1978–1983. (b) Webb, M. R. *Biochemistry* **1980**, *19*, 4744–4748. (c) Lowe, G.; Sproat, B. S.; Tansley, G.; Cullis, P. M. *Biochemistry* **1983**, *22*, 1229–1236.

(20) Romers, C.; Ketelaar, J. A. A.; MacGillavry, C. H. *Acta Crystallogr.* **1951**, *4*, 114–120.

that both ^{17}O atoms of the starting material are retained during replacement of sulfur by an oxygen atom from the solvent water. hence, AMP (or AMPS) arises by cleavage of the O–P_β bond of ADPαS.

Discussion

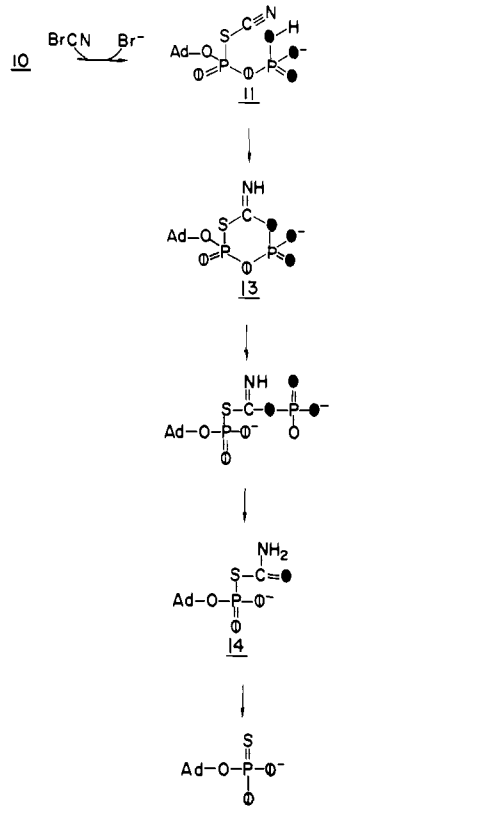
The results detailed in this paper exclude the possibility of a simple mechanism in the conversion of ADPαS and ATPβS to ADP and ATP, respectively. The process almost certainly begins with the reaction of the phosphorothioate groups in these molecules with BrCN to form cyanated intermediates, in which the sulfur has been transformed into the thiocyanato group. The intermediates exhibit high electrophilic reactivity because of the absence of negative charge on the thiocyanato phosphoryl group and because of the strong electron-withdrawing properties of the -SCN substituent. Thiocyanate is displaced because it is the most stable leaving group from the reacting center in aqueous solutions at neutral to alkaline pHs. Its stability is reflected in the low pK_a of thiocyanic acid, which is reported to be 0.85.[21] The other prospective leaving groups under these conditions would be phosphate dianion, phosphate trianion, and AMP dianion. The pK_a values for their conjugate acids range from 7 to 12, reflecting their low stabilities as leaving groups relative to thiocyanate.

The mechanistic complexities in these reactions arise in the hydrolysis of the thiocyanato intermediates to ADP and ATP. This process is accompanied by a rearrangement of oxygens in the polyphosphate systems of the nucleotides. The results presented in this paper clearly show that in the course of hydrolysis oxygens bridging the terminal phosphoryl groups and the phosphorothioate groups can migrate out of the bridges and into positions replacing the departing sulfur. This migration cannot be compulsory because part of the bridging oxygen remains bridging in the product, so the mechanism is permissive on this point. The transfer of bridging oxygens to nonbridging positions is clearly demonstrated by the ^{31}P NMR spectra in Figures 4 and 5.

The mass spectral data obtained from the desulfurization of ADPαS at pH 7.0 and 10.2 using cyanogen bromide show that the reaction involves intramolecular oxygen transfer, presumably via an intermediate *cyclo*-diphosphate (Schemes III and IV). The initially formed thiocyanato compound is partitioned between two reaction pathways, direct displacement by H_2O, and intramolecular displacement to form the *cyclo*-diphosphate. Hydrolytic opening of the intermediate *cyclo*-diphosphate occurs by attack of water at both P_α and P_β. Based on the hydrolysis of alkyl and dialkyl *cyclo*-triphosphates, which occurs by attack of water at the phosphorus atom that would lead to the generation of the most stable leaving group,[19a-c] one would have predicted exclusive reaction of water at P_β of the *cyclo*-diphosphates. However, in this case, it is not unreasonable for a highly reactive intermediate to be less discriminating toward hydrolytic ring opening. The peak of mass 100 observed in the mass spectral analysis of the triethyl phosphate derived from P_β of [^{17}O,^{18}O]ADP (Table II) is most simply accounted for by spontaneous hydrolysis of the *cyclo*-diphosphate intermediate **8** by attack at P_α (pathway **8a**). Interestingly, the data at pH 10.2 and 7.0 indicate that ring opening by attack at P_α and P_β appears to be pH independent, but partitioning of the thiocyanato intermediate between direct nucleophilic displacement of thiocyanate by water and intramolecular displacement shows a significant pH dependence. The acid dissociation state of the terminal phosphoryl group probably determines the extent to which neighboring-group participation competes with direct nucleophilic displacement. Thus at higher pH, where the fully ionized dianionic phosphate group is predominant, intramolecular displacement is favored.

The ^{31}P NMR and mass spectral results provide complementary and fully consistent data supporting the involvement of *cyclo*-diphosphates as intermediates in these reactions. The NMR data show that bridging oxygen in ADPαS and ATPβS is in part transferred to a nonbridging position in the product in place of the departing sulfur. This transfer would not be revealed by the mass spectral analysis used here. The mass spectral data conclusively show that oxygen transfer from the terminal phosphoryl group to the thiocyanato center cannot be intermolecular, and so must be intramolecular. The mass spectral data also show that bridging oxygen in ADPαS appears in part as nonbridging oxygen at P_β of ADP, the terminal phosphoryl group. This transfer, which was not detected in the NMR experiments, also supports the involvement of a *cyclo*-diphosphate as an intermediate. The partition ratios for intermediates in Schemes III and IV also arise from the mass spectral data.

Scheme V. Hypothetical Mechanism for the Formation of AMPS during Desulfurization of ADPαS



Experiments on the desulfurization of ADPαS at pH 7.0 and 10.2 by cyanogen bromide showed that the yield of ADP increased at higher pH (Table VI). At lower pH the major product was initially AMPS, which underwent further desulfurization to AMP. Mass spectral analysis of the [^{17}O]AMP obtained from cyanogen bromide desulfurization of [α-$^{17}O_2$,β-$^{18}O_3$]ADPαS at pH 7.0 (Table V) showed that both oxygen atoms at P_α were retained in the product.

A mechanism that rationlizes the formation of AMPS at pH 7.0 is presented in Scheme V. The first step involves formation of the cyanated intermediate **11**. The protonated oxygen atom of the terminal phosphate undergoes an intramolecular four-centered reaction with the π orbitals of the C≡N triple bond to form the cyclic six-membered diphosphate **13**. Spontaneous ring opening followed by subsequent hydrolysis results in the formation of the thiocarbamate **14**, which decomposes to product [α-$^{17}O_2$]AMPS. The salient feature of this mechanism involves a protonated oxygen atom of P_β. At higher pH this phosphate would be predominately in its dianionic form. In the reaction of ATPβS at pH 7 ADPβS is a minor product, suggesting that the pathway in Scheme V is a minor pathway in its reaction as well.

According to Scheme II sulfur is displaced as thiocyanate; however, colorimetric tests for thiocyanate failed to confirm that it was produced in more than trace amounts. We also found that a minumum of 2 mol of BrCN per mol of ATPβS is required to consume all of the substrate and produce the maximum yield of ATP. These facts suggested that the final sulfur-containing

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product is not thiocyanate but either the product of its further reaction with BrCN according to eq 1 or a further decomposition product of $S(CN)_2$.

$$BrCN + {}^{-}SCN \rightarrow Br^{-} + S(CN)_2 \quad (1)$$

The reactions of ADPαS and ATPβS are not useful for synthesizing adenosine 5′-[^{18}O]diphosphates or -triphosphates with chiral P_α or P_β because of the oxygen rearrangements in the polyphosphate systems described herein. These can be prevented by protecting the terminal phosphoryl groups with removable alkyl substituents, as demonstrated by the reactions of the R_P and S_P epimers of β-(cyanoethyl)-ADPαS with BrCN in $H_2{}^{18}O$ to produce high yields of the S_P and R_P epimers of β-(cyanoethyl)-[α-^{18}O]ADP.[22] The displacement of thiocyanate by $H_2{}^{18}O$ proceeded with inversion of configuration at P_α, and the β-cyanoethyl groups were easily removed by treatment with base to produce the S_P and R_P epimers of [α-^{18}O]ADP.[22]

Eckstein and Lowe and their collaborators have been able to desulfurize nucleoside phosphorothioates with electrophilic brominating agents in acidic solutions with inversion of configuration and without rearrangements in polyphosphates.[19c,23] At neutral pHs these reactions also involved rearrangements, suggesting that our observations do not represent an isolated phenomenon observable only in the special case of reactions of BrCN with ADPαS or ATPβS.

This paper and our earlier communication[4] provide the first evidence for involvement of *cyclo*-diphosphates in chemical reactions. Dimeric phenylphosphonic anhydride is the only four-membered ring organophosphorus compound reported in the literature that has two P–O–P bonds.[24,25] Recently a cyclic phosphoric acid anhydride was postulated as a possible intermediate in the equilibration of the phosphorus in $H_3{}^{32}PO_4$ with that of $POCl_3$.[26]

cyclo-Dephosphates have never been proposed as intermediates in enzymatic reactions. In view of their involvement in nonenzymatic reactions proceeding to completion within a few minutes in aqueous solutions at physiological pHs and moderate temperatures, they should be considered as possible intermediates in enzymatic reactions. However, the substrates for most nucleoside di- and triphosphate-dependent enzymes are the metal complexes of the nucleotides rather than the nucleotides themselves, and it is not known what effect metal complexation would have on the rearrangements described here. It is possible that these rearrangements would be inhibited or prevented by metal chelation. Moreover, there is no reason to consider invoking *cyclo*-diphosphates as intermediates in ordinary phosphotransferase or nucleotidyl transferase reactions. Nevertheless, the ease and speed with which *cyclo*-diphosphates appear and react under mild conditions demonstrate their potential as transient intermediates that might be involved in more complex biological reactions.

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Registry No. 4, 91711-81-8; **5**, 91741-70-7; **10**, 91741-71-8; cyanogen bromide, 506-68-3; (S_P)-[β-^{18}O]ATPBS, 87226-46-8; (S_P)-[β,γ-bridging-^{18}O]ATPBS, 87883-26-9; (R_P)-[β-^{18}O]ADPBS, 69182-10-1; (S_P)-[β-^{18}O]ADPBS, 68973-41-1; (S_P)-ATPBS, 59261-36-8; (S_P)-ADPαS, 59286-20-3; (S_P)-[α-$^{18}O_2$]ADPαS, 91711-80-7; [α-^{18}O,β-^{18}O]ADP, 91711-82-9; [β-^{18}O,γ-^{18}O]ATP, 91711-83-0.

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Solvent-Induced Fragmentation of Prostaglandin Endoperoxides. New Aldehyde Products from PGH_2 and a Novel Intramolecular 1,2-Hydride Shift during Endoperoxide Fragmentation in Aqueous Solution[1]

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Abstract: The prostaglandin (PG) endoperoxide nucleus, 2,3-dioxabicyclo[2.2.1]heptane, is three orders of magnitude less stable in aqueous vs. cyclohexane solution. Water-induced fragmentation of 2,3-dioxabicyclo[2.2.1]heptane-*1*-d_1 exhibits a deuterium kinetic isotope effect $k_H/k_D = 1.5$. Although rate-determining cleavage of a C–D bond occurs, deuterium is not lost to the protic solvent. Rather, the bridgehead deuterium migrates intramolecularly to the incipient methyl group of the levulinaldehyde product. Dimethyl sulfoxide (Me_2SO) also induces rapid decomposition of the PG endoperoxide nucleus at 37 °C. However, a different mechanism is suggested by $k_H/k_D = 3.3$ for the fragmentation reaction induced by Me_2SO. The PG endoperoxide PGH_2 [9α,11α-epidioxy-15(*S*)-hydroxy-5(*Z*),13(*E*)-prostadienoic acid] rearranges spontaneously at 37 °C in Me_2SO or aqueous solution to form levulinaldehyde derivatives, levuglandin (LG) E_2 [8(*R*)-acetyl-9(*R*)-formyl-12(*S*)-hydroxy-5(*Z*),10(*E*)-heptadecadienoic acid] and LGD_2 [9(*R*)-acetyl-8(*R*)-formyl-12(*S*)-hydroxy-5(*Z*),10(*E*)-heptadecadienoic acid]. The Me_2SO-induced fragmentation affords levuglandins in 70 to 80% yield according to ^{1}H NMR analysis of the decomposition product mixture while the yield of levuglandins in aqueous solution is about 22% at pH 7.9.

Prostaglandin (PG) endoperoxides, e.g., PGH_2 (**1a**), are pivotal intermediates in the biosynthesis of a vast array of natural products (Scheme I).[2] Most of the primary derivatives, **2a–10a**, exhibit potent physiological activities.[3] With the exception of **6a**, they

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