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Stereochemical Course of the 3′→5′-Exonuclease Activity of DNA Polymerase I[†]

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ABSTRACT: (S_P)-2′-Deoxyadenosine 5′-*O*-[1-^{17}O,1-^{18}O,1,2-^{18}O]triphosphate has been synthesized by desulfurization of (S_P)-2′-deoxyadenosine 5′-*O*-(1-thio[1,1-$^{18}O_2$]diphosphate) with *N*-bromosuccinimide in [^{17}O]water, followed by phosphorylation with phosphoenolpyruvate-pyruvate kinase. A careful characterization of the product using high-resolution ^{31}P NMR revealed that the desulfurization reaction proceeded with approximately 88% direct in-line attack at the α-phosphorus and 12% participation by the β-phosphate to form a cyclic α,β-diphosphate. The latter intermediate underwent hydrolysis by a predominant nucleophilic attack on the β-phosphate. This complexity of the desulfurization reaction, however, does not affect the stereochemical integrity of the product but rather causes a minor dilution with nonchiral species. The usefulness of the (S_P)-2′-deoxyadenosine 5′-*O*-[1-^{17}O,1-^{18}O,1,2-^{18}O]triphosphate in determining the stereochemical course of deoxyribonucleotidyl-transfer enzymes is demonstrated by using it to delineate the stereochemical course of the 3′→5′-exonuclease activity of DNA polymerase I. Upon incubation of this oxygen-chiral substrate with Klenow fragment of DNA polymerase I in the presence of poly[d(A-T)] and Mg^{2+}, a quantitative conversion into 2′-deoxyadenosine 5′-*O*-[^{16}O,^{17}O,^{18}O]monophosphate was observed. The stereochemistry of this product was determined to be R_P. Since the overall template-primer-dependent conversion of a deoxynucleoside triphosphate into the deoxynucleoside monophosphate involves incorporation into the polymer followed by excision by the 3′→5′-exonuclease activity and since the stereochemical course of the incorporation reaction is known to be inversion, it can be concluded that the stereochemical course of the 3′→5′-exonuclease is also inversion.

The question of the number and kind of reaction intermediates in an enzyme-catalyzed reaction is best addressed by determining the stereochemical course of that reaction (Knowles, 1980). The determination of the stereochemical course of a given enzyme-catalyzed reaction requires the existence of a suitable chiral substrate or substrate analogue. A stereochemical investigation of the 3′→5′-exonuclease reaction of DNA polymerase I and other deoxyribonucleotidyltransferase reactions depends on a convenient stereospecific synthesis of oxygen isotopically substituted dATP. Although several methods have been described for the synthesis of ^{17}O- and ^{18}O-substituted ribonucleoside triphosphates (Connolly et al., 1982; Lowe et al., 1982; Sammons & Frey, 1982), none of these methods has been applied to the synthesis of oxygen chiral deoxyribonucleoside triphosphates. In the work described here, a synthesis of the S_P diastereomer of 2′-deoxyadenosine 5′-*O*-[1-^{17}O,1-^{18}O,1,2-^{18}O]triphosphate [(S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP][1] has been achieved. This synthesis is based on the recent development of a method to stereospecifically substitute an oxygen isotope for sulfur in nucleoside phosphorothioates (Connolly et al., 1982). A thorough characterization of the substrate (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP by high-resolution ^{31}P NMR spectroscopy reveals the complex nature of the desulfurization reaction and sheds light on its mechanism.

The 3′→5′-exonuclease activity of DNA polymerase I, as in other prokaryotic polymerases, has been implicated in determining its overall fidelity of replication by carrying out a proofreading function (Kornberg, 1980; Hopfield, 1974; Galas & Branscomb, 1978; Clayton et al., 1979). This activity, therefore, is closely related to the polymerase function and is coexpressed during DNA replication. One of the consequences of this coexpression of the polymerase and exonuclease activities is that when one of the complementary deoxynucleoside triphosphates is omitted during DNA replication, an idling–turnover process results, causing the repretitive incorporation and excision of a given nucleotide. The net result of such a process is the conversion of a given deoxynucleoside triphosphate pool into the corresponding deoxynucleoside monophosphate (Gupta et al., 1982, 1984). This idling–turnover process provides a convenient method for the determination of the stereochemical course of the 3′→5′-exonuclease activity. Thus, (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP was converted into [^{16}O,^{17}O,^{18}O]dAMP in the presence of poly[d(A-T)] template-primer with the Klenow fragment of DNA polymerase I, and the stereochemistry of the product [^{16}O,^{17}O,^{18}O]dAMP was determined by the method of Jarvest et al. (1981). Since the stereochemistry of the polymerization reaction of DNA polymerase I is known to be inversion (Burgers & Eckstein, 1979; Brody & Frey, 1981), the stereochemistry of the 3′→5′-exonuclease can be readily deduced.

Experimental Procedures

Materials and Methods. (*1*) *Enzymes*. Klenow fragment of DNA polymerase I was purified from a cloned overproducer of that enzyme according to the method of Joyce & Grindley (1983). The cell line CJ155, containing plasmid pCJ55 (Klenow fragment under P_L control) in *Escherichia coli* strain

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[1] Abbreviations: [$^{18}O_2$]dAMPS, 2′-deoxyadenosine 5′-*O*-[$^{18}O_2$]phosphorothioate; (S_P)-[α-$^{18}O_2$]dATPαS, the S_P diastereomer of 2′-deoxyadenosine 5′-*O*-(1-thio[1,1-$^{18}O_2$]triphosphate); (S_P)-[α-$^{18}O_2$]dADPαS, the S_P diastereomer of 2′-deoxyadenosine 5′-*O*-(1-thio[1,1-$^{18}O_2$]diphosphate); (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dADP, the S_P diastereomer of 2′-deoxyadenosine 5′-*O*-[1-^{17}O,1-^{18}O,1,2-^{18}O]diphosphate; (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP, the S_P diastereomer of 2′-deoxyadenosine 5′-*O*-[1-^{17}O,1-^{18}O,1,2-^{18}O]triphoshate; [^{16}O,^{17}O,^{18}O]dAMP, 2′-deoxyadenosine 5′-*O*-[^{16}O,^{17}O,^{18}O]monophosphate of undetermined configuration at the phosphorus; DEAE, diethylaminoethyl; NMR, nuclear magnetic resonance; poly[d(A-T)], double-stranded alternating copolymer of deoxyadenylate and deoxythymidylate; DMF, dimethylformamide; TEAB, triethylammonium bicarbonate; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; PEP, phosphoenolpyruvate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; Me_2SO, dimethyl sulfoxide.

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N4830 was a generous gift of the authors. The purification was carried out through all the steps, including the Sephacryl S-200 gel filtration step. The enzyme so obtained was of greater than 95% purity as judged by SDS–polyacrylamide gel electrophoresis and had a specific activity of 10 000 units/mg of protein. Adenylate kinase, pyruvate kinase, hexokinase,and DNase I were purchased from Sigma Chemical Co.

(*2*) *Chemicals.* Tris (TRIZMA base), Hepes, dithiothreitol, ATP, phosphoenolpyruvate, D-glucose, deoxyadenosine, and diadenosine tetraphosphate were purchased from Sigma. Poly[d(A-T] was purchased from P-L Biochemicals and activated with DNase I according to the procedure of Modrich & Lehman (1970). [^{17}O]Water and [^{18}O]water were purchased from KOR-Isotopes. The isotopic composition of the [^{17}O]water was 12.5% ^{16}O, 35% ^{18}O, and 52.5% ^{17}O. The [^{18}O]water had an isotopic enrichment of greater than 95%. Nuclease-free BSA was purchased from Boehringer Mannheim. Thiophosphoryl chloride was purchased from Ventron and redistilled before use. DEAE-Sephadex A-25 was from Pharmacia and poly(ethylenimine)–cellulose plates were from Merck. All other organic reagents, buffers, and inorganic salts were commercially available reagent grade chemicals. Doubly distilled deionized water was used throughout.

(*3*) *Analytical Procedures.* Thin-layer chromatography was carried out on PEI–cellulose plates containing fluorescent indicator with either 1 M KCl or 0.75 M K_2HPO_4 (pH 3.5). Nucleotides were readily visualized by UV light at 254 nm. The phosphorothioate nucleotides were detected upon spraying with 2,6-dibromobenzoquinone 4-chlorimidate (DBQ: 1% in glacial acetic acid) (Stenersen, 1968).

High-performance liquid chromatography was performed on an Altex Model 100 instrument using either a reverse-phase column (Whatman Partisil PXS5/25 ODS2) or a strong anion-exchange column (Whatman Partisil PXS10/25 SAX). A UV detector was used to monitor absorbance at 254 nm. For the reverse-phase column, the elution procedure usually consisted of a 20-min linear gradient at a flow rate of 2 mL/min where the initial solvent is 50 mM potassium phosphate at pH 7.0 and the final solvent is 50 mM potassium phosphate containing 25% methanol. Isocratic elution with 7 mM ammonium phosphate buffer, pH 3.5, at a flow rate of 3 mL/min was employed to elute the anion-exchange column.

^{31}P NMR spectra were recorded at 145.81 Hz on a Bruker Instrument, Inc., WM 360 spectrometer with quadrature detection and proton broad-band decoupling. Field frequency locking was provided by the deuterium resonance of D_2O or Me_2SO-d_6. Positive chemical shifts reported are downfield from external 85% phosphoric acid. The characterization spectra were measured by using 16K data points, a sweep width of 10 000–30 000 Hz, a 70° radio-frequency pulse, and an overall repetition rate of 2–3 s. Several hundred to several thousand scans were acquired for each spectrum, depending on the concentration of the sample. The spectra were resolution enhanced by using a Gaussian window function (GB = 0.1 Hz, LB = –1.5 Hz). The high-resolution, isotopic shift analysis spectra were measured by using 8K or 16K data points, a sweep width of 600–3000 Hz, an 85° radio-frequency pulse, and an overall repetition rate of 3–6 s. Approximately 2000 scans were acquired for each spectrum. The data acquired with 8K data points were usually zero filled to 16K data points. All spectra were resolution enhanced by using a Gaussian window function (GB = 0.1 Hz, LB = –0.6 to –1.5 Hz). Samples were 1–5 mM in nucleotide that had been passed through a short column of Chelex-100 followed by a 0.25-μm filter. The samples contained 50% D_2O (or 50% Me_2SO-d_6), and the pH was 9–10 for aqueous samples.

NOE-free ^{31}P NMR spectra of dATP and (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP were obtained by using the following automated sequence (inverse gated proton decoupling): (1) ZE (zero memory), (2) DO (decoupler off between pulses), (3) D1 (first delay, set at 2 s to allow NOE to decay), (4) BB (decoupler on during acquisition), (5) D2 (second delay, set at 0.004 s), (6) GO = 2 (acquire, go to 2), and (7) exit.

Syntheses. (*1*) *Synthesis of* [$^{18}O_2$]*dAMPS.* [$^{18}O_2$]dAMPS was synthesized from deoxyadenosine and thiophosphoryl chloride in anhydrous triethyl phosphate followed by hydrolysis with [^{18}O]water, as described earlier (Brody & Frey, 1981; Murray & Atkinson, 1968). A total 1.15 mmol of [$^{18}O_2$]dAMPS was recovered from 2.0 mmol of starting deoxyadenosine (58% yield) after chromatography on DEAE-Sephadex A-25. The compound was judged to be pure by both TLC and HPLC. The proton-decoupled ^{31}P NMR consisted of a singlet at 43.1 ppm. The ^{18}O enrichment of [$^{18}O_2$]dAMPS was judged to be greater than 95% since no ^{16}O-containing species were detected.

(*2*) *Synthesis of* (S_P)-[α-$^{18}O_2$]*dATPαS.* (S_P)-[α-$^{18}O_2$]dATPαS was synthesized from [$^{18}O_2$]dAMPS by the coupled activities of adenylate kinase and pyruvate kinase as described by Brody & Frey (1981). A total of 360 μmol of (S_P)-[α-$^{18}O_2$]dATPαS was obtained from 0.5 mmol of starting [$^{18}O_2$]dAMPS (72% yield). The compound was found to be chemically and stereochemically pure by TLC and HPLC. The proton-decoupled ^{31}P NMR (sweep width 30 000 Hz) consisted of a P_α doublet at 42.95 ppm ($J_{\alpha,\beta}$ = 26.03 Hz), a P_β doublet of doublets centered at –22.89 ppm, and a P_γ doublet at –6.04 ppm ($J_{\beta,\gamma}$ = 24.16 Hz). For an accurate determination of the isotopic enrichment, a highly resolved spectrum of P_α was obtained by using a small spectral window (sweep width 1500 Hz). In this spectrum, two additional sets of doublets, each amounting in area to approximately 3% of the main doublet, were detected. These doublets were displaced 0.023 and 0.036 ppm downfield relative to the main doublet and represent the bridging and nonbridging (S_P)-[α-^{16}O,α-^{18}O]dATPαS respectively based on the difference in the magnitudes of ^{18}O-induced upfield shifts caused by bridging and nonbridging oxygens (Tsai & Chang, 1980). The doublet due to the (S_P)-[α-$^{16}O_2$]dATPαS species would be expected to be less than 1% of the main doublet and was not detected. The overall ^{18}O enrichment of (S_P)-[α-$^{18}O_2$]dATPαS was therefore calculated to be 94%.

(*3*) *Synthesis of* (S_P)-[α-$^{18}O_2$]*dADPαS.* (S_P)-[α-$^{18}O_2$]dADPαS was synthesized by degradation of (S_P)-[α-$^{18}O_2$]dATPαS with hexokinase (Stahl et al., 1974). In a total volume of 100 mL, the reaction mixture contained 70 mM TEA-HCl (pH 8), 40 mM D-glucose, 3.6 mM (S_P)-[α-$^{18}O_2$]dATPαS, 12 mM $MgCl_2$, 10 μM diadenosine tetraphosphate, and 4 μg/mL hexokinase. The reaction mixture was incubated at 25 °C, and the progress of the reaction was monitored by TLC on PEI–cellulose in 1 M KCl. Only a trace of the starting material could be detected at the end of 3 h. The reaction mixture was diluted to a total volume of 250 mL, and applied to a DEAE-Sephadex A-25 column(2.5 cm × 30 cm), HCO_3^- form. Elution was carried out by a linear gradient of TEAB buffer from 0.05 to 0.8 M (1.5 L of each), and 20-mL fractions were collected. (S_P)-[α-$^{18}O_2$]dADPαS was detected in fractions 86–106 which were pooled and evaporated to dryness. The excess TEAB buffer was removed by coevaporation with ethanol (3 × 20 mL). The yield was 4700 A_{260} units (307 μmol, 85%), and the material was found to be chemically and stereochemically pure by TLC and HPLC. The proton-decoupled ^{31}P NMR (sweep width 30 000 Hz) consisted of a P_α doublet at 40.94 ppm ($J_{\alpha,\beta}$ = 31.5 Hz) and a P_β doublet at −6.73 ppm ($J_{\alpha,\beta}$ = 31.2 Hz). A high-resolution spectrum of the α region (sweep width 1500 Hz) again revealed two additional sets of doublets, 0.024 and 0.036 ppm downfield relative to the main doublet, each approximating 3% in area of the main doublet. These were readily attributed to the bridging and nonbridging ^{18}O isomers of (S_P)-[α-^{16}O,α-^{18}O]dADPαS, respectively. This observation confirmed that the ^{18}O enrichment at P_α is approximately 94%.

(*4*) *Synthesis of* (S_P)-*[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dADP.* (S_P)-[α-$^{18}O_2$]dADPαS was converted into (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dADP with *N*-bromosuccinimide in [^{17}O]water–dioxane by using a slight modification of the procedure described by Connolly et al. (1982). (S_P)-[α-$^{18}O_2$]dADPαS (100 μmol) was lyophilized and dried by several coevaporations with absolute ethanol followed by dry dioxane (freshly distiled from sodium/benzophenone). The material was then placed under vacuum over P_2O_5 (0.01 mmHg for 3 days). Anhydrous dioxane (0.4 mL) and [^{17}O]water (200 μL) were added under a dry argon atmosphere. *N*-Bromosuccinimide (72 mg, 0.4 mmol), freshly recrystallized from water and thoroughly dried under vacuum over P_2O_5 (0.01 mmHg, 58 °C for 3 days), was dissolved in 400 μL of dry dioxane and added slowly to the above solution which was maintained at 0 °C by an ice bath. After 5 min 100 μL of β-mercaptoethanol was added in order to destroy the excess *n*-bromosuccinimide. Water (5 mL) was added, immediately followed by triethylamine to bring the pH to 8.0. The mixture was further diluted with 20 mL of water and applied to a DEAE-Sephadex A-25 column (3 cm × 30 cm), HCO_3^- form. Elution was carried out with a linear gradient of TEAB buffer (pH 7.5) from 0.05 to 0.8 M (1 L of each). One hundred fractions (20 mL each) were collected. (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dADP was eluted in fractions 61–76, which were pooled and evaporated to dryness. The excess TEAB buffer was removed by coevaporation with ethanol (3 × 20 mL). A total of 430 A_{260} units (28.1 μmol, 28% yield) of the product was obtained. The product was pure by TLC and HPLC analysis. The high-resolution proton-decoupled ^{31}P NMR spectrum (sweep width 1000 Hz) consisted of a P_β doublet at −6.40 ppm ($J_{\alpha,\beta}$ = 22.8 Hz) and three sets of P_α doublets at −10.82 ($J_{\alpha,\beta}$ = 22.8 Hz), −10.83 ($J_{\alpha,\beta}$ = 22.8 Hz), and −10.85 ($J_{\alpha,\beta}$ = 22.8 Hz). The maximum upfield signal for P_α was assigned to [α-$^{18}O_2$,$\alpha\beta$-^{18}O]dADP, the next signal (~0.02 ppm downfield) to [α-$^{18}O_2$]dADP, and the third signal (~0.03 ppm downfield) to [α-^{18}O,$\alpha\beta$-^{18}O]dADP. These assignments are based on the perturbation magnitudes caused by bridging and nonbridging ^{18}O atoms on a phosphorus signal and are in good agreement with the published values (Lowe et al., 1979). The [α-$^{18}O_2$]dADP is 12% of the visible P_α species. All the species containing ^{17}O on P_α do not contribute to the intensity of the P_α signal due to electric quadrupolar broadening of phosphorus by ^{17}O nucleus (Tsai, 1979).

(*5*) *Synthesis of* (S_P)-*[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP.* (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dADP was converted into (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP with pyruvate kinase and phosphoenolpyruvate. In a total volume of 6 mL, the reaction mixture contained 4.7 mM (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dADP, 10 mM phosphoenolpyruvate, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.0), 10 mM KCl, and 4 units/mL pyruvate kinase. The reaction mixture was incubated at 25 °C, and the progress of the reaction was monitored by TLC on PEI–cellulose in 1 M KCl. The conversion of the diphosphate into the triphosphate was judged to be complete at the end of 2 h. The reaction mixture was diluted to a total volume of 25 mL and applied to a DEAE-Sephadex A-25 column (1.5 cm × 30 cm), HCO_3^- form. Elution was carried out with a linear gradient of TEAB buffer (pH 7.5) from 0.05 to 0.6 M (1 L of each). One hundred fractions (20 mL) were collected, and the appropriate fractions containing the desired product were pooled. The pooled fractions were evaporated to dryness, and the excess TEAB buffer was removed by coevaporation with ethanol (3 × 20 mL). A total of 25 μmol (89% yield) of (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP was recovered. The compound was judged to be pure by TLC and HPLC analysis. The high-resolution proton-decoupled ^{31}P NMR consisted of a P_γ doublet at −5.89 ppm ($J_{\beta,\gamma}$ = 20.4 Hz), a P_β doublet of doublets at −21.80 ppm, and three sets of doublets for P_α. As in the case of (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dADP, the P_α signals were assigned to [α-$^{18}O_2$,$\alpha\beta$-^{18}O]dATP at −10.98 ppm, [α-$^{18}O_2$]dATP at −10.96 ppm, and [α-^{18}O,$\alpha\beta$-^{18}O]dATP at −10.95 ppm. The ^{31}P NMR spectra of (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP are shown in Figures 1 and 2.

(*6*) *Poly[d(A-T)]-Dependent Idling–Turnover of* (S_P)-*[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP with Klenow Fragment.* In a total volume of 30 mL, the reaction mixture contained, per milliliter, 50 μmol of Hepes (pH 7.4), 5 μmol of $MgCl_2$, 2 μmol of β-mercaptoethanol, 67 μg of nuclease-free BSA, 100 nmol of DNase-activated poly[d(A-T)] (total nucleotide), 500 nmol of (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP, and 6.8 μg of Klenow fragment. The reaction mixture was incubated at 37 °C. The progress of the reaction was monitored by withdrawing 10-μL aliquots and subjecting them to analysis by HPLC. The HPLC analysis was carried out using a reverse-phase column and a 20-min linear gradient where the initial buffer was 50 mM potassium phosphate (pH 7.0), and the final buffer was 50 mM potassium phosphate (pH 6.5) containing 25% methanol. At a flow rate of 2 mL/min, dATP had a retention time of 4.8 min and dAMP had a retention time of 7.6 min under these conditions. The reaction was judged to be complete at the end of 12 h as only a trace of the starting material was detected. The reaction mixture was diluted to 100 mL and applied to a DEAE-Sephadex A-25 column (1.5 cm × 25 cm), HCO_3^- form. The column was eluted with 1200 mL of a linear gradient of 0.05–0.5 mM TEAB buffer (pH 7.5). One hundred fractions (12 mL each) were collected at a flow rate of 1 mL/min. The desired product, [^{16}O,^{17}O,^{18}O]dAMP, was eluted in fractions 33–54. These fractions were pooled, evaporated to dryness and coevaporated with ethanol (3 × 20 mL) to remove the TEAB buffer. A total of 222 A_{260} units (14.5 μmol, 97% yield) of product were recovered. An HPLC separation revealed a small amount of contamination from dTMP from the degradation of poly[d(A-T)]. However, since the contamination with dTMP would not interfere with the analysis of the chiral [^{16}O,^{17}O,^{18}O]dAMP product, it was not removed. The high-resolution proton-decoupled ^{31}P NMR spectrum (sweep width 1500 Hz) consisted of three singlets at 3.952, 3.928, and 3.905 ppm, assigned to species [$^{16}O_3$]dAMP, [$^{16}O_2$,,^{18}O]dAMP, and [^{16}O,$^{18}O_2$]dAMP, respectively. An additional signal at 2.71 ppm corresponding to the dTMP contamination was observed. The [$^{16}O_3$]dAMP species, like dTMP, is a degradation product of poly[d(A-T)].

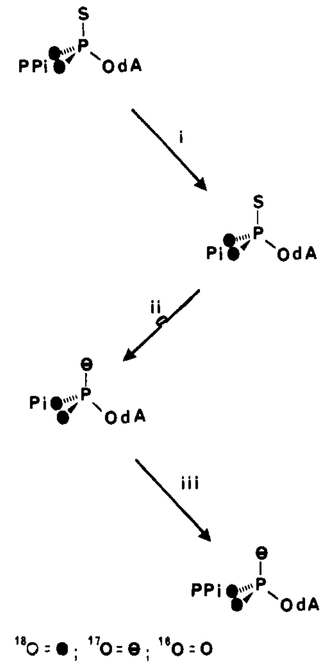
(*7*) *Configurational Analysis of [^{16}O,^{17}O,^{18}O]dAMP.* [^{16}O,^{17}O,^{18}O]dAMP was cyclized by reaction with diphenyl phosphorochloridate followed by potassium *tert*-butoxide and methylated with MeI/18-crown-6 using a slight modification of the procedures of Jarvest et al. (1981). The triethylammonium salt of [^{16}O,^{17}O,^{18}O]dAMP (14.5 μmol) dissolved in 5 mL of water was converted into the pyridinium salt by passage of the solution through a 3-mL column of pyridinium–Dowex. The solution was recycled through the column several times to ensure completion of ion exchange. The resin was finally rinsed with 5 mL of water, and the combined solution and washing were lyophilized to dryness. The pyridinium salt was coevaporated with anhydrous methanol (3 × 0.5 mL) to remove traces of water. Methanol (200 μL) was added, followed by 16 μmol of tri-*n*-octylamine. The mixture was stirred until all the pyridinium salt dissolved. Methanol was evaporated off, and the resulting tri-*n*-octylammonium salt was rendered anhydrous by coevaporation with dry DMF (3 × 200 μL).

The tri-*n*-octylammonium salt of [^{16}O,^{17}O,^{18}O]dAMP was dissolved in a 1:1 mixture of dioxane and DMF (200 μL). Diphenyl phosphorochloridate (16 μmol) and tri-*n*-butylamine (32 μmol) were added, and the mixture was stirred for 40 min in a dry argon atmosphere. Dioxane (0.5 mL) followed by potassium *tert*-butoxide (100 μL, 1 M solution in *tert*-butyl alcohol) was added, and the mixture was stirred another 10 min. The reaction was quenched with 10 mL of water, and the pH of the solution was adjusted to 8–9 by bubbling in CO_2. After the pH was adjusted to 3.5, an aliquot was analyzed by HPLC (anion-exchange column, eluted with 7 mM ammonium phosphate, pH 3.5, at a flow rate of 3 mL/min). Approximately 50% conversion of the starting [^{16}O,^{17}O,^{18}O]dAMP (retention time 10 min) into cyclic [^{16}O,^{17}O,^{18}O]dAMP (retention time 4.5 min) was evident from the HPLC profile. The latter was purified by chromatography on a DEAE-Sephadex A-25 column (1.5 × 20 cm). Elution was carried out with a linear gradient (0–0.25 M, 600 mL each) of TEAB buffer. One hundred fractions (12 mL) were collected, and the desired material was eluted in fractions 25–39. These fractions were pooled and evaporated to dryness. Methylation with MeI/18-crown-6 to give N^1-methyldeoxyadenosine cyclic 3′,5′-phosphate methyl esters was carried out as described by Jarvest et al. (1981). The ^{31}P NMR spectrum obtained is shown in Figure 4 and discussed under Results.

Results

Synthesis of (S_P)-[α-^{17}*O*,α-^{18}*O*,$\alpha\beta$-^{18}*O*]*dATP*. The synthesis of (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP is based on a stereospecific replacement of sulfur by ^{17}O in (S_P)-[α-$^{18}O_2$]dADPαS with *N*-bromosuccinimide. The method used was similar to that described by Connolly et al. (1982) for replacement of sulfur with ^{18}O in ribonucleoside phosphorothioates. The overall strategy for the synthesis of (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP is depicted in Scheme I. (S_P)-[α-$^{18}O_2$]dATPαS was synthesized by the method of Brody & Frey (1981). The ^{18}O enrichment in this compound was judged to be ~94% as determined by ^{31}P NMR spectroscopy. It has been shown (Connolly et al., 1982; Lowe et al., 1982) that any attempt to substitute the sulfur with an oxygen isotope in ATPαS results in a distribution of the isotope between the γ- and α-phosphorus sites. Such a scrambling of the isotope has been attributed to the intermediacy of adenosine 5′-trimetaphosphate (Glonek et al., 1974; Knorre et al., 1976). As a consequence, the direct displacement of sulfur in (S_P)-[α-$^{18}O_2$]dATPαS by ^{17}O to give the desired target molecule was not deemed feasible; instead, the (S_P)-[α-$^{18}O_2$]dATPαS was converted to (S_P)-[α-$^{18}O_2$]dADPαS by hexokinase (Scheme I). The (S_P)-[α-$^{18}O_2$]dADPαS was then treated with *N*-bromosuccinimide in [^{17}O]water–dioxane, and the desired (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dADP was isolated in 28% yield after chromatography on DEAE-Sephadex. Some of the major byproducts of the reaction were identified as adenine, [$^{18}O_2$]dAMPS, and [^{17}O,^{18}O]dAMP. The large amount of [^{17}O,^{18}O]dAMP (40–50%) undoubtedly results from the excellent leaving ability conferred on the α-phosphate through bromination, thereby facilitating loss of the β-phosphate. This major competing pathway accounts for the low yield of the desired product.

Scheme I[a]



[a] Reagents: i, hexokinase; ii, NBS and $H_2{}^{17}O$/dioxane (1:4); iii, phosphoenolpyruvate and pyruvate kinase.

The stereochemical course of the desulfurization reaction was determined by carrying out the reaction in H_2O (results not shown) and determining the stereochemistry of the resulting [α-^{16}O,α-^{18}O,$\alpha\beta$-^{18}O]dADP by forming a cobalt(III) complex and examining its ^{31}P NMR (Coderre & Gerlt, 1980). In agreement with earlier results (Connolly et al., 1982), desulfurization had proceeded stereospecifically with a greater than 90% inversion of configuration (within the limits of detection by ^{31}P NMR spectroscopy). There is a question as to whether desulfurization with *N*-bromosuccinimide is completely stereospecific or whether a small amount of racemization accompanies desulfurization. Desulfurization of thymidine cyclic 3′,5′-phosphorothioate with *N*-bromosuccinimide in dioxane/[^{18}O]water gave 23% racemic product at phosphorus (Lowe et al., 1982) whereas a completely stereospecific substitution of sulfur by ^{18}O in (S_P)-GDPαS was observed when the reaction was carried out for 2 rather than 0.25 min (Senter et al., 1983). The stereospecificity of the present reaction was confirmed when the ultimate (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP product was utilized by DNA polymerase in the idling reaction and the resulting [^{16}O,^{17}O,^{18}O]dAMP analyzed by the method of Lowe et al. (1981). Quantitative analysis at that stage indicated that no (R_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP had been present in the substrate. The (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dADP was converted to the desired (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP with phosphoenolpyruvate and pyruvate kinase.

The final enrichment of ^{17}O in (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP was determined by ^{31}P NMR spectroscopy as follows. Since ^{17}O directly bonded to ^{31}P broadens the ^{31}P signal by electric quadrupolar relaxation (Tsai, 1979), only those species which have either ^{16}O or ^{18}O directly attached to the α-P will yield a ^{31}P signal for the α-P. A ^{31}P NMR spectrum of (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP should therefore

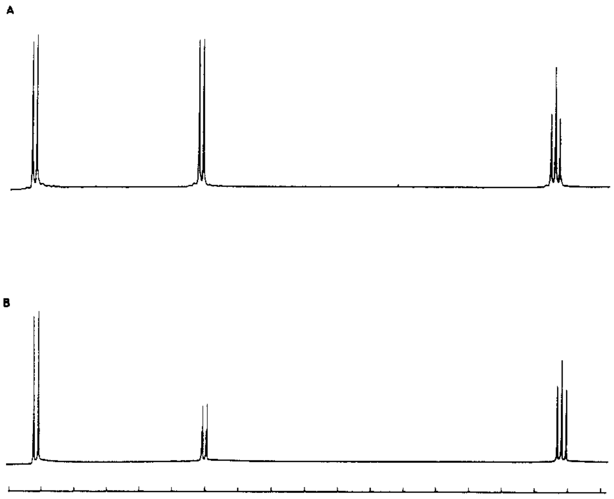


FIGURE 1: 145.8-MHz ^{31}P NMR spectrum of (A) dATP and (B) (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP in 50% D_2O at pH 9.5. Both spectra are inverse gated proton decoupled (see Experimental Procedures). Other ^{31}P NMR parameters were the following: offset 93 060 Hz, sweep width 2994 Hz, acquisition time 2.74 s, pulse width (angle) 24 μs (85°), Gaussian multiplication (line broadening −1.5 Hz, Gaussian broadening 0.1), data collection in 16K, 1000 transients. Scale 1 ppm/division.

show a diminished signal intensity for the α-P compared to that of either the β-P or the γ-P. The amount by which the α-P signal is decreased is a direct measure of the ^{17}O species. However, the signal intensities for α-, β-, and γ-P in an ordinary proton-decoupled spectrum of dATP are not equal, owing to a NOE that causes an enhancement of the α-P signal relative to the β- and γ-P. Since the magnitude of this enhancement is variable and dependent on the condition of NMR measurement, a more accurate estimate of the amount of ^{17}O species was obtained by recording the spectrum of (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP under conditions of inverse gated decoupling (Freeman, 1970) where the decoupler is switched on only during data acquisition and a delay is allowed between pulses to permit decay of any accumulated NOE. Figure 1A records the ^{31}P NMR spectrum of dATP with inverse gated proton decoupling. The peak areas for the α-, β, and γ-P were within 5% of each other, which denotes the limit of accuracy of ^{31}P measurements. Figure 1B records the ^{31}P NMR spectrum of (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP under the same conditions. An internal comparison of the α-P signal intensity with the γ-P signal intensity indicates that the P_α–^{17}O species must account for 47% of the total species.

Further information about the different species present in the product mixture is obtained from the high-resolution ^{31}P NMR spectrum of the α- and β-P regions (Figure 2). For the α-P, the spectrum consists of three major sets of doublets indicating the presence of three different species that are readily identified as labeled in Figure 2A owing to the observed isotope shifts of 0.075, 0.058, and 0.046 ppm in accord with published values of ^{18}O substitution. The presence of species 1 and 3 is expected and readily accounted for since the $H_2{}^{17}O$ employed in the desulfurization reaction contained 12.5% ^{16}O and 35% ^{18}O. However, the presence of species 2, whose relative intensity equals 12% of all the visible species in the α-P spectrum and ~6% of total phosphorus in the β-P spectrum, requires further explanation.

Since the starting (S_P)-[α-$^{18}O_2$]dADPαS contained only 3% of the corresponding (S_P)-[α-^{18}O,$\alpha\beta$-^{16}O]dADPαS, an uncomplicated simple displacement reaction during desulfurization should have resulted in ca, 2% of species 2 in the α-P spectrum. The origin of species 2 is clearly due to the desulfurization reaction since the ^{31}P NMR of α-P in (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dADP showed the same pattern. Moreover, the observed ratio of species 3 to species 1 is 1.85, considerably lower than the expected ratio of 2.8 based on the original composition of the $H_2{}^{17}O$ employed. Although this increase in the amount of species 1 relative to species 3 can be explained by a dilution of the [^{17}O]water with moisture during the desulfurization reaction, such a rationale does not explain the considerable increase in the amount of (2) relative to the $\alpha\beta$-^{18}O content in the dADPαS.

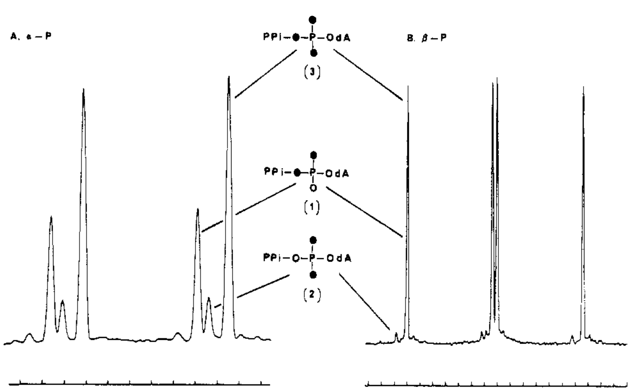


FIGURE 2: 145.8-MHz ^{31}P NMR spectrum of (A) P_α region and (B) P_β region of (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP in 50% D_2O at pH 9.5. Other ^{31}P NMR parameters were the following: (A) offset 93 510 Hz, sweep width 749.6 Hz, acquisition time 5.46 s, pulse width (angle) 24 μs (85°), Gaussian multiplication (line broadening −0.6 Hz, Gaussian broadening 0.1), data collection in 16K, 1391 transients, scale 0.02 ppm/division; (B) offset 92 200 Hz, sweep width 600.2 Hz, acquisition time 13.65 s, pulse width (angle) 24 μs (85°), Gaussian multiplication (line broadening −0.6 Hz, Gaussian broadening 0.1), data collection in 16K, 2815 transients, scale 0.02 ppm/division.

Clearly, a mechanism is operative in the desulfurization reaction that results in enrichment of species 1 and 2 relative to species 3. Recently, Sammons & Frey (1982) presented evidence for the intermediacy of a four-membered cyclic diphosphate during desulfurization of ADPαS with cyanogen bromide at pH 10.5. In [^{18}O]water under these conditions approximately equal amounts of the ^{18}O label appeared in the α- and β-P of the ADP product owing to attack by water on the β-P of the cyclic diphosphate as well as the α-P of ADPαS. The distribution of the various species found for (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP can be accounted for quantitatively on the basis of a similar mechanism as shown in Scheme II. The first step involves bromination of the sulfur of ADPαS with *N*-bromosuccinimide to give a cationic intermediate that predominantly (88%) undergoes a direct nucleophilic attack on the α-P with [^{17}O]water to give the expected product (species arising from oxygen isotopes other than ^{17}O have been omitted for clarity). The remaining 12% of the intermediate partitions to the cyclic diphosphate species that ring opens following exclusive nucleophilic attack on the β-P by [^{17}O]-water, to give equal amounts of the dADP species analogous to (1) and (2) in Figure 2. Since product 2 is unique to the cyclization pathway, the initial partitioning of 88:12 can be obtained from an iterative calculation with its peak area in Figure 2 as the basis. This mechanism is in principle the same as the one proposed by Sammons & Frey, (1982) except that in this case only 12% of the cationic intermediate cyclizes as opposed to 50% observed by those authors. This difference most probably arises from the lower reaction pH in our case. As seen in Table I the observed relative intensities for various (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP species can be readily calculated on the basis of the proposed mechanism (Scheme II). An accurate estimation of these species is necessary for a

Scheme II

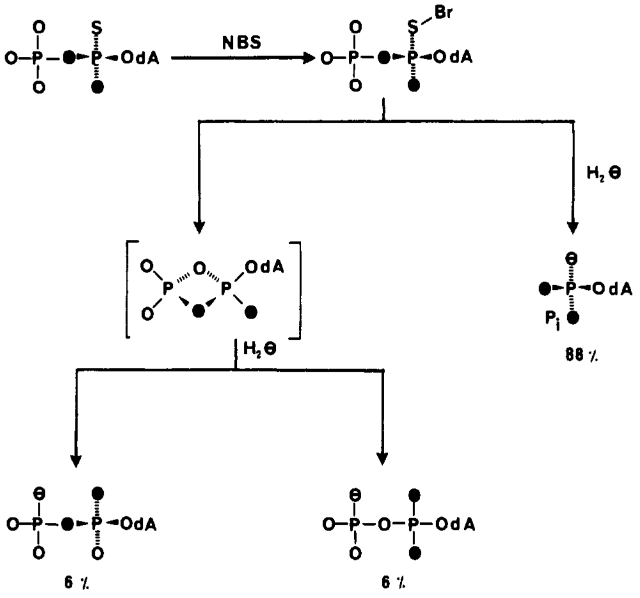
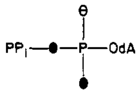
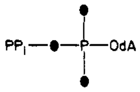
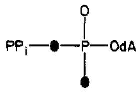
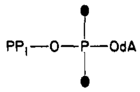


Table I: Analysis of (S_P)-[α-^{17}O,α-^{18}O$_2$,$\alpha\beta$-^{18}O]dATP by ^{31}P NMR

	α-P[a]			β-P[a]		
species	obsd	calcd[b]	calcd[c]	obsd	calcd[b]	calcd[c]
 $PP_i-\bullet-\overset{\ominus}{\underset{\bullet}{P}}-OdA$	–	49	47			
 $PP_i-\bullet-\overset{\bullet}{\underset{\bullet}{P}}-OdA$	30	33	30	90	97	94
 $PP_i-\bullet-\overset{O}{\underset{\bullet}{P}}-OdA$	16.2	12.8	17			
 $PP_i-O-\overset{\bullet}{\underset{\bullet}{P}}-OdA$	6.4	<1.5	6.5	5	3	6

[a] Peak area as percent of γ-P. [b] Based on the original [^{17}O]water composition: 52.5% ^{17}O, 35% ^{16}O, and 12.5% ^{16}O. [c] Based on the proposed mechanism in Scheme II of 88% direct attack on α-P and 12% cyclization followed by β-P attack.

quantitative determination of the stereoselectivity of a reaction when this substrate is utilized.

Poly[d(A-T)]-Dependent Idling–Turnover of (S_P)-[α-^{17}*O*,α-^{18}*O*,$\alpha\beta$-^{18}*O*]*dATP.* The idling–turnover reaction catalyzed by Klenow fragment was monitored by HPLC analysis of periodically withdrawn aliquots. At the end of 12 h, none of the starting material was detected, and the only products of the reaction were labeled dAMP and a small amount of dTMP from the degradation of poly[d(A-T)]. Since the idling reaction involves repeated incorporation–excision cycles on a poly[d(A-T)] template-primer, any competitive degradation of the latter results in only a partial turnover of the triphosphate pool. For this reason, intact *Pol*I could not be employed in this reaction because of its 5′→3′-exonuclease activity. Also, any extraneous nuclease activities must be rigorously excluded, a condition satisfied by the highly purified preparation of Klenow fragment obtained readily from the overproducer strain (Joyce & Grindley, 1983).

The [^{16}O,^{17}O,^{18}O]dAMP of unknown configuration obtained from the reaction was purified by anion-exchange chromatography on DEAE-Sephadex A-25. A total of 14.5 μmol of nucleotide (97% yield) was isolated, which was found to be contaminated wth approximately 10% dTMP from degradation of the poly[d(A-T)] template-primer. Consequently, the [^{16}O,^{17}O,^{18}O]dAMP likewise is diluted by 10% with dAMP from the template-primer. The ^{31}P NMR of this product is shown in Figure 3. The spectrum consists of three signals centered at 3.9 ppm downfield from phosphoric acid which are readily assigned on the basis of the ^{18}O content. An additional signal (not shown) at 2.7 ppm was observed and attributed to the dTMP contamination. The ratio of the unlabeled dAMP signal to the sum of the signals for the ^{18}O-containing dAMP species and the invisible ^{17}O species (calculated from the known ^{17}O) is 1:10 as expected.

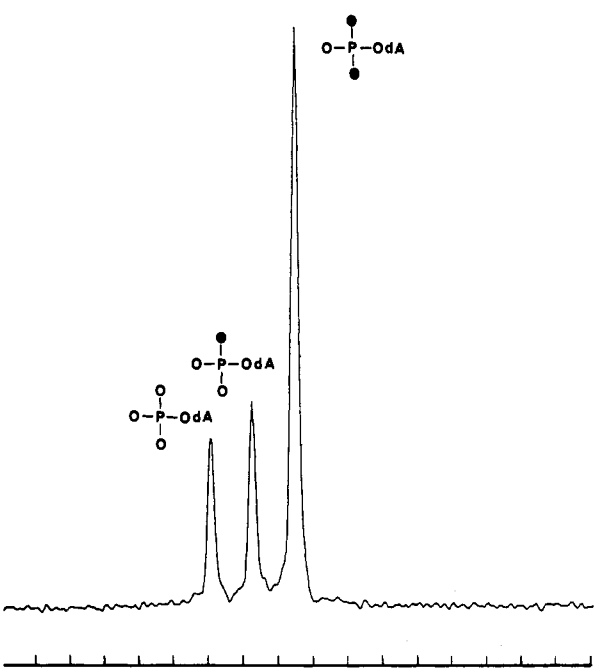


FIGURE 3: 145.8-MHz ^{31}P NMR spectrum of [^{16}O,^{17}O,^{18}O]dAMP isolated from the idling reaction in 50% D$_2$O at pH 9.5. Other ^{31}P NMR parameters were the following: offset 95 560 Hz, sweep width 1501.5 Hz, acquisition time 5.46 s, pulse width (angle) 22 μs (80°), Gaussian multiplication (line broadening –0.6 Hz, Gaussian broadening 0.1), data collection in 16K, 1219 transients, scale 0.02 ppm/division.

Configurational Analysis of [^{16}*O*,^{17}*O*,^{18}*O*]*dAMP.* The configurational characterization of [^{16}O,^{17}O,^{18}O]dAMP obtained from the idling reaction was carried out by the method of Jarvest et al., (1981). The 5′-monophosphate was converted into the cyclic 3′,5′-monophosphate by activation with diphenyl phosphorochloridate followed by cyclization with potassium *tert*-butoxide. After purification on a DEAE-Sephadex A-25 column, the latter was converted into the K$^+$ salt and treated with MeI/18-crown-6. The proton-decoupled high-resolution ^{31}P NMR spectrum of the resulting axial and equatorial N^1-methyldeoxyadenosine cyclic 3′,5′-monoposphate methyl triesters in Me$_2$SO is shown in Figure 4. From the known distribution of all the major species in the starting (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP (Table I) and the extent of dilution of label from the degradation of poly[d(A-T)], it is possible to calculate the expected relative peak intensities of the ^{31}P NMR signals from the equatorial and axial triesters, corresponding to retention and inversion stereochemistries in the idling reaction. A comparison of the observed and calculated relative peak intensities (Table II) clearly indicates that the overall idling–turnover process results in a net retention of stereochemistry at phosphorus.

Discussion

In recent years, several syntheses for nucleotides chirally labeled by the stable isotopes of oxygen have been developed,

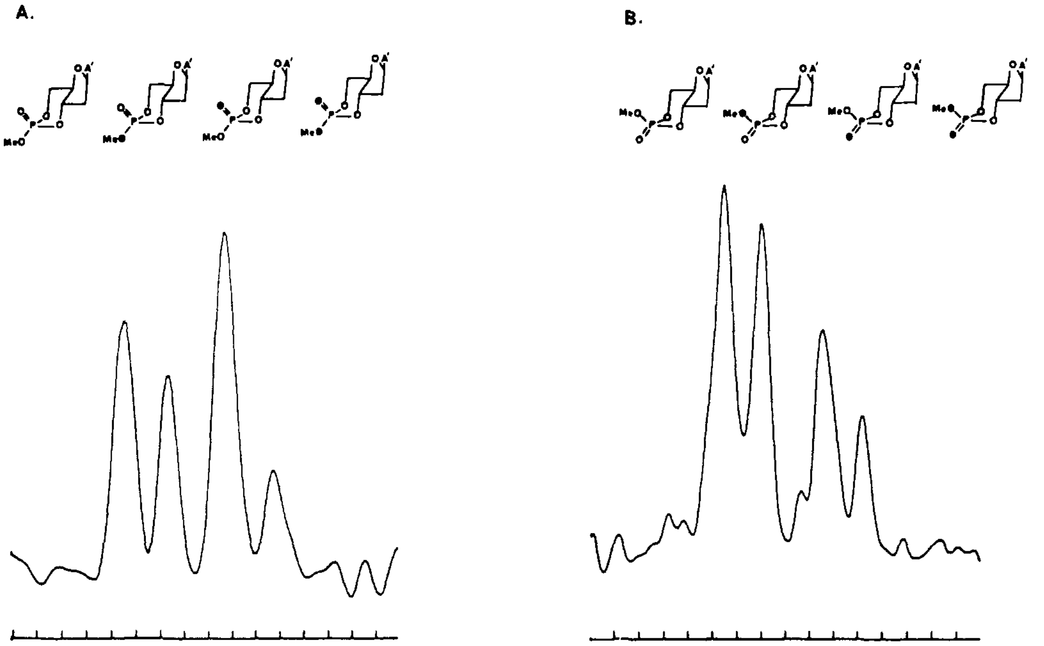


FIGURE 4: 145.8-MHz ^{31}P NMR spectrum of (A) equatorial and (B) axial triesters derived by cyclization and methylation of [^{16}O,^{17}O,^{18}O]dAMP obtained from the idling–turnover of (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]dATP with Klenow fragment. The spectra were obtained in Me_2SO-d_6–Me_2SO (1:1 v/v). Other parameters were the following: (A) offset 94 900 Hz, sweep width 749.6 Hz, acquisition time 5.46 s, pulse width (angle) 24 μs (85°), Gaussian multiplication (line broadening −3 Hz, Gaussian broadening 0.1), data collection in 8K, 5624 transients; (B) offset 94 900 Hz, sweep width 1000 Hz, acquisition time 4.1 s, pulse width (angle) 24 μs (85°), Gaussian multiplication (line broadening −3 Hz, Gaussian broadening 0.1), data collection in 16K, 13 503 transients, scale 0.01 ppm/division.

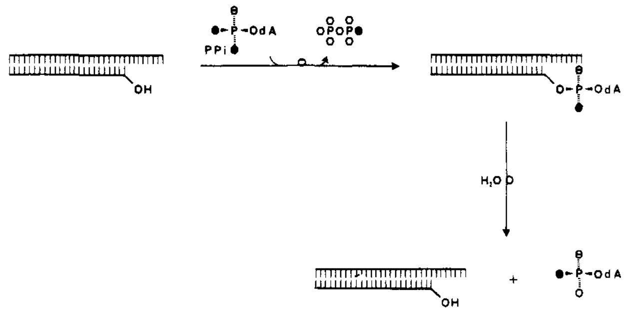
Table II: Configurational Analysis of [^{16}O,^{17}O,^{18}O]dAMP

	equatorial triester			axial triester		
		calcd			calcd	
	obsd	inversion	retention	obsd	inversion	retention
MeO—P=O	0.695	0.47	0.47	1.1	0.47	0.47
Me●—P=O	0.507	1.00	0.535	1.00	0.535	1.00
MeO—P=●	1.00	0.535	1.00	0.7	1.00	0.535
Me●—P=●	0.315	0.37	0.37	0.47	0.37	0.37

although most of the activity has been limited to the ribo derivatives (Lowe et al., 1979; Connolly et al., 1982; Sammons & Frey, 1982; Senter et al., 1983). In this paper we describe the first successful synthesis of isotopically chiral 2′-deoxyadenosine 5′-triphosphate using a modification of the desulfurization reaction described earlier (Connolly et al., 1982). The conditions employed for the desulfurization of (S_P)-[α-$^{18}O_2$]dATPαS in [^{17}O]water were chosen carefully to minimize depurination due to low pH conditions generated during the course of the reaction. The use of low pH during the reaction, however, is necessary in order to avoid complications caused by the neighboring group participation by the terminal phosphate group during the hydrolysis of the bromothiophosphoryl intermediate at a higher pH (Sammons et al., 1982; Lowe et al., 1983).

A careful characterization of (S_P)-[α-^{17}O,α-^{18}O,$\alpha\beta$-^{18}O]-dATP by high-resolution ^{31}P NMR spectroscopy revealed that a minor dilution of label in the P_α–O–P_β bridging position had occurred during the desulfurization reaction. This dilution indicates that the P_α–O–P_β bridging ^{18}O had partially migrated out of the bridge (Sammons et al., 1982). This dilution, together with the apparent change in the $^{18}O/^{16}O$ dATP ratio relative to the composition of the [^{17}O]water after the reaction as well as slight reduction in the P_β intensity relative to P_γ in the inverse gated proton-decoupled ^{31}P NMR spectrum (not shown), supports the mechanism indicated in Scheme II. This complication, however, does not compromise the chiral integrity of the substrate since the cyclic diphosphate intermediate (Scheme II) undergoes ring opening by an exclusive nucleophilic attack at β-P.

Scheme III



As depicted in Scheme III, the stereochemical investigation of the 3′→5′-exonuclease activity was carried out by determining the overall stereochemical course of the idling–turnover process. For T4 DNA polymerase, it has been shown that idling–turnover is a direct consequence of repeated incorporation–excision cycles (Gupta et al., 1982). Similar evidence was obtained to show that the same must be true for DNA polymerase I (Gupta et al., 1984). Since the idling–turnover proceeds with a net retention of configuration (Scheme III and Table II) and given that polymerization proceeds with inversion of configuration (Burgers & Eckstein, 1979; Brody & Frey, 1981), the stereochemical course of the 3′→5′-exonuclease must also be inversion. Inversion stereochemistry at phosphorus in an enzymatic reaction implies the occurrence of a single or odd number of direct displacement steps in the catalytic mechanism (Knowles, 1980). The simplest and thus favored mechanism of the 3′→5′-exonuclease activity of DNA polymerase I is therefore a single, direct displacement by water on the 3′ terminus of the DNA chain to yield a 5′-nucleotide.

Acknowledgments

We thank Dr. Katherine Joyce for providing us with her cloned overproducer of Klenow fragment, Paul Domanico for help with recording ^{31}P NMR spectra, and Dr. Douglas Sammons for helpful discussions.

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