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Unambiguous Determination of the Stereochemistry of Nucleotidyl Transfer Catalyzed by DNA Polymerase I from *Escherichia coli*†

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ABSTRACT: Nucleotidyl transfer catalyzed by DNA polymerase I from *Escherichia coli* proceeds with greater than 97% inversion of configuration at P_α of the α-phosphorothioate analogue of dATP. This is shown by experiments in which dAMPS,$^{18}O_2$ is stereospecifically phosphorylated to (*Sp*)-dATPαS,$\alpha^{18}O_2$, which is then copolymerized with dTTTP by DNA polymerase. The product of the polymerization is degraded to dAMPS,^{18}O by methods that do not affect the configuration of the phosphorothioate. After the dAMPS,^{18}O is stereospecifically phosphorylated, the resulting (*Sp*)-dATPαS,$\alpha^{18}O$ is copolymerized as before with dTTP. The ^{18}O is found in the displaced pyrophosphate by mass spectral analysis and so must have been in the pyrophosphate bridge of (*Sp*)-dATPαS,$\alpha^{18}O$. Since this ^{18}O was originally nonbridging in (*Sp*)-dATPαS,$\alpha^{18}O_2$, the phosphorothioate configuration must have been inverted in the polymerization reaction. This confirms the determination of P. M. J. Burgers & F. Eckstein [(1979) *J. Biol. Chem. 254*, 6889–6893], who used kinetic correlations based on the stereoselectivity of snake venom phosphodiesterase to deduce the stereochemistry of this reaction.

DNA polymerase I from *Escherichia coli* catalyzes the polymerization of deoxynucleotide triphosphates in the presence of primer template to produce DNA-like polymers (Kornberg & Kornberg, 1974). The incorporation of a deoxynucleotide into the growing polymer involves either a single bond cleavage at the α-phosphorus of the nucleotide triphosphate as the 3′-hydroxyl of the growing chain displaces pyrophosphate or two bond cleavages if a covalent nucleotidyl enzyme is involved. The use of chiral phosphorothioate nucleotides, already extensively employed as stereochemical probes of phospho- and nucleotidyltransferases (Pliura et al., 1980; Gerlt et al., 1980; Webb & Trentham, 1980; Burgers & Eckstein, 1979a–c; Sheu et al., 1979; and references found therein), can distinguish between these two reaction possibilities. As the accumulated evidence indicates that all cleavages of phosphate bonds in enzymatic reactions occur with inversion of configuration, a single bond cleavage will result in inversion and two bond cleavages will yield retention of the configuration about the phosphorothioate [see Westheimer (1980) and Knowles (1980) for discussions of the stereochemistry of nonenzymatic and enzymatic reactions of phosphate esters].

The polymerization of dATPαS[1] catalyzed by DNA polymerase I has recently been investigated by Burgers & Eckstein (1979c). They found that the *Sp* diastereomer of dATPαS is polymerized in the presence of a poly(dT) template to poly(deoxyadenylic acid) containing phosphorothioate internucleotide linkages. The kinetic constants for the polymerization of (*Sp*)-dATPαS are similar to those for dATP, while the *Rp* diastereomer is not a substrate for the enzyme. The chirality of the poly[d(A_s-A)] produced in the polymerization reaction was assigned by using the stereoselectivity of the exonuclease activity of snake venom phosphodiesterase for phosphorothioate diesters having the *Rp* configuration (Burgers & Eckstein, 1978). In a dinucleotide model system, Burgers & Eckstein (1979b) found that 3′-uridyl 5′-adenosyl *Rp* diastereomer hydrolyzed by the diesterase 110 times faster than the *Rp* diastereomer of 3′-*O*-uridyl-5′-*O*-adenosyl phos-

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[1] Abbreviations used: dAMPS, 2′-deoxyadenosine 5′-*O*-phosphorothioate; dAMPS,^{18}O, 2′-deoxyadenosine 5′-*O*-[^{18}O]phosphorothioate; dATPαS, 2′-deoxyadenosine 5′-*O*-(1-thiotriphosphate); dATPαS,$\alpha^{18}O$, 2′-deoxyadenosine 5′-*O*-(1-thio[1-^{18}O]triphosphate); pdTp_sdA, 3′-*O*-(5′-phospho-2′-deoxythymidyl)-5′-*O*-(2′-deoxyadenosyl) phosphorothioate; poly[d(T_s-A)], alternating copolymer of 2′-deoxyadenosine 5′-*O*-phosphorothioate and 2′-deoxythymidine 5′-phosphate; poly[d(A-T)], alternating copolymer of deoxyadenylate and deoxythymidylate; poly[d-(T)], poly(deoxythymidylic acid); poly[d(A)], poly(deoxyadenylic acid); poly(rA), poly(adenylic acid); poly[r(A_s-A)], poly(adenylic acid) containing phosphorothioate internucleotide linkages; *Rp*, the *R* configuration of the chiral phosphorus in a nucleotide; *Sp*, the *S* configuration of the chiral phosphorus in a nucleotide; A_{260} units, absorbance at 260 nm if entire sample were in 1 mL; Me_2SO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; GCMS, gas chromatograph interfaced with a mass spectrometer; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TLC, thin-layer chromatography.

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phorothioate and 185 000 times faster than the *Sp* diastereomer. Snake venom phosphodiesterase also hydrolyzes poly(rA) 15 times faster than it hydrolyzes the *Rp* diastereomer of poly[r(A_sA)]. As the enzyme catalyzes the hydrolysis of poly(dA) 9 times faster than the hydrolysis of the poly[d(A_sA)] produced by the action of DNA polymerase, Burgers & Eckstein (1979c) assigned the configuration of the poly[d(A_sA)] to be *Rp*. This means that an inversion of the configuration of the α-phosphorothioate of (*Sp*)-dATPαS occurs during the polymerization reaction. No polymer with phosphorothioate linkages in the *Sp* configuration has been made, however, and so it cannot be confirmed that snake venom phosphodiesterase catalyzes the hydrolysis of an (*Sp*)-phosphorothioate polymer at a rate substantially slower than one-tenth the rate of a phosphate polymer. The stereochemical determination thus rests on the assumption that the relative rates of the hydrolyses of phosphate diesters, (*Rp*)-phosphorothioate diesters, and (*Sp*)-phosphorothioate diesters catalyzed by snake venom phosphodiesterase are invariant to whether the leaving group of the reaction is a mononucleotide or a polymer. While this assumption is reasonable, it is by no means certain. Burgers & Eckstein (1978) and Bryant & Benkovic (1979) have assigned the absolute configurations of the diastereomers of ATPαS by using similar rate correlations. However, in this case, both diastereomers of ATPαS existed, and the rates of their hydrolyses could be compared to the rates for model phosphorothioates of known configuration. Jarvest & Lowe (1979) have confirmed the assignment of chirality to the isomers of ATPαS with an independent determination.

In this paper, we describe a completely unambiguous determination of the stereochemical course of nucleotidyl transfer catalyzed by DNA polymerase I. We make use of previous work that showed that chiral phosphorothioate analogues of ATP can be synthesized enzymatically (Sheu & Frey, 1977; Jaffe & Cohn, 1978), DNA polymerase will accept the *Sp* diastereomer of dATPαS as a substrate (Burgers & Eckstein, 1979c), DNA polymerase will polymerize dATP and dTTP into an alternating poly[d(A-T)] copolymer (Schachman et al., 1960), and treatment of a deoxynucleic acid with hydrazine and base will yield purine sequences (Takemura, 1959; Habermann, 1963; Chargaff et al., 1963).

Experimental Procedures

Enzymes. *E. coli* DNA polymerase I was purchased from Worthington. Calf intestinal mucosa adenosine deaminase, rabbit muscle adenylate kinase and pyruvate kinase, and *E. coli* alkaline phosphatase were purchased from Sigma.

Chemicals. [^{14}C]dTTP was purchased from Schwarz/Mann. All other nucleosides and nucleotides were purchased from Sigma, as were poly[d(A-T)], Hepes, Tris, DEAE-Sephadex A-25, and 1-[3′-(dimethylamino)propyl]-3-ethylcarbodiimide. Thiophosphoryl chloride and sodium phosphorothioate were purchased from Ventron. AG 1-X2 anion-exchange resin and 99.8% D_2O were purchased from Bio-Rad. Sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ was purchased from Merck. Activated magnesium silicate (Florisil) was purchased from J. T. Baker. Sulfur trioxide–pyridine complex and *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine were purchased from Aldrich.

Analytical Procedures. Thin-layer chromatography was done on Eastman silica gel plates containing a fluorescent indicator with system A (1-propanol/concentrated ammonia/water, 6:3:1) and on Baker cellulose poly(ethylenimine) plates with system B (1.0 M lithium chloride). Nucleotides were visualized with UV light. Phosphorothioate nucleotides could be distinguished by the fact that they, as well as being visible with UV light, could be stained with iodine. Inorganic phosphate and pyrophosphate were visualized on poly(ethylenimine) plates by using Hanes–Isherwood spray (1949).

Nucleotides were detected after column chromatography by the absorbance of the fractions at 260 nm. All evaporation was done by rotary flash evaporation under vacuum with a bath temperature below 35 °C.

DNA polymerase activity was assayed with [^{14}C]dTTP, dATP, and poly[d(A-T)] according to the method of Richardson et al. (1964). The amount of [^{14}C]dTTP incorporated into polymer was also followed by the paper chromatographic method of Burgers & Eckstein (1979c). Buffers for DNA polymerase reactions were routinely made up just before they were needed with boiled water.

The concentrations of adenine and thymidine nucleotides were determined by measuring the absorbance at 260 nm and using 15.3×10^3 cm^{-1} M^{-1} and 9.3×10^3 cm^{-1} M^{-1} as the respective extinction coefficients (Dunn & Hall, 1970). UV measurements were made with a Pye-Unicam SP 1800 spectrophotometer.

dAMPS was determined in reaction mixtures by using TLC (system A), Ellman's reagent (Ellman, 1959; Goody & Eckstein, 1971), and a coupled assay system containing in 1 mL at room temperature 0.5 M Hepes buffer (pH 7.5), 3 units of alkaline phosphatase, 0.5 unit of adenosine deaminase, and 5 mM 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide. Samples were first combined with the buffer, alkaline phosphatase, and adenosine deaminase. Under these conditions, dAMP is converted to deoxyadenosine and then to deoxyinosine. The amount of deoxyadenosine and dAMP can be determined from the decrease in absorbance at 265 nm (Zielke & Suelter, 1971). dAMPS is a poor substrate for alkaline phosphatase (Chlebowski & Coleman, 1974) and is not degraded under these conditions. The decrease in absorbance at 265 nm after the carbodiimide is added is a measure of the dAMPS in solution. The carbodiimide removes sulfur from dAMPS (Mikolajczyk, 1966; Eckstein, 1967), yielding dAMP which is then converted to deoxyinosine.

Inorganic phosphate was detected by the procedure described by Ames (1966). Inorganic pyrophosphate was heated in 1 M HCl at 100 °C for 10 min and then assayed by the same procedure. Organic phosphates were ashed (Ames, 1966) and then assayed for inorganic phosphate.

^{31}P nuclear magnetic resonance spectra were obtained on a Bruker HX-90 spectrometer operating at 36.43 MHz and equipped with a Fourier transform accessory, a Bruker BS-V-3B decoupler, and a Nicolet DNC-12 computer. The spectrometer was field frequency locked at the deuterium resonance with 33% D_2O used as the solvent and 85% H_3PO_4 as the external standard. One-milliliter aqueous samples were made up at pH 8.5 with 5 mM EDTA and then added to 0.5 mL of D_2O.

^{1}H nuclear magnetic resonance spectra were obtained on the Bruker HX-90 spectrometer described above. The spectrometer was field frequency locked at the deuterium resonance with 99% D_2O used as the solvent. The spectra were referenced to the trimethylsilyl peak of sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ (δ 0.00).

Mass Spectral Analysis. The ^{18}O content of pyrophosphate and dAMPS was determined by conversion of these molecules to volatile phosphate derivatives (Midelfort & Rose, 1976) and analysis with a Du Pont 21-490 mass spectrometer (electron impact ionization, single focusing magnetic sector) coupled to a Perkin-Elmer 990 gas chromatograph. The column was

packed with 10% SE-30 on chromosorb G (Richard et al., 1978). The mass spectrometer was modified to allow for selective ion detection (McFadden, 1973). When our procedure was used, the enrichment of 0.01-μmol samples could be determined, although typically 0.1–1.0 μmol was used in each analysis. The phosphates were ethylated by the addition of an ether solution of diazoethane that was generated from *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine by the addition of ether and 40% potassium hydroxide (McKay, 1948). Diazoethane was made in small quantities and distilled just prior to its use.

Pyrophosphate. Inorganic pyrophosphate (typically 0.5 μmol) that had been isolated from a DNA polymerase reaction was dried under vacuum and dissolved in 0.5 mL of 0.5 M HCl in methanol. The acid solution was made by bubbling HCl gas (dried by passage through sulfuric acid, activated charcoal, and silica gel) into dry methanol (the methanol was refluxed with magnesium and iodine and then distilled). The reaction mixture was heated at 65 °C for 20 min and evaporated to dryness. Ethanol (0.5 mL) was then added, and the mixture of inorganic phosphate and methyl phosphate was ethylated with diazoethane.[2] The sample was then concentrated to approximately 20 μL with a stream of nitrogen. Samples were injected into the GC/MS at a column temperature of 75 °C. Triethyl phosphate came off the column at ~16 min and diethyl methyl phosphate came off at ~13 min. The fragmentation pattern of diethyl methyl phosphate [*m/e* 168 (2), 153 = (4), 141 = (70), 113 = (100)] is as expected from the fragmentations of trimethyl phosphate and triethyl phosphate (Bafus et al., 1966). The ^{18}O enrichment was determined by taking 40 scans a minute of *m/e* 140–144 and comparing the intensities of *m/e* 141 and 143. This fragment contains all the oxygens bound to phosphorus. At least 10 scans across the top of the GC peak were used for the calculation of the isotope enrichment.

dAMPS. dAMPS was ethylated with diazoethane and then oxidized by the method of Parikh & Doering (1967). The oxidation leads to the sponanteous elimination of diethyl phosphorothioate. Samples of dAMPS from 0.05 to 1.0 μmol were dissolved in 0.5 mL of methanol, and 10 μL of a 1.2 M solution of pyridinium hydrochloride in methanol was added. An ether solution of diazoethane was then added until the solution turned yellow. After 5 min, the diazoethane and ether were blown off with nitrogen, and a second equal addition of pyridinium hydrochloride and diazoethane was made. After a third addition of pyridinium hydrochloride and diazoethane, the sample was blown dry and further dried under vacuum for 1 h. The resulting diethyl dAMPS was dissolved in Me_2SO (0.1 mL, dried over sieves) and triethylamine (0.03 mL). A solution of sulfur trioxide–pyridine complex (0.014 g, 90 μmol) in 0.1 mL of Me_2SO was then added and the solution kept at room temperature for 30 min. 2-Propanol (0.04 mL) was then added, and, after another 30 min, the sample was diluted with 3 mL of water and applied to a 0.7 × 15 cm column of DEAE-Sephadex A-25 (bicarbonate form). The column was washed with 12 mL of 0.02 M triethylammonium bicarbonate buffer (pH 7.5), and the product was eluted with 18 mL of a 0.1 M solution of the same buffer. The product was evaporated to dryness, ethanol was added, and the product was again evaporated to dryness. The yields, determined by the ashing procedure of Ames (1966), were greater than 70%. After the product was ethylated by the same procedure used for dAMPS, it was further purified by the method of Shafik et al. (1971). The sample was dissolved in 1.5 mL of water and extracted 5 times with 1.5-mL portions of hexane. The hexane fractions were combined, applied to a column made from a Pasteur pipet, and packed with 0.5 g of activated magnesium silicate. The column was washed with 5 mL of hexane and then with 5 mL of benzene. The product was then eluted with 5 mL of ethyl acetate–benzene (4:6 v/v) and concentrated to ~20 μL with a stream of nitrogen. Samples were injected into the GC/MS at a column temperature of 115 °C. *O,O,S*-Triethyl phosphorothioate came off the column at ~12 min and had a fragmentation pattern [*m/e* 198 (41), 170 (42), 138 (100), 111 (86)] consistent with the patterns of *O,O,S*-trimethyl phosphorothioate (Santoro, 1973) and triethyl phosphate (Bafus et al., 1966).[3] *O,O,S*-Triethyl phosphorothioate resulting from treatment of phosphorothioic acid with diazoethane had the same GC retention time and MS fragmentation pattern. Multiple scans were made of *m/e* 193–203, and the intensities of the parent ion (*m/e* 198) were compared with those of the *m* + 2 and *m* + 4 ions to determine the ^{18}O enrichment. At least 10 scans across the top of the GC peak were used for the calculation of the isotope enrichment.

Syntheses. dAMPS. dAMPS,$^{18}O_2$ was synthesized by a modification of the procedures described by Murray & Atkinson (1968) and Richard et al. (1978). All manipulations before the chromatography were carried out in a nitrogen atmosphere or under vacuum. Deoxyadenosine (2 mmol, 0.535 g) was dried at 110 °C under vacuum for 8 h. Triethyl phosphate (10 mL; dried by passage over a Woelm basic alumina column) and pyridine (4.1 mmol, 0.33 mL; distilled over a calcium hydride) were then added, and the mixture was stirred at 100 °C until most of the deoxyadenosine dissolved (~5 min). After the solution had been cooled in an ice bath, freshly distilled thiophosphoryl chloride (8 mmol, 0.8 mL) was added and the reaction stirred at 0 °C for 1 h. Unreacted thiophosphoryl chloride was then removed by a 15-min vacuum distillation (~0.1 mm) at 30 °C. The remaining material was again cooled in ice, and a solution of pyridine (8 mmol, 0.66 mL) in $H_2{}^{18}O$ (0.5 mL, 99.12%) was added. After the mixture was stirred at room temperature for 2 h, a solution of pyridine (2 mL) in water (100 mL) was added. The pH was then raised from 5 to 9 with 1 M NaOH and the solution was applied to a 2.3 × 41 cm column of DEAE-Sephadex A-25 (bicarbonate form). The column was eluted at 4 °C with a linear gradient of 1.5 L of 0.1 M and 1.5 L of 0.4 M triethylammonium bicarbonate buffers (pH 7.5). The fractions that contained the product eluted at ~0.26 M buffer and were pooled and dried by evaporation. The residue was dissolved in ethanol and again evaporated to dryness to remove traces of buffer. The yield was 18 000 A_{260} units (1.18 mmol, 60%) of material that was pure by TLC (system A, R_f 0.45) and had the adenosine UV chromophore. The proton-decoupled ^{31}P NMR consisted of a singlet at −43.1 ppm.

dATPαS. (A) (*Sp*)-dATPαS,α$^{18}O_2$ was synthesized from dAMPS,$^{18}O_2$ by the coupled activities of adenylate kinase and pyruvate kinase. The procedure was a modification of that

[2] When this procedure is used, the presence of an inorganic phosphate contamination of 10 μmol does not affect the enrichment of the diethyl methyl phosphate. In samples with large inorganic phosphate contaminations, the diazoethylation must be done in ethanol rather than methanol to prevent dilution of the ^{18}O. When no phosphate contamination is present, the diazoethylation can be done in ethanol or methanol and analysis of the triethyl phosphate gives the same enrichment as analysis of the diethyl methyl phosphate.

[3] The *m/e* 138 peak results from loss of a thioacetaldehyde fragment and shows that the sulfur has been ethylated by diazoethane (Shafik et al., 1970). However, the GC peak may also contain some *O,O,O*-triethyl phosphorothioate. This will not affect the results, as both compounds will have the same ^{18}O enrichment.

of Sheu & Frey (1977). The reaction mixture contained in 50 mL at room temperature 5.0 mM dAMPS,$^{18}O_2$ (0.25 mmol), 5.0 mM ATP, 15 mM phosphoenol pyruvate, 15 mM magnesium chloride, 60 mM potassium chloride, 42 mM Tris-HCl buffer (pH 8.0), 1 mM dithiothreitol, 145 units/mL adenylate kinase, and 6.5 units/mL pyruvate kinase. Before the addition of the enzymes, the pH of the reaction mixture was adjusted to 8 with 0.3 M potassium hydroxide. The reaction was followed by TLC (system A). After 7 days, the chromatograms showed only traces of dAMPS (R_f 0.45) while the spot for dATPαS (R_f 0.12) was approximately the same intensity as that for ATP (R_f 0.04). No dADPαS or ADP was detected. The reaction mixture was then applied to a 2.3 × 42 cm column of DEAE-Sephadex A-25 (bicarbonate form) and eluted at 4 °C with a linear gradient of 1.5 L of 0.2 M and 1.5 L of 0.75 M triethylammonium bicarbonate buffers (pH 7.5). The fractions that contained dATPαS came off the column at ~0.6 M buffer and were pooled and evaporated to dryness. Ethanol was added and the solution again dried by evaporation. The yield was 2650 A_{260} units (173 mmol, 69%) of material that was pure by TLC (system A) and had the adenosine UV chromophore. The proton-decoupled ^{31}P NMR spectrum consisted of a P_α doublet at −42.87 ppm ($J_{\alpha,\beta}$ = 28.08 Hz), a P_β doublet of doublets at 23.02 ppm, and a P_γ doublet at 6.15 ppm ($J_{\beta,\gamma}$ = 20.75 Hz).

(B) A modification of the method described in (A) was used to phosphorylate the dAMPS,^{18}O that was isolated from the hydrazine degradation. dATPαS,$\alpha^{18}O$ was synthesized in a reaction mixture that contained in 9.2 mL at room temperature 0.33 mM dAMPS,^{18}O (3 μmol), 0.33 mM ATP, 1 mM phosphoenol pyruvate, 1 mM magnesium chloride, 60 mM potassium chloride, 42 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 790 units/mL adenylate kinase, and 35 units/mL pyruvate kinase. Both enzymes were supplied as ammonium sulfate solutions and were dialyzed at 4 °C against a solution of 42 mM Tris-HCl buffer (pH 8), 60 mM potassium chloride, and 1 mM dithiothreitol before use. The procedures described in part A were used to follow the reaction. The reaction reached completion in 11 h at which time it was applied to a 1 × 42 cm column of DEAE-Sephadex A-25 (bicarbonate form) and eluted at 4 °C with a linear gradient of 400 mL of 0.2 M and 400 mL of 0.75 M triethylammonium bicarbonate buffers (pH 7.5). (*Sp*)-dATPαS,$\alpha^{18}O$ was isolated as described in part A in 90% yield.

DNA Polymerase Reaction. Procedure A. The reaction mixture contained in 140 mL at 37 °C 0.5 mM (*Sp*)-dATPαS,$\alpha^{18}O_2$ (70 μmol), 0.5 mM dTTP (70 μmol), 0.003 mM poly[d(A-T)], 60 mM Hepes buffer (pH 7.4), 10 mM magnesium chloride, 1 mM 2-mercaptoethanol, and 0.7 unit/mL DNA polymerase I. Aliquots of 25 μL were removed during the reaction, and, after dilution to 1 mL, their absorbances at 260 nm were measured. The absorbance at 260 nm decreased to 85% of the initial value after 50 h, reflecting a change in hypochromicity as polymer was formed (Radding & Kornberg, 1962). At 60 h, the absorbance began increasing, and the reaction was stopped by the addition of 20 mL of 0.1 M EDTA. The mixture was stirred until all the precipitate dissolved, and the resulting solution was lyophilized to dryness.

Purification of Poly[d(T_s-A)]. The lyophilized DNA polymerase reaction mixture was dissolved in 10 mL of 0.05 M triethylammonium bicarbonate buffer (pH 7.5), applied to a 2.3 × 42 cm column of Sephadex G-50, and eluted at 4 °C with the same buffer. A total of 154 A_{260} units (17% of theory after correcting for the polymer's hypochromicity) of poly-[d(T_s-A)] came off in the exclusion volume and was lyophilized to dryness. In other polymerization experiments, it was found that the same amount of polymer was excluded from Sephadex G-200 as was excluded from G-50.

Purification of Pyrophosphate. A second peak containing 1100 A_{260} units came off the Sephadex G-50 column in one column volume and was dried by evaporation. Ethanol was added and the sample again evaporated to dryness. The sample was dissolved in 50 mL of water, and 10 mL of a 1 M solution of magnesium chloride was added. After the sample had been stirred at 50 °C for 10 min, the precipitated magnesium pyrophosphate was pelleted in a centrifuge. The supernatant fluid was decanted, and the precipitate was redissolved in 0.1 M EDTA (pH 7) and applied to a 1 × 42 cm column of AG 1-X2 anion-exchange resin (bicarbonate form). The column was eluted at 4 °C with a linear gradient of 400 mL of 0.2 M and 400 mL of 0.7 M triethylammonium bicarbonate buffers (pH 7.5). The fractions that contained pyrophosphate eluted at ~0.5 M buffer and were pooled and evaporated to dryness. After ethanol was added, the sample was again evaporated to dryness, and a phosphate analysis (Ames, 1966) showed there was 94 μmol of pyrophosphate (67%). TLC (system B) showed a single spot that cochromatographed with authentic pyrophosphate.

Purification of pdTp$_s$dA. The supernatant from the magnesium chloride precipitation was applied to a 2.3 × 42 cm column of DEAE-Sephadex A-25 (bicarbonate form). The column was eluted at 4 °C with a linear gradient of 1.5 L of 0.2 M and 1.5 L of 0.45 M triethylammonium bicarbonate buffers (pH 7.5). All fractions that had absorbances at 260 nm were concentrated by rotoevaporation and assayed for dAMPS by TLC and Ellman's reagent. No dAMPS was found in any fraction. The only major nucleotide peak off the column eluted at ~0.35 M buffer and contained 705 A_{260} units (40% of theory for pdTp$_s$dA). This compound had a single spot by TLC (system A) that could be detected by both UV light and iodine staining. The migration of the compound on TLC (R_f 0.33) was between that of dAMPS (R_f 0.45) and dATPαS (R_f 0.12); ^1H NMR (D_2O) δ 8.49 (s, 1 H), 8.17 (s, 1 H), 7.56 (s, 1 H), 6.44 (t, 1 H, J = 6 Hz), and 6.09 (m, 1 H); proton-coupled ^{31}P NMR (D_2O:H_2O, 1:2) δ −3.7 (t, 1 P, $J_{P,H}$ = 4.3 Hz) and −55.0 (m, 1 P). ^1H NMR (D_2O) of commercial (Sigma) 3′-(2′-deoxythymidyl)-5′-(2′-deoxyadenosyl) phosphate: δ 8.41 (s, 1 H), 8.15 (s, 1 H), 7.36 (s, 1 H), 6.42 (t, 1 H, J = 6 Hz), and 5.98 (m, 1 H). See also Davies & Danyluk (1974) and Ts'o et al. (1969) for the ^1H chemical shifts of similar compounds. For the ^{31}P shifts of similar compounds, see Cozzone & Jardetsky (1976a,b), Sheu & Frey (1977), Jaffe & Cohn (1978), and Burgers & Eckstein (1979b).

After treatment of a sample of pdTp$_s$dA with alkaline phosphatase (6 units of alkaline phosphatase, 40 A_{260} units of pdTp$_s$dA, 1 mL of 0.1 M triethylammonium bicarbonate buffer, pH 8) and purification of the UV-absorbing material on a DEAE-Sephadex A-25 column (product eluted at ~0.1 M triethylammonium bicarbonate buffer), the ^{31}P NMR spectrum contained only one peak at δ −55.4.

Purification of (pdTp$_s$dA)$_2$. The DEAE-Sephadex A-25 column that was used to purify pdTp$_s$dA was eluted at 4 °C with a second linear gradient consisting of 800 mL of 0.45 M and 800 mL of 0.75 M triethylammonium bicarbonate buffers (pH 7.5). The only nucleotide peak eluted at ~0.6 M buffer and was dried by evaporation. Ethanol was added, and the solution was again evaporated to dryness. This compound (240

A_{260} units, 14%) had a single spot on TLC (system A, R_f 0.2) that could be detected by both UV light and iodine staining; ^{1}H NMR (D_2O) δ 8.52 (s, 1 H), 8.45 (s, 1 H), 8.15 (s, 2 H), 7.56 (s, 1 H), 7.41 (s, 1 H), 6.38 (m, 2 H), and 6.09 (m, 2 H); proton-decoupled ^{31}P NMR (D_2O:H_2O, 1:2) δ −55.5 (s, 1 P), −55.1 (s, 1 P), −3.7 (s, 1 P), and 1.1 (s, 1 P).

Procedure B. A modification of procedure A was used to polymerize (*Sp*)-dATPαS,α^{18}O that was synthesized from the dAMPS,^{18}O isolated from the hydrazine degradation. (*Sp*)-dATPαS,α^{18}O (2.8 μmol) was polymerized at 37 °C in a 5.6-mL reaction mixture that contained 5.4 units/mL DNA polymerase I. All other compounds were in the same concentrations as used in procedure A. After 20 h, the absorbance at 260 nm had decreased to 80% of the initial reading. The absorbance then began to increase, and, after 40 h, it was 94% of the initial value. The reaction mixture was then combined with 0.65 mL of 1 M magnesium chloride, and the precipitated magnesium pyrophosphate was pelleted by centrifugation and purified as described in procedure A. The supernatant was applied at 4 °C to a 1 × 42 cm column of DEAE-Sephadex A-25 (bicarbonate form) and eluted with a linear gradient of 400 mL of 0.2 M and 400 mL of 0.45 M triethylammonium bicarbonate buffers (pH 7.5). The pdTp$_s$dA that was isolated (35 A_{260} units, 50%) was identical by TLC (system A) with that isolated in procedure A. TLC (system A) before the chromatography showed no spot corresponding to (pdTp$_s$dA)$_2$.

Conversion of (pdTp$_s$dA)$_2$ to pdTp$_s$dA. The reaction mixture contained in 1 mL at 37 °C the following: 0.3 mM (pdTp$_s$dA)$_2$, 100 mM Hepes buffer (pH 7.4), 10 mM magnesium chloride, 1 mM 2-mercaptoethanol, and 1 unit of DNA polymerase I. After 21 h, the (pdTp$_s$dA)$_2$ was completely converted to pdTp$_s$dA as indicated by TLC (system A). No trace of dAMPS was detected. A second unit of DNA polymerase was added and the reaction allowed to proceed for 48 more h. Again, analysis by TLC showed no dAMPS.

Conversion of Poly[d(T$_s$A)] to pdTp$_s$dA. The reaction mixture contained in 1 mL at 37 °C the following: 6.7 A_{260} units of poly[d(T$_s$A)], 100 mM Hepes buffer (pH 7.4), 10 mM magnesium chloride, 1 mM 2-mercaptoethanol, and 5 units of DNA polymerase I. The initial TLC of the reaction mixture (system A) contained only the spot for poly[d(T$_s$A)] (R_f 0.0). After 22 h, the only major TLC spot chromatographed with pdTp$_s$dA (R_f = 0.33).

Hydrazinolysis and Base Degradation of pdTp$_s$dA. The degradation of pdTp$_s$dA with hydrazine and base was done with procedures derived from those used by Türler & Chargaff (1969) and Cashmore & Peterson (1969) to degrade DNA. A major difference in our procedure is that the sugar hydrazone was not converted to the aldehyde prior to base elimination.[4] pdTp$_s$dA (7.2 μmol) was dried under vacuum (~0.1 mm) in the presence of phosphorus pentoxide at 80 °C for 2 h. Anhydrous hydrazine (0.5 mL; prepared as described by Brown (1967) and sealed in glass for storage) was added, and the reaction mixture was sealed with a tight-fitting septum and heated at 37 °C for 24 h. The solution was then frozen and the hydrazine removed under vacuum with a sulfuric acid trap. Immediately after the lyophilization was complete, 1 mL of a freshly made solution of 0.3 M potassium hydroxide was added and the reaction mixture heated at 100 °C for 40 min. The reaction was quenched by the addition of a solution containing 1 mL of 1 M Tris-HCl buffer (pH 8) and 0.3 mL of 0.23 M HCl. The solution was then applied at 4 °C to a 1 × 42 cm column of DEAE Sephadex A-25 (bicarbonate form) and eluted with a linear gradient of 300 mL of 0.15 M and 300 mL of 0.35 M triethylammonium bicarbonate buffers (pH 7.5). The product eluted at ~0.26 M buffer and was identical with dAMPS by the criteria of UV, TLC, Ellmans reagent, and the carbodiimide–alkaline phosphatase–adenine deaminase coupled assay. The yield was 56 A_{260} units (3.7 μmol, 51%). Other hydrazinolyses described in the text were done with the same procedure. However, the yields were variable and some were as low as 30%.

K^{18}OH Degradation of the Hydrazinolysis Product of pdTp$_s$dA. The hydrazinolysis of pdTp$_s$dA was carried out as described above except that 0.3 mL of a 0.3 M K^{18}OH solution (95% ^{18}O) was used in the base degradation. The K^{18}OH was prepared from 0.35 mL of a 0.3 M KOH solution that was lyophilized to dryness. The resulting powder was dissolved in 0.025 mL of $H_2{}^{18}O$ (95.8% ^{18}O) and lyophilized. After a second addition of 0.025 mL of $H_2{}^{18}O$ and lyophilization, 0.35 mL of $H_2{}^{18}O$ was added to the sample. The enrichment of the K^{18}OH was determined by the addition of 0.5 μL of freshly distilled thiophosphoryl chloride to 0.05 mL of the K^{18}OH solution. The mixture was heated at 100% for 2 min and then allowed to sit at room temperature for 24 h. Triethylammonium bicarbonate buffer (1 M, pH 7.5, 0.2 mL) was then added, and the sample was evaporated to dryness. Ethanol was added, and the sample was again evaporated to dryness. After ethylation with diazoethane and pyridinium hydrochloride and analysis by GC/MS, the ^{18}O enrichment of the triethyl phosphorothioate was found to be 95%.

Results and Discussion

The mass spectral techniques described in this paper can be used to determine the ^{18}O enrichment of as little as 0.01 μmol of a volatile phosphate derivative. With samples of this size, we found it practical to develop degradations of pyrophosphate and dAMPS that would not be affected by contamination with inorganic phosphate. The acid-catalyzed methanolysis of pyrophosphate yields methylphosphoric acid and phosphoric acid. This mixture can be ethylated with diazoethane and analyzed by GC/MS. The ^{18}O enrichment of diethyl methyl phosphate will not be affected by contamination as any inorganic phosphate will be converted to triethyl phosphate. The ^{18}O enrichment of dAMPS could not be determined via a periodate oxidation followed by a base-catalyzed elimination of inorganic phosphate as was done with AMPS (Midelfort & Sarton-Miller, 1978). However, Pfitzner & Moffat (1965) found that when 2′-deoxythymidine 5′-phosphate is oxidized to 3′-keto-2′-deoxythymidine 5′-phosphate by dicyclohexylcarbodiimide and Me_2SO, inorganic phosphate is released by a spontaneous β elimination. The phosphate is subsequently converted to trimetaphosphate by excess carbodiimide. The analogous release of phosphorothioate in our system would lead to the removal of sulfur (Mikolajczyk, 1966; Eckstein, 1967). After protection of the sulfur via ethylation, however, the diethyl-dAMPS could be oxidized and diethyl phosphorothioate eliminated without the

[4] When the hydrazinolysis product of pdTp$_s$dA was treated with benzaldehyde to convert the sugar hydrazone to the aldehyde, subsequent base elimination yielded dAMP instead of dAMPS. Preliminary results indicate that treatment of pdTp$_s$dA with benzaldehyde removes sulfur from the phosphorothioate diester, yielding pdTpdA. This reaction is being investigated in the synthesis of chiral ^{16}O,^{18}O-phosphate diesters.

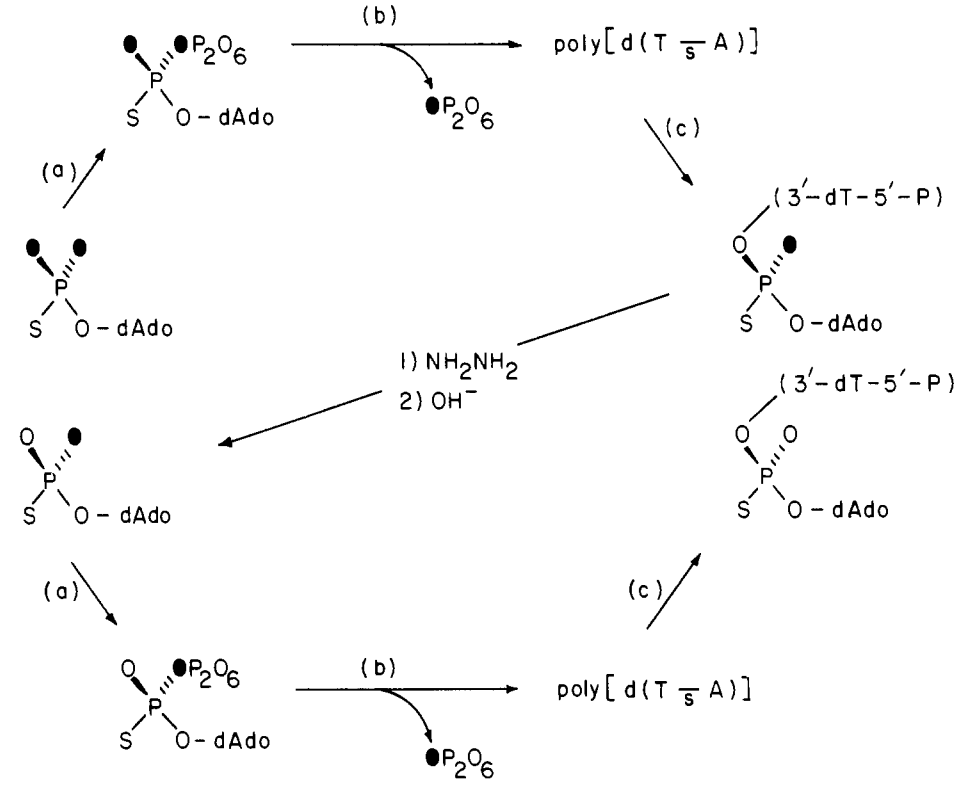

FIGURE 1: Elucidation of the stereochemistry of polymerization by DNA polymerase I. (○) ^{16}O; (●) ^{18}O. (a) Adenylate kinase, pyruvate kinase, ATP, and phosphoenolpyruvate. (b) DNA polymerase, dTTP, and poly[d(A-T)]. (c) DNA polymerase. The figure was drawn assuming that the polymerization catalyzed by DNA polymerase proceeds with inversion of the configuration at P_α of dATPαS.

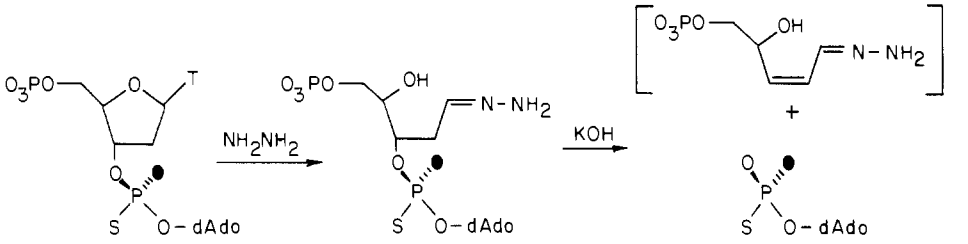

FIGURE 2: Degradation of pdTp$_s$dA to dAMPS.

loss of sulfur or ^{18}O. It was convenient to use the more rapid Me_2SO and sulfur trioxide–pyridine oxidation of Parikh & Doering (1967) rather than the Me_2SO and DCC reaction.

Other workers have determined the ^{18}O content of phosphate esters and whether the ^{18}O is bridging or nonbridging in triphosphates, using high-frequency ^{31}P NMR (Cohn & Hu, 1980, and references found therein; Gerlt & Wan, 1979; Webb & Trentham, 1980). The NMR method requires much larger samples than those needed in this study.

The overall reaction sequence described in this paper is shown in Figure 1. Adenylate kinase phosphorylates dAMPS to give, after phosphorylation to the triphosphate with pyruvate kinase, one diastereomer of dATPαS as indicated by ^{31}P NMR. In analogy with the stereospecific phosphorylation of AMPS by the coupled activities of adenylate kinase and pyruvate kinase (Sheu & Frey, 1977), the dATPαS is assumed to be the *Sp* diastereomer (Burgers & Eckstein, 1978). The absolute configuration of the dATPαS does not affect the conclusions drawn in this paper. Since adenylate kinase phosphorylates dAMPS at a much slower rate than it phosphorylates AMPS, the reaction requires more adenylate kinase, higher concentrations of ATP, and longer reaction times to reach completion.

DNA polymerase I from *E. coli* polymerizes dTTP and the *Sp* diastereomer of dATPαS into a larger polymer that is excluded from both Sephadex G-50 and Sephadex G-200 gels. In analogy with the polymerization of dATP and dTTP (Schachman et al., 1960), this polymer is assumed to be a copolymer with alternating phosphate (3′-*O*-adenosyl-5′-*O*-thymidyl) and phosphorothioate (3′-*O*-thymidyl-5′-*O*-adenosyl) linkages. The polymer is degraded by the nuclease activity of DNA polymerase (Kornberg & Kornberg, 1974) into nucleotide tetramers and dimers that have the structures (pdTp$_s$dA)$_2$ and pdTp$_s$dA, respectively. Upon further treatment with polymerase, the tetramer is quantitatively degraded to the dimer. As the structure of the dimer is firmly based on ^1H and ^{31}P NMR data, this degradation sequence offers evidence that the polymer is composed of pdTp$_s$dA sequences.

No dAMPS is detected in the degradation of either the polymer of the tetramer. Thus, under conditions of these experiments, the nuclease activity does not hydrolyze phosphorothioate diester bonds. This is consistent with the finding of Burgers & Eckstein (1979c) that the polymerase does not degrade poly[d(A$_s$A)]. The ability of the nuclease activity of DNA polymerase to excise dimers and oligomers has been previously demonstrated (Cozzarelli et al., 1969; Kelly et al., 1969).

The thymidine moiety of pdTp$_s$dA is degraded to a sugar hydrazone by the action of anhydrous hydrazine as shown in Figure 2. A base-catalyzed elimination then releases dAMPS.

Table I: ^{18}O Enrichment of dAMPS,$^{18}O_2$ and the Products of Its Polymerization and Degradation[a]

compound	origin of compound	mass % ^{18}O[b]
dAMPS,$^{18}O_2$	starting material	92 ± 1
pyrophosphate	first polymerization	45 ± 1.5
dAMPS,^{18}O	first hydrazine degradation	46 ± 1
pyrophosphate	second polymerization	45 ± 1.5
dAMPS	second hydrazine degradation	1.5 ± 1

[a] All enrichments were corrected for the natural abundances of ^{34}S and ^{18}O. The synthesis of dAMPS,$^{18}O_2$ allows for the incorporation of ^{18}O into two oxygens of the phosphorothioate. The percent ^{18}O enrichment of these two oxygens in the triethyl phosphorothioate obtained from the degradation of dAMPS samples is then calculated from $[(m + 2) + 2(m + 4)]/[2[m + (m + 2) + (m + 4)]]$. The polymerization of dTTP and dATP catalyzed by DNA polymerase yields an alternating poly[d(A-T)] copolymer (Schachman et al., 1960). For every molecule of pyrophosphate obtained from dATPαS,α^{18}O, a pyrophosphate molecule is obtained from dTTP. In the analysis, these two pyrophosphates are degraded to phosphates. The ^{18}O enrichment of the α–β bridging oxygen of dATPαS,α^{18}O is therefore obtained by multiplying the phosphate enrichment by four. The enrichment of this bridging oxygen is expressed relative to the original enrichment of the two oxygens of dAMPS,$^{18}O_2$ and is calculated by multiplying the previous equation by 4 and setting $(m + 4) = 0$. The percent enrichment is thus, at most, half the enrichment of dAMPS,$^{18}O_2$. [b] Errors are expressed as ±1 standard deviation.

Hydrazinolysis followed by base treatment has been used previously to degrade DNA into purine sequences (Habermann, 1963; Chargaff et al., 1963; Sedat & Sinsheimer, 1964; Türler & Chargaff, 1969; Türler et al., 1969), and limited hydrazinolysis has been used in sequencing DNA (Maxam & Gilbert, 1977). Other workers, however, have questioned the use of this method for the quantitative determination of purine sequences because some degradation of adenosine occurs in the hydrazinolysis and nonnucleotide organic phosphates are produced in the base elimination (Cape & Spencer, 1968; Cashmore & Petersen, 1969; Jones et al., 1968; Brammer et al., 1968). The hydrazone that is produced from thymidylic acid and hydrazine has been characterized by Temperli et al. (1964). However, the base-elimination step, while quite plausible, has not been investigated. In particular, the conjugated ribose residue that presumably results from the elimination has not been isolated. Our conclusions require that the configuration of the phosphorothioate remain unchanged during the degradation of pdTp$_s$dA to dAMPS. The only mechanism by which the phosphorothioate stereochemistry can be altered and dAMPS produced is for a solvent molecule to attack the phosphorus of the hydrazinolysis product of pdTp$_s$dA. Therefore, if the degradation of unenriched pdTp$_s$dA by hydrazine and K^{18}OH yields dAMPS that does not contain ^{18}O, then the configuration of the phosphorothioate has not been inverted in the reaction. In fact, when base hydrolysis is done in 0.3 M KOH that is 95% enriched in ^{18}O, only 0.5 ± 0.5% of the dAMPS molecules contain ^{18}O.

Figure 1 shows the distribution of ^{18}O into the products of the reaction sequence that would occur if the DNA polymerization reaction proceeds with inversion of configuration about the α-phosphorus of dATPαS. The *pro-R* ^{18}O of the starting dAMPS,$^{18}O_2$ is not phosphorylated by the stereospecific action of adenylate kinase (Sheu & Frey, 1977) and so will be in the dAMPS,^{18}O that is isolated after the first polymerization and degradation steps. Since the configuration about the phosphorus is now inverted, this ^{18}O is now in the *pro-S* position and will be phosphorylated by adenylate kinase. The pyrophosphate from the second polymerization will then contain ^{18}O. If the polymerization proceeds via retention of configuration, this pyrophosphate will not be enriched, and the ^{18}O will be in pdTp$_s$dA. The results shown in Table I clearly show that the reaction proceeds with inversion of the phosphorothioate's configuration. The enrichments of the pyrophosphate molecules that are expelled during the first and second polymerizations are the same while the enrichment of pdTp$_s$dA isolated from the second polymerization is almost zero.[5]

The calculation of the pyrophosphate enrichment is based on the assumption that DNA polymerase synthesizes an alternating copolymer, incorporating equal amounts of (*Sp*)-dATPαS and dTTP. When this assumption is used, all the ^{18}O of the starting dAMPS,$^{18}O_2$ can, within error, be accounted for in the pyrophosphate and the pdTp$_s$dA.

The results described in the text show that nucleotidyl transfer catalyzed by DNA polymerase I proceeds with inversion of the configuration about the α phosphorus of the *Sp* diastereomer of dATPαS. This confirms the determination of Burgers & Eckstein (1979c) who used kinetic correlations based on the stereoselectivity of snake venom phosphodiesterase to establish the reaction's stereochemistry. The simplest interpretation of this result is that nucleotidyl transfer occurs directly between the donor and acceptor substrates in the enzyme's active site and not via a covalent enzyme intermediate.

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[5] The small amount of ^{18}O that was found in the dAMPS from the second polymerization and hydrazine degradation is most likely an artifact due to the similar GC retention times of the product and an impurity having a mass spectral fragment at m/e 200. This hypothesis is supported by the fact that, unlike the other GC/MS runs where the ^{18}O enrichments were constant over the GC peak, the apparent percentage of ^{18}O in this experiment decreased across the peak. This variation in mass ratios across the GC peak caused the large relative standard deviation for the ^{18}O enrichment of dAMPS.

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