Syntheses and Configurational Analyses of Thymidine 4-Nitrophenyl [^{17}O,^{18}O]Phosphates and the Stereochemical Course of a Reaction Catalyzed by Bovine Pancreatic Deoxyribonuclease I†

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ABSTRACT: The syntheses of both diastereomers of thymidine 3′-(4-nitrophenyl [^{17}O,^{18}O]phosphate) ([^{17}O,^{18}O]TpNP) and of thymidine 5′-(4-nitrophenyl [^{17}O,^{18}O]phosphate) ([^{17}O,^{18}O]NPpT) and the R_P diastereomer of thymidine 3′-(4-nitrophenyl [^{17}O,^{18}O]phosphate) 5′-(4-nitrophenyl phosphate) ([^{17}O,^{18}O]NPpTpNP) are described. The absolute configurations of the chiral phosphorus atoms have been determined unambiguously by ^{31}P and ^{17}O NMR spectroscopy. The R_P diastereomer of [^{17}O,^{18}O]NPpTpNP was hydrolyzed in the presence of bovine pancreatic deoxyribonuclease I to yield the R_P diastereomer of thymidine 3′-[^{16}O,^{17}O,^{18}O]-phosphate 5′-(4-nitrophenyl phosphate), demonstrating that the enzyme-catalyzed reaction occurs by a mechanism in which a single displacement reaction occurs at the chiral phosphorus atom.

Perhaps the most fundamental mechanistic question that can be asked about the reactions catalyzed by phosphoryl- and nucleotidyltransferases is whether catalysis involves the necessary formation of covalent adduct between an active site functional group and the phosphoryl group of the substrate. The pioneering work of Usher and Eckstein on the reaction catalyzed by pancreatic ribonuclease A (Usher et al., 1970) demonstrated the considerable utility of stereochemical studies in probing the nature of the displacement reactions catalyzed by phosphotransferases. Whereas these experiments were used to investigate whether ligand reorganization processes involving potential pentacoordinate intermediates were involved in the reaction catalyzed by ribonuclease, subsequent studies in a number of laboratories have used stereochemical studies to ascertain the number of nucleophilic displacement reactions that occur during the conversion of substrate to product: an inversion of configuration at the chiral phosphorus atom indicating a single displacement reaction, which can be most simply interpreted as the direct transfer of the phosphoryl group from the donor group in the substrate to the acceptor molecule, and a retention of configuration indicating two displacement reactions, which can be most simply interpreted as the necessary formation of a phosphorylated enzyme intermediate during the transfer reaction. These studies have been the subject of a number of recent reviews (e.g., Eckstein, 1983; Gerlt et al., 1983).

Because no naturally occurring phosphate ester contains an asymmetric phosphorus atom, the chirality necessary for stereochemical studies has been introduced by two types of substitution of the nonesterified phosphoryl oxygens. Replacement of an oxygen with a sulfur generates a diastereotopic phosphorus atom in the case of phosphodiesters and the internal anhydrides of nucleoside polyphosphates; in monoesters and the terminal anhydrides of nucleoside polyphosphates, such substitution generates a prochiral phosphorus atom which can be made diastereotopic by further substitution of the remaining phosphoryl oxygens with ^{17}O and/or ^{18}O, the two stable heavy isotopes of oxygen. Alternatively, the required substitutions can be accomplished solely with the heavy isotopes of oxygen.

The use of phosphorothioates for stereochemical studies often is the technically easier approach, since the diastereomers of phosphorothioate diesters frequently can be separated by chromatographic procedures and the prochiral oxygens of some phosphorothioate monoesters can be distinguished enzymatically. However, phosphorothioates are processed at rates significantly lower than the analogous phosphates, and this rate difference caused a concern that stereochemical studies employing phosphorothioates may not give reliable mechanistic information; this concern, now known to be unfounded given the comparative studies that have been performed on a number of enzymes, provided the incentive to develop methodology for the syntheses and configurational analyses of phosphate esters chiral by oxygen isotope substitution.

In this paper we describe in detail the syntheses and configurational analyses of three acyclic phosphate diesters that are chiral at phosphorus due to substitution of the phosphoryl oxygens with ^{17}O and ^{18}O: thymidine 3′-(4-nitrophenyl [^{17}O,^{18}O]phosphate) ([^{17}O,^{18}O]TpNP), thymidine 5′-(4-nitrophenyl [^{17}O,^{18}O]phosphate) ([^{17}O,^{18}O]NPpT) and thymidine 3′-(4-nitrophenyl [^{17}O,^{18}O]phosphate) 5′-(4-nitrophenyl phosphate) ([^{17}O,^{18}O]NPpTpNP). These esters were chosen since the unlabeled esters had been shown to be substrates for a number of phosphodiesterases and nucleases. Previous reports from this laboratory have described the use of [^{17}O,^{18}O]NPpT to determine the stereochemical consequences of the reactions catalyzed by snake venom phosphodiesterase (Mehdi & Gerlt, 1981a) and Staphylococcal nuclease (Mehdi & Gerlt, 1982) and of [^{17}O,^{18}O]TpNP to determine the stereochemical consequence of the reaction catalyzed by bovine spleen exonuclease (Mehdi & Gerlt, 1981b). In this paper we also describe a stereochemical study of the hydrolysis of [^{17}O,^{18}O]NPpTpNP catalyzed by bovine pancreatic deoxyribonuclease I (DNase I); the hydrolysis of this substrate is catalyzed by the Ca^{2+}- and Mn^{2+}-dependent enzyme with inversion of configuration at phosphorus.

Materials and Methods

$H_2{}^{17}O$ (13% ^{16}O, 52% ^{17}O, and 35% ^{18}O) was purchased from Monsanto Research Corp., and $C^{18}O_2$ (99% ^{18}O) was the product of Prochem. Unlabeled TpNP and NPpT were obtained from Sigma, and unlabeled NPpTpNP was from Ash

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Stevens. Staphylococcal nuclease was the product of Worthington, and DNase I from bovine pancrease was obtained from Boehringer-Mannheim. All other chemicals were the best grades commercially available.

NMR Measurements. Phosphorus-31 NMR spectra at 32 MHz were obtained with a Varian CFT-20 NMR spectrometer equipped with a phosphorus probe. Phosphorus-31 NMR spectra at 81 MHz were obtained with a Varian XL-200 NMR spectrometer located at Wesleyan University and with the generous cooperation of Professor Philip H. Bolton. Phosphorus-31 NMR spectra at 145.7 MHz were obtained with the Bruker WH-360 NMR spectrometer located in the NIH Middle Atlantic High Resolution NMR Facility at the University of Pennsylvania. Phosphorus-31 NMR chemical shifts are measured relative to an external standard of 85% H_3PO_4, with positive values being downfield of the reference.

Proton NMR spectra at 270 MHz were obtained with the Bruker HX-270 spectrometer located in the NSF Northeast NMR Facility at Yale University. The same instrument was used to obtain ^{17}O NMR spectra at 36.6 MHz and 95 °C; for these experiments a tunable probe equipped for ^{31}P decoupling was employed (Coderre et al., 1981a). Oxygen-17 NMR chemical shifts are measured relative to the natural abundance ^{17}O in the 20% D_2O used as the solvent (0.57 ppm upfield from natural abundance ^{17}O in H_2O).

All glassware used in the preparation of samples for high-resolution ^{31}P NMR was soaked in a 1:1 mixture of concentrated nitric and sulfuric acids, rinsed with deionized water, and dried. The samples for high-resolution ^{31}P NMR were dissolved in a mixture of H_2O and D_2O and percolated through a small column of Chelex-100 (Na^+) contained in a Pasteur pipet plugged with a glass-fiber filter disk (Whatman 934-AH) into an NMR tube. The resin was washed with additional H_2O and D_2O, and 0.4 mL of a 0.1 M solution of ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) was filtered through a glass fiber filter into the NMR tube. The total sample volume was approximately 2.5 mL.

Samples for ^{17}O NMR were dissolved in D_2O, shaken with Chelex-100 (Na^+), and centrifuged. The centrifugate was transferred to an NMR tube, and the resin was washed with additional solvent and transferred to the NMR tube. The total sample volume was approximately 2.5 mL, and the sample concentration was approximately 30 mM.

Syntheses. (1) 4-Nitrophenyl-N-phenyl[^{17}O]phosphoramidic Chloride. Phosphorus [^{17}O]oxychloride ([^{17}O]$POCl_3$) was prepared by a literature procedure (Abbott et al., 1979). The isotopic composition was determined by the addition of 50 μL of [^{17}O]$POCl_3$ to 5 mL of methanol followed by evaporation of excess methanol and gas chromatography/mass spectrometry of the residue of trimethyl phosphate. The isotopic composition of the [^{17}O]$POCl_3$ used for the syntheses of [^{17}O,^{18}O]TpNP and [^{17}O,^{18}O]NPpTpNP was 19% ^{16}O, 49% ^{17}O, and 32% ^{18}O; the composition of the [^{17}O]$POCl_3$ used for the synthesis of [^{17}O,^{18}O]NPpT was 16% ^{16}O, 51% ^{17}O, and 33% ^{18}O.

A mixture of [^{17}O]$POCl_3$ (4.2 g, 27 mmol), dry 4-nitrophenol (3.5 g, 25 mmol), and $AlCl_3$ (0.1 g, catalyst) was refluxed under nitrogen until HCl evolution ceased (Zielinski & Lesnikowski, 1976). 4-Nitrophenyl [^{17}O]phosphorodichloridate was isolated by distillation at 0.05 mmHg using a short-path condenser: yield, 46% (3.2 g, 12.5 mmol); bp 130–150 °C. A solution of freshly distilled aniline (2.3 g, 24.6 mmol) in dry benzene (8 mL) was added dropwise to a refluxing solution of 4-nitrophenyl [^{17}O]phosphorodichloridate (3.15 g, 12.3 mmol) in dry benzene (20 mL). After the addition was completed, the reaction was refluxed for 2 h and then allowed to cool to room temperature; the precipitated anilinium hydrochloride was removed by filtration and washed with benzene. The filtrate and washings were reduced in volume, and the concentrated solution was stored overnight at 4 °C. The pale yellow crystals of 4-nitrophenyl-*N*-phenyl[^{17}O]phosphoramidic chloride were collected by filtration and washed with cyclohexane: yield 95% (3.0 g, 11.7 mmol, two crops); mp 124–126 °C; ^{31}P NMR ($CDCl_3$) +0.5 ppm.

(2) Thymidine 3'-(4-Nitrophenyl [^{17}O,^{18}O]Phosphates). Dry pyridine (40 mL) was distilled into a flask containing 4-nitrophenyl-*N*-phenyl[^{17}O]phosphoramidic chloride (1.9 g, 6.1 mmol) and 5'-(monomethoxytrityl)thymidine (2.9 g, 6.1 mmol) prepared according to the literature procedure (Schaller et al., 1963). The reaction mixture was stirred at room temperature for 36 h, and the reaction was quenched by the addition of aqueous sodium acetate (1 g in 75 mL of H_2O). The product was extracted into CH_2Cl_2, and after being dried with $MgSO_4$, the solvent was removed. The residue was solidified by dropwise addition of a concentrated solution in CH_2Cl_2 to vigorously stirred cyclohexane (600 mL). The pale orange solid was collected by suction filtration: yield 78% (3.9 g, 4.8 mmol); ^{31}P NMR ($CDCl_3$) −5.79 and −6.02 ppm of approximately equal intensity.

The diastereomers (epimers at phosphorus) were separated by chromatography on silica gel. A slurry of 180 g of silica gel 60H (TLC grade; Merck) in 25:1 CH_2Cl_2/2-propanol was allowed to settle on a bed of sand in a 3 × 90 cm jacketed glass column maintained at 4 °C; gentle pressure was applied with a small aquarium air pump. The mixture of diastereomers (1.5 g in each separation) was dissolved in a small volume of the column solvent and applied to the column. The column was eluted under pressure provided by the aquarium pump, and fractions of 4–5 mL were collected. TLC on silica of the fractions showed that the diastereomers were cleanly separated. Appropriate fractions were combined and evaporated to yield the separated diastereomers: recovery 83% (from 3.0 g of mixture, 1.3 g of the high R_f diastereomer and 1.2 g of the low R_f diastereomer); ^{31}P NMR ($CDCl_3$) high R_f diastereomer −5.9 ppm, low R_f diastereomer −5.5 ppm. The high R_f diastereomer has the S_P configuration, and the low R_f diastereomer has the R_P configuration (Gerlt et al., 1980).

Each dried *N*-phenyl[^{17}O]phosphoramidate (0.41 g, 0.5 mmol) was dissolved in dry pyridine (2.0 mL) in a thick-walled flask fitted with a side arm and a vacuum stopcock (Ace Glass, 7412-07). Sodium hydride (72 mg of a 50% dispersion in oil, 1.5 mmol) in dry pyridine (2 mL) was added, and the dark yellow reaction mixture was allowed to stir under dry nitrogen for 20 min. The reaction mixture was then frozen in liquid nitrogen, and the flask was attached to a vacuum line via the side arm. $C^{18}O_2$ (5 mmol) was dispensed from a storage bulb on the vacuum line and condensed into the reaction flask. The flask was sealed, removed from the vacuum line, and allowed to thaw to room temperature behind a protective shield. After the contents of the flask were stirred for 4 h, the reaction mixture was frozen in liquid nitrogen, attached to the vacuum line, and allowed to thaw to −37 °C in a dry ice/anisole bath. The unreacted $C^{18}O_2$ was transferred to a second storage bulb on the vacuum line. Excess sodium hydride was destroyed by the addition of dilute acetic acid, and the yellow solution was transferred to a round-bottom flask and evaporated to dryness. The orange residue was dissolved in 25 mL of 80% acetic acid, and the reaction mixture was stirred at 60 °C until removal of the trityl group was completed; the acetic acid was removed

by rotary evaporation. An aqueous solution of the residue was extracted with CH_2Cl_2, and the water was evaporated to yield a yellow glassy solid which was purified by one of the following two chromatographic procedures: (1) The residue was dissolved in water (10 mL) and applied to a column (3 × 30 cm) of Amberlite XAD-2 which had been washed previously with both water and methanol. The column was first eluted with water (200 mL) and then with a linear gradient (1.0 L total) of 50% methanol in water to pure methanol. The fractions containing the product were evaporated to yield the labeled thymidine 3′-(4-nitrophenyl phosphate). (2) The residue was dissolved in water (10 mL), the pH was adjusted to 8 with ammonium hydroxide, and the solution was applied to a column of DEAE-Sephadex A-25 (2.5 × 40 cm). The column was eluted with a linear gradient (1.8 L total) of water to 0.4 M triethylammonium bicarbonate, pH 7.5: yield 90% (450 μmol based on ϵ_{270} = 16 250 M^{-1} cm^{-1}); ^{31}P NMR (D_2O, pH 7.0) −6.3 ppm, doublet with J_{HP} = 8.0 Hz with ^1H coupling; ^1H NMR [Na^+ salt in dimethyl sulfoxide-d_6 (Me_2SO-d_6)], identical with that of commercially obtained material.

(3) *Thymidine 5′-(4-Nitrophenyl [^{17}O,^{18}O]Phosphates).* 3′-(Monomethoxytrityl)thymidine was prepared by a modification of literature procedures (Ogilvie & Letsinger, 1967; Arentzen & Reese, 1971). (4-Chlorophenoxy)acetyl chloride (4.3 mL, 27 mmol) in dry CH_3CN (5 mL) was added dropwise to a suspension of thymidine (6.0 g, 25 mmol) in dry CH_3CN (35 mL) and dry 2,6-lutidine (20 mL). After being stirred at room temperature for 24 h, the reaction mixture was filtered; the residue was washed with CH_3CN, $CHCl_3$, and diethyl ether and then crystallized from ethanol to afford 5′-*O*-[(4-chlorophenoxy)acetyl]thymidine: yield 40% (3.8 g, 10 mmol). The 5′-protected thymidine and monomethoxytrityl chloride (3.2 g, 10. 1 mmol) were dissolved in dry pyridine (60 mL). The reaction mixture was stirred at 95 °C for 8 h and, after cooling, was quenched by the addition of 10 mL of methanol. After the solvent was removed, the residue was dissolved in 25 mL of 10% diethylamine in methanol, and the reaction was stirred at room temperature for 12 h. The solvent was evaporated, and the residue was chromatographed on a column of alumina (400 g deactivated with 40 mL of water). The column was washed with CH_2Cl_2 (400 mL), and the product was eluted with 5% methanol in CH_2Cl_2 (1 L). Further purification was accomplished by washing a solution of the product in CH_2Cl_2 with a 10% sodium bicarbonate solution followed by precipitation with cyclohexane. The yields of this preparation of 3′-(monomethoxytrityl)thymidine were variable.

Phosphorylation of 3′-(monomethoxytrityl)thymidine (3.3 g, 7 mmol) with 4-nitrophenyl-*N*-phenyl[^{17}O]phosphoramidic chloride (2.50 g, 8 mmol) and isolation of the diastereomeric mixture of 3′-(monomethoxytrityl)thymidine 5′-(4-nitrophenyl *N*-phenyl[^{17}O]phosphoramidate) were performed as previously described for the isomeric 3′-phosphoramidates: yield 75% (4.25 g, 5.1 mmol); ^{31}P NMR ($CDCl_3$) −5.11 and −5.33 ppm of approximately equal intensity.

This mixture of diastereomers was not as easily separated as was the mixture of isomeric 3′-phosphoramidates; only 1.0 g of the mixture could be applied to the previously described column of silica gel 60H, and this chromatography did not cleanly resolve the diastereomers. Fractions containing the pure diastereomers were combined and evaporated; fractions containing mixed diastereomers were rechromatographed: recovery 80% (from 4.25 g of mixture, 1.96 g of the high R_f diastereomer, and 1.36 g of the low R_f diastereomer); ^{31}P NMR ($CDCl_3$) high R_f diastereomer −4.3 ppm, low R_f diastereomer −4.7 ppm. The high R_f diastereomer has the S_P configuration, and the low R_f diastereomer has the R_P configuration (Gerlt et al., 1980).

Each dried *N*-phenyl[^{17}O]phosphoramidate was reacted with $C^{18}O_2$, detritylated, and purified according to the procedures described for the isomeric 3′-phosphates: yield 90% (450 μmol based on ϵ_{271} = 15 600 M^{-1} cm^{-1}); ^{31}P NMR (D_2O, pH 7.0) −5.71 ppm, unresoved triplet of doublets with ^1H coupling; ^1H NMR (Na^+ salt in Me_2SO-d_6) identical with that of commercially obtained material.

(4) *Thymidine 3′-(4-Nitrophenyl [^{17}O,^{18}O]Phosphate) 5′-(4-Nitrophenyl Phosphate).* The S_P diastereomer of 5′-(monomethoxytrityl)thymidine 3′-(4-nitrophenyl *N*-phenyl-[^{17}O]phosphoramidate) (0.5 g, 0.6 mmol) was detritylated by stirring with 80% acetic acid at room temperature for 11 h. The acetic acid was removed by evaporation. The residue was chromatographed on a column (3 × 30 cm) of silica gel 60 with 10% methanol in CH_2Cl_2 as the eluent. The fractions containing product were pooled and evaporated. Unlabeled 4-nitrophenyl *N*-phenylphosphoramidic chloride was added to the flask, and dry pyridine (15 mL) was added. After 10 h at room temperature, the reaction was quenched by the addition of aqueous sodium acetate (0.5 g in 10 mL of water). The solvent was removed by evaporation. The residue was dissolved in CH_2Cl_2 and washed with water, and the organic layer was evaporated to give an orange solid. The solid was dissolved in dry dioxane and transferred to the thick wall flask used for the $NaH/C^{18}O_2$ reaction. The solvent was removed by lyophilization, and the residue was dried by repeated lyophilization with dioxane. Following dissolution in pyridine, the bis(*N*-phenylphosphoramidate) was reacted with 10 mmol of $C^{18}O_2$ as previously described. The product was isolated by chromatography on DEAE-Sephadex A-25 using a linear gradient of water to 0.5 M triethylammonium bicarbonate as the eluent: yield 56% (335 μmol based on ϵ_{274} = 23 600 M^{-1} cm^{-1}); ^{31}P NMR (D_2O, pH 7.0) −5.99 ppm for the 5′-phosphate and −6.53 and −6.56 ppm for the 3′-phosphate, with the three signals having an intensity ratio of 100:26:40; ^1H NMR (Na^+ salt in Me_2SO-d_6) identical with that of commercially obtained material. The bis-ester product is racemically labeled with ^{16}O and ^{18}O at the 5′-phosphorus and chirally labeled with ^{17}O and ^{18}O at the 3′-phosphorus; discussion of this material in the text will note only the stereochemically pertinent labeling at the 3′-phosphorus.

Configurational Analyses. (1) [^{17}O,^{18}O]TpNP and [^{17}O,^{18}O]NPpT by Hydrogenolysis, Cyclization, Methylation, and ^{31}P NMR Spectroscopy. The labeled 4-nitrophenyl ester (100 μmol) was dissolved in anhydrous ethanol (25 mL) in a thick-walled bottle. Concentrated HCl (100 μL) and Adams's catalyst (20 mg) were added, and hydrogenation was carried out at 55 psi. Additional catalyst was added if the rate of the reaction subsided, and the reactions were typically completed in 8 h. The catalyst was removed by filtration, and the solvent was evaporated. Labeled thymidine 3′- or 5′-phosphate (Tp or pT) was obtained in quantitative yield following chromatography on DEAE-Sephadex A-25 using a linear gradient of water to 0.4 M triethylammonium bicarbonate as the eluent.

The labeled Tp or pT (100 μmol) was converted to the pyridinium salt by percolation through a small column of Dowex 50 (pyridinium). Following evaporation of the solvent, the salt was suspended in methanol (5 mL), 1.1 equiv of tri-*n*-octylamine was added, and the mixture was warmed until dissolution was complete. The methanol was evaporated, and the residue was dried overnight under vacuum. The residue

was further dried by repeated evaporation with dry dioxane and was finally dissolved in 1.5 mL of dry dioxane containing a few drops of dry dimethylformamide (DMF) to clarify the solution. A few freshly activated 4-Å molecular sieves were added, and the solution was stirred in a glove bag with a dry argon atmosphere for 4 h. Freshly distilled diphenyl phosphorochloridate (19 μL, 0.9 equiv) and dry tri-*n*-butylamine (25 μL, 1.0 equiv) were added with syringes, and the reaction was stirred for 30 min. Potassium *tert*-butoxide (0.15 g) in dry DMF (20 mL) was added, and the reaction was stirred for an additional 10 min. The reaction was removed from the glove bag and quenched by the addition of 20 mL of wet Amberlite IR-120 (pyridinium). The solids were removed by filtration and thoroughly washed with water. The combined filtrate and washings were evaporated, and an aqueous solution of the residue was extracted with diethyl ether. The solution containing labeled thymidine cyclic 3′,5′-phosphate (cyclic TMP) was applied to a DEAE-Sephadex A-25 column, and the cyclic diester was eluted with a linear gradient of water to 0.4 M triethylammonium bicarbonate: yield of cyclic TMP 40%: (40 μmol); ^{31}P NMR (D_2O, pH 7.0) −2.2 ppm (doublet with apparent J_{HP} = 20.0 Hz with ^{1}H coupling).

The isolated triethylammonium salt of labeled cyclic TMP was dissolved in methanol (5 mL). Concentrated HCl (5 μL) was added followed by a solution of diazomethane in diethyl ether (0.5 M) until the yellow color persisted. The addition of HCl and diazomethane was repeated two more times before the solvent was evaporated. Phosphorus-31 NMR analysis (D_2O, pH 7.0) of the residue obtained from unlabeled cyclic TMP showed three peaks in an intensity ratio of approximately 2:1:1 at −2.2 (cyclic TMP), −2.7 (equatorial methyl ester), and −3.5 ppm (axial methyl ester).

(2) [^{17}O,^{18}O]TpNP and [^{17}O,^{18}O]NPpT by Cyclization and ^{17}O NMR Spectroscopy. The labeled 4-nitrophenyl ester (100 μmol) was dried by repeated evaporation from dry DMF. Potassium *tert*-butoxide (0.14 g) in dry Me_2SO (10 mL) was added (Borden & Smith, 1966). After the solution was stirred at room temperature (10 min for TpNP and 2 h for NPpT), the reactions were quenched by the addition of Amberlite IR-120 (pyridinium), and the labeled cyclic TMPs were purified as described in the previous section. The samples were then used for analysis by ^{17}O NMR spectroscopy.

(3) Methylation of [^{17}O,^{18}O]TpNP. The triethylammonium salt of the labeled 4-nitrophenyl ester (60 μmol) was methylated by a procedure analogous to that used to methylate cyclic TMP except that excess HCl was neutralized by the addition of triethylammonium bicarbonate 10 min after the final addition of diazomethane. ^{31}P NMR analysis (2:1 methanol/D_2O) of unlabeled material showed three peaks in an intensity ratio of approximately 2:1:1 at −6.8 (TpNP), −7.35 (methyl ester of the *R* configuration), and −7.38 ppm (methyl ester of the *S* configuration).

(4) [^{17}O,^{18}O]NPpTpNP. [^{17}O,^{18}O]NPpTpNP (70 μmol) was incubated with 40 000 units of Staphylococcal nuclease at 45 °C in 10 mL of 0.05 M sodium borate buffer, pH 8.8, containing 10 mM $CaCl_2$. The removal of the 5′-(4-nitrophenyl phosphoryl) group was complete in 4 h as judged by TLC. The reaction mixture was washed with diethyl ether, and the [^{17}O,^{18}O]TpNP was isolated by chromatography on DEAE-Sephadex A-25 followed by purification on Amberlite XAD-2. The chiral diester was methylated as described in the previous section, and the methyl esters were analyzed by ^{31}P NMR spectroscopy.

Hydrolysis of [^{17}O,^{18}O]NPpTpNP by DNase I. The reaction mixture (12.5 mL) contained the R_P diastereomer of [^{17}O,^{18}O]NPpTpNP (16 mM), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (25 mM, pH 7.2), $CaCl_2$ (1 mM), $MnCl_2$ (10 mM), and DNase I (5 mg, 2144 Kunitz units). The release of 4-nitrophenolate was monitored spectrophotometrically, and the hydrolysis was judged to be approximately 70% complete in 40 h. 4-Nitrophenol was extracted from the reaction mixture with diethyl ether, and the solution was applied to a DEAE-Sephadex A-25 column and eluted with a linear gradient of water to 0.7 M triethylammonium bicarbonate. The substrate ([^{17}O,^{18}O]NPpTpNP) and product ([^{16}O,^{17}O,^{18}O]NPpTp) coeluted from this column. Fractions containing the nucleotides were combined and evaporated. Analysis by high-performance liquid chromatography (HPLC) (anion-exchange column eluted isocratically with 0.1 M phosphate buffer, pH 3.3, at 1 mL/min: NPpTpNP at 9.5 min and NPpTp at 13.3 min) showed that the substrate and product (192 μmol total) were present in a ratio of 1:2.

The mixture of NPpTp and NPpTpNP was incubated with Staphylococcal nuclease in a reaction mixture (192 mL) which contained 1 mM total nucleotide, sodium borate buffer (pH 8.8, 0.05 M), $CaCl_2$ (10 mM), and Staphylococcal nuclease (51 300 units). The nuclease catalyzed reaction was followed by HPLC (anion-exchange column as previously described). No NPpTpNP was detected after 4 h, and measurement of the absorbance at 400 nm revealed that only 2–3% of 4-nitrophenolate was formed. The solution was extracted with ether, and the solvent was removed. The residue was evaporated several times from methanol, dissolved in water, and chromatographed on a column of Dowex 1-X2-400 (bicarbonate) (2 × 30 cm) by using a linear gradient of water to 0.5 M triethylammonium bicarbonate as eluent. The recovered [^{16}O,^{17}O,^{18}O]Tp was further purified by chromatography on DEAE-Sephadex A-25. The Tp so isolated (114 μmol) was converted to the tri-*n*-octylammonium salt, activated, and cyclized as previously described.

The cyclic TMP obtained from the cyclization (43 μmol) was converted to the potassium salt with Dowex 50 (K^+) and then to a mixture of equatorial and axial methyl esters by using the reaction described by Lowe and his co-workers (Jarvest et al., 1981). The product-containing solution (in 0.8 mL of Me_2SO) was filtered into an NMR tube, and the reaction flask was rinsed with a mixture of dry Me_2SO and Me_2SO-d_6; the total sample volume was 2.5 mL. In Me_2SO the ^{31}P NMR chemical shift of equatorial methyl ester is −1.86 ppm and that of the axial methyl ester is −3.23 ppm.

Results and Discussion

The stereospecific incorporation of ^{18}O to form the ^{17}O,^{18}O chiral phosphodiesters was accomplished with the Wittig–Staudinger reaction in which a carbonyl-containing compound is reacted with the N anion of an *N*-phenylphosphoramidate. Stec very recently reviewed the elegant studies performed in his laboratory which demonstrated that *N*-phenylphosphoramidates can be stereospecifically converted to phosphorothioates and phosphoroselenates with retention of configuration at phosphorus (Stec, 1983). In subsequent simultaneous and independent studies Stec's laboratory (Baraniak et al., 1980) and our own (Gerlt & Coderre, 1980) found that oxygen chiral cyclic phosphodiesters could be prepared analogously with retention of configuration at phosphorus. In our studies we have employed $C^{18}O_2$ as the carbonyl compound since it is commercially available in high isotopic enrichment (99%); Stec's work utilized [^{18}O]benzaldehyde. Both the mass spectral method of configurational analysis used by Stec and our less precise but more convenient method based on ^{18}O perturbations

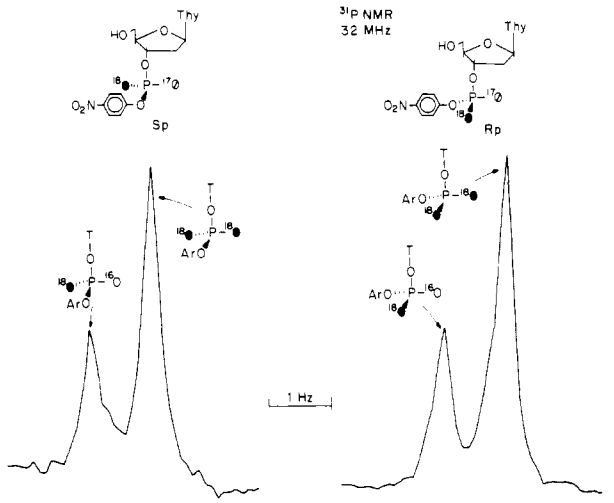


FIGURE 1: Phosphorus-31 NMR spectra at 32 MHz of the diastereomers of [^{17}O,^{18}O]TpNP.

of ^{31}P NMR chemical shifts demonstrated that oxygen chiral cyclic phosphodiesters are formed with a high degree of stereospecificity from diastereomerically pure *N*-phenylphosphoramidates; Stec's group reported the stereospecificity to be about 95%.

Phosphorus-31 NMR spectra at 32 MHZ of the diastereomers of [^{17}O^{18}O]TpNP are shown in Figure 1. Resonances associated with molecules containing ^{17}O are not seen in these spectra due to the extensive line broadening of the associated ^{31}P nucleus caused by scalar relaxation of the second kind (Tsai, 1979); ^{17}O is a quadrupolar nucleus with a spin of $^5/_2$. The smaller downfield resonance in each spectrum corresponds to material having an ^{16}O in the position designated by ^{17}O in the structural formulas and ^{18}O in the position designated by ^{18}O; the more intense upfield resonance corresponds to material having an ^{18}O in the position designated ^{17}O and ^{18}O in the position designated by ^{18}O. The difference in chemical shifts between these two resonances, 0.020 ppm, is due to the increased magnitude of the ^{18}O perturbation on the ^{31}P NMR chemical shift caused by the presence of additional bonding to ^{18}O (Lowe et al., 1979; Cohn & Hu, 1980). Addition of unlabeled esters to these samples resulted in the appearance of a third resonance 0.020 ppm downfield of the less intense resonance. The relative magnitudes of the resonances shown in the figure are consistent with the isotopic composition of the [^{17}O]$POCl_3$ used in the synthesis. Analogous spectra and conclusions regarding isotopic composition were obtained for the diastereomers of [^{17}O,^{18}O]NPpT.

Configurational Analyses. Although the stereochemical course of the Wittig–Staudinger reaction in the preparation of oxygen chiral cyclic phosphodiesters was demonstrated to be with retention of configuration, with evidence for only a small contribution by a pathway accompanied by inversion of configuration, we deemed it necessary to ascertain the stereochemical purity of our oxygen chiral acyclic phosphodiesters before they were used in stereochemical studies of enzyme-catalyzed reactions. This was accomplished by three procedures.

The first method is based upon the observation that hydrogenolysis of phenyl esters of phosphoric acids proceeds by C–O bond cleavage (Brigl & Müller, 1939). Hydrogenolysis of the diastereomers of [^{17}O,^{18}O]TpNP and [^{17}O,^{18}O]NPpT yields samples of chiral [^{16}O,^{17}O,^{18}O]Tp and [^{16}O,^{17}O,^{18}O]pT, with the ^{16}O being derived from the ester oxygen. These samples can be chemically cyclized by initial activation with diphenylphosphorochloridate followed by exposure to *tert*-butoxide. The diphenylphosphorochloridate reacts randomly with the three phosphoryl oxygens of Tp or pT, and subsequent ring closure results in the loss of one of the phosphoryl oxygens to form a mixture of three types of doubly labeled and chiral cyclic TMP, i.e., ^{16}O,^{17}O, ^{17}O,^{18}O, and ^{16}O,^{18}O labeled. Work performed in Lowe's laboratory (Cullis et al., 1981; Jarvest et al., 1981) and our own (Coderre et al., 1981b) has allowed the conclusion that this chemical cyclization procedure proceeds essentially exclusively with the expected inversion of configuration at phosphorus (Westheimer, 1980); ^{16}O,^{17}O, methylation of the mixture of labeled TMPs yields a mixture of labeled equatorial and axial methyl esters whose ^{31}P NMR chemical shifts have been assigned.

The configuration of the ^{16}O,^{18}O-labeled cyclic TMP present in the sample used to prepare the mixture of methyl esters can be established by inspection of the ^{31}P NMR spectrum of the methyl esters. This analysis, first used by this laboratory to determine the configurations of samples of cyclic [^{16}O,^{18}O]-dAMP (Gerlt & Coderre, 1980), is based upon the demonstration that the magnitude of the upfield change in chemical shift induced by the presence of ^{18}O increases with the number of bonds between ^{18}O and ^{31}P (Lowe et al., 1979; Cohn & Hu, 1980): ^{18}O located in a singly bonded ester oxygen results in an upfield change in chemical shift of 0.015 ppm relative to the unlabeled ester whereas ^{18}O located in the double bonded (nonesterified) oxygen results in an upfield change in chemical shift of 0.035 ppm. For a given sample of chiral cyclic [^{16}O,^{18}O]TMP, the magnitudes of the ^{18}O perturbations on the resonances for the equatorial and axial esters should differ and yield complementary configurational information. However, in practice the configurational analyses of the mixtures of isotopically labeled samples of cyclic TMP obtained from the acyclic ^{17}O,^{18}O-labeled diesters are complicated by the fact that the ^{17}O enrichment is less than 100%; additional ^{16}O,^{18}O-labeled species are present in the mixture which contribute a racemic background of single and double bond ^{18}O perturbations on which the signals from the ^{16}O,^{18}O-labeled species of interest are superimposed. (Resonances due to ^{16}O,^{16}O- and ^{18}O,^{18}O-labeled species will also be present, but these provide convenient internal reference signals.) A simple calculation reveals that the predominant resonances will be associated with the ^{16}O,^{18}O-labeled ester of interest and the ratio of the resonances arising from species having one and two bonds between ^{18}O and ^{31}P can be used to quantitate the diastereomeric purity of the cyclic ester.

A ^{31}P NMR spectrum at 145.7 MHz of the methyl esters of labeled cyclic TMP obtained from the presumed S_P diastereomer of [^{17}O,^{18}O]TpNP is shown in Figure 2. In this spectrum the signals associated with the axial ester most clearly reveal that the starting labeled acyclic phosphodiester is chiral, since the resonances associated with species having one and two bonds between ^{18}O and ^{31}P are of unequal intensity; in fact, this spectrum and the knowledge that the chemical cyclization reaction proceeds with inversion of configuration at phosphorus allow the conclusion that the configuration of the acyclic ester is S_P, thereby demonstrating that the Wittig–Staudinger reaction on acyclic P-anilidates is (at least partially) stereospecific and proceeds with the predicted retention of configuration at phosphorus. A quantitative analysis of the spectrum in Figure 2 indicates that the Wittig–Staudinger reaction is not completely stereospecific, since the ratio of the intensities of the resonances associated with the species having one and two bonds between ^{18}O and ^{31}P is not 2.0 as would be expected if the conversion of the P-anilidate to the labeled phosphodiester had proceeded with quantitative retention of

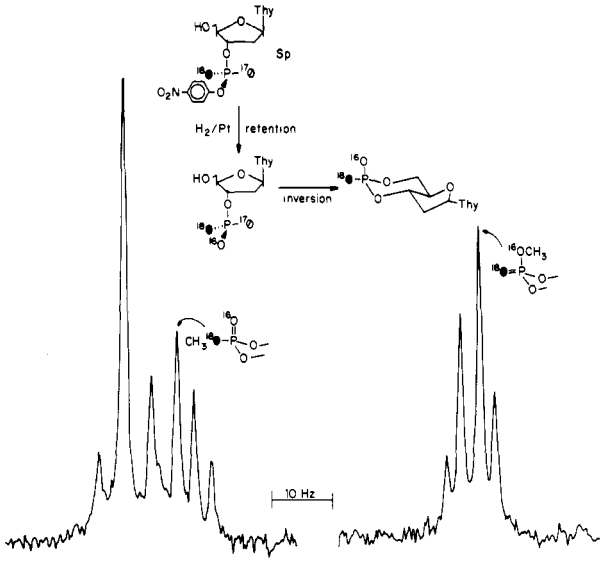


FIGURE 2: Phosphorus-31 NMR spectrum at 145.7 MHz of the methyl esters of cyclic TMP obtained following hydrogenolysis of the S_P diastereomer of [^{17}O,^{18}O]TpNP, chemical cyclization, and methylation.

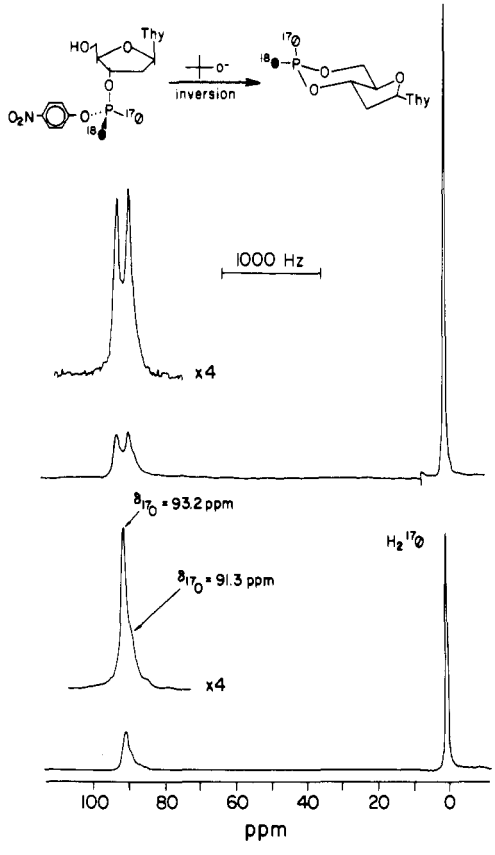


FIGURE 3: Oxygen-17 NMR spectra at 36.6 MHz of cyclic [^{17}O,^{18}O]TMP obtained by chemical cyclization of the R_P diastereomer of [^{17}O,^{18}O]TpNP. The bottom spectrum was obtained with broad-band ^{31}P decoupling; the top spectrum was obtained without ^{31}P decoupling.

configuration at phosphorus; the measured ratio is 1.5 which corresponds to 80% configurational purity (or 60% diastereomeric excess) of the chiral diesters. Equivalent spectra on cyclic esters obtained from the presumed R_P diastereomer of [^{17}O,^{18}O]TpNP and both diastereomers of [^{17}O,^{18}O]NPpT yielded analogous configurational information.

The second method of configurational analysis of the acyclic ^{17}O,^{18}O-labeled phosphodiesters is based on their direct conversion to samples of cyclic [^{17}O,^{18}O]TMP in the presence of

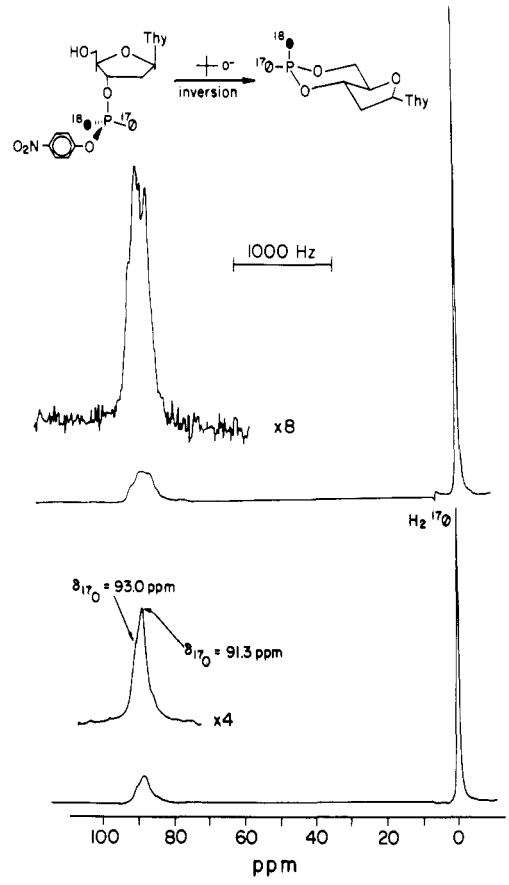


FIGURE 4: Oxygen-17 NMR spectra at 36.6 MHz of cyclic [^{17}O,^{18}O]TMP obtained by chemical cyclization of the S_P diastereomer of [^{17}O,^{18}O]TpNP. The bottom spectrum was obtained with broad-band ^{31}P decoupling; the top spectrum was obtained without ^{31}P decoupling.

tert-butoxide; the accepted principles of nucleophilic displacement reactions at tetrahedral phosphorus allow the prediction that this cyclization reaction should occur with at least predominant, if not exclusive, inversion of configuration at phosphorus (Westheimer, 1980). This transformation places the nonesterified phosphoryl oxygens of the 4-nitrophenyl esters in either the axial or equatorial exocyclic positions of the phosphodiester ring in cyclic TMP, thereby facilitating their isotopic identification. The most direct approach for determining the isotopic identify of the exocyclic phosphoryl oxygens is to use ^{17}O NMR spectroscopy, since in previously published work this laboratory has demonstrated that both the ^{17}O NMR chemical shifts and one bond ^{31}P–^{17}O coupling constants for the axial and equatorial exocyclic oxygens in 3′,5′-cyclic nucleotides are sufficiently different that they can be distinguished (Coderre et al., 1981a). The spectra recorded for samples of cyclic [^{17}O,^{18}O]TMP obtained from the R_P and S_P diastereomers of [^{17}O,^{18}O]TpNP are shown in Figures 3 and 4, respectively. The bottom spectrum in each figure was obtained with broad-band ^{31}P decoupling, and in each case a predominant resonance with a small upfield (Figure 3) or downfield (Figure 4) shoulder is observed. The chemical shift of the major resonance in Figure 3 can be associated with ^{17}O located in the axial exocyclic position, and the shift of the major resonance in Figure 4 can be associated with ^{17}O located in the equatorial exocyclic position; the chemical shifts estimated for the shoulders are those for ^{17}O in the other exocyclic position. The top spectrum in each figure was obtained without broad-band ^{31}P decoupling, and in these the resulting signals appear as somewhat asymmetric doublets. The measured coupling constants, 129 Hz in Figure 3 and 103 Hz in Figure

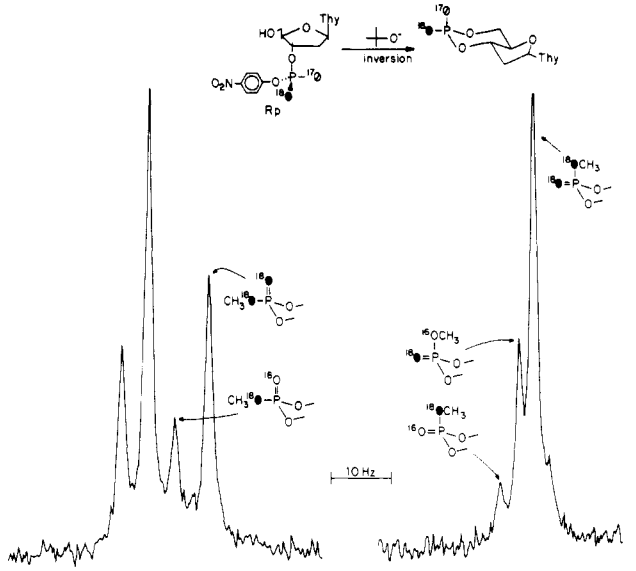


FIGURE 5: Phosphorus-31 NMR spectrum at 145.7 MHz of the methyl esters of cyclic [^{17}O,^{18}O]TMP obtained by chemical cyclization of the R_P diastereomer of [^{17}O,^{18}O]TpNP.

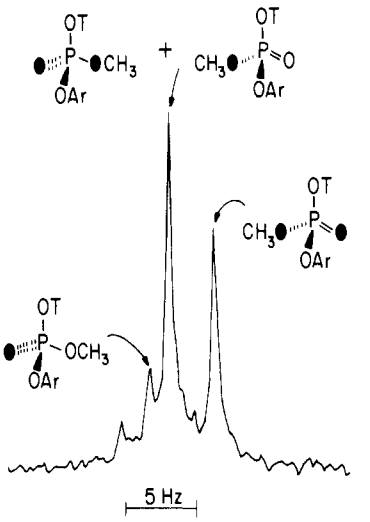


FIGURE 6: Phosphorus-31 NMR spectrum at 81 MHz of the methyl esters of the S_P diastereomer of [^{17}O,^{18}O]TpNP.

4, provide additional confirmation of the predominant location of the ^{17}O nucleus. The locations of the ^{17}O nuclei in these samples of cyclic TMP are those expected if the Wittig–Staudinger reaction proceeded with predominant retention of configuration at phosphorus. The relatively large line widths for the ^{17}O resonances preclude any quantitative assessment of the relative amounts of ^{17}O-labeled species present in the samples. Similar results were obtained following the cyclization of the diastereomers of [^{17}O,^{18}O]NPpT.

In order to confirm the configurational assignments based on ^{17}O NMR spectroscopy and also to quantitate the configurational purity of the samples of cyclic [^{17}O,^{18}O]TMP, the samples were methylated with diazomethane, and their ^{31}P NMR spectra were obtained at 145.7 MHz. The spectrum recorded on the sample of the methyl esters of cyclic [^{17}O,^{18}O]TMP obtained from the R_P diastereomer of [^{17}O,^{18}O]TpNP is shown in Figure 5. Examination of the resonances associated with the axial esters most clearly allows assessment of the configurational purity of this sample. The largest resonance is associated with ester labeled with ^{18}O in both the axial and equatorial exocyclic oxygens; the two more downfield resonances associated with the axial esters demonstrate the presence of ^{16}O (and therefore ^{17}O) in both exocyclic positions. The relative intensities of the downfield resonances provide confirmation for the configurational assignments made by ^{17}O NMR spectroscopy and also allow the configurational purity of this sample to be approximated as 80%. If it is *assumed* that the *tert*-butoxide-mediated cyclization reaction occurs with complete inversion of configuration at phosphorus, this spectrum provides additional evidence that the Wittig–Staudinger reaction proceeds with predominant but not exclusive retention of configuration. Analogous results were obtained with the methyl esters prepared from the S_P diastereomer of [^{17}O,^{18}O]TpNP and both diastereomers of [^{17}O,^{18}O]NPpT.

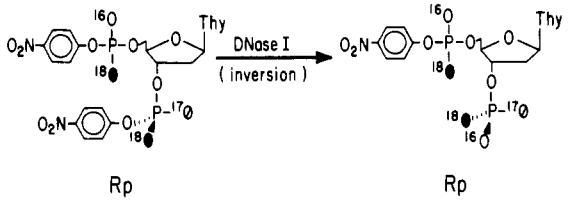
Because both of these methods of configurational analysis rely on a cyclization reaction of somewhat uncertain stereochemical integrity, the configurational purity (but not absolute configuration) of the R_P and S_P diastereomers of [^{17}O,^{18}O]-TpNP was assessed by methylation with diazomethane followed by analysis by ^{31}P NMR spectroscopy; the spectrum obtained for the S_P diastereomer is shown in Figure 6. In 2:1 methanol/D_2O as solvent, the chemical shift difference between the two diastereomeric methyl esters is only 0.03 ppm, but the spectrum is sufficient to demonstrate less than complete diastereomeric purity of the acyclic diesters. The largest resonance is associated with the R_P methyl ester having three bonds between ^{18}O and ^{31}P (as well as a contribution from the S_P methyl ester having one bond between ^{18}O and ^{31}P). The two resonances downfield of this signal are associated with R_P methyl ester having one and two bonds between ^{18}O and ^{31}P, and both of these are present because the sample of [^{17}O,^{18}O]TpNP was not diastereomerically pure; if the Wittig–Staudinger reaction had proceeded with complete retention of configuration, only the resonance associated with a double bond between ^{18}O and ^{31}P would have been present. Complementary results were obtained with the R_P diastereomer. The ^{31}P NMR resonances associated with the methyl esters of NPpT could not be resolved.

Thus, the conclusion can be reached that the samples of [^{17}O,^{18}O]TpNP and [^{17}O,^{18}O]NPpT that were prepared were not configurationally pure but were partially racemic by virtue of a lack of stereochemical integrity in the Wittig-Staudinger reaction. This observation stands in contrast to those made both in Stec's laboratory (Niewiarowski et al., 1980) and in our own (Gerlt et al., 1980) on the conversion of acyclic P-anilidates to chiral phosphorothioate diesters; when CS_2 is used in the Wittig–Staudinger reaction, the products appear to be produced with complete retention of configuration at phosphorus. However, the incomplete configurational purity of the acyclic phosphodiesters does not prevent their use in determining the stereochemical consequences of enzyme-catalyzed displacement reactions.

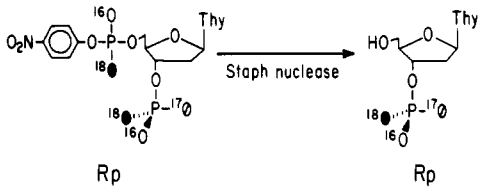
The configuration of the sample of [^{17}O,^{18}O]NPpTpNP was established by converting it with Staphylococcal nuclease to a sample of [^{17}O,^{18}O]TpNP whose configuration could be assigned by methylation and ^{31}P NMR spectroscopy. A comparison of the resulting spectrum (data not shown) with those obtained by methylation of the diastereomers of [^{17}O,^{18}O]TpNP revealed that the configuration at the 3′-phosphorus was R_P, as expected based on the known configuration of the 3′-anilidate and the knowledge that the Wittig–Staudinger reaction proceeds with predominant retention of configuration with acyclic P–anilidates.

Hydrolysis of [^{17}*O*,^{18}*O*]*NPpTpNP by Bovine Pancreatic DNase I.* Pancreatic DNase I is an endonuclease that hydrolyzes DNA to yield 5′-mononucleotides (Moore, 1981); divalent metal ions are required for catalysis, with optimal

Scheme I



Scheme II



activity being produced by a mixture of either Ca^{2+} and Mg^{2+} or Ca^{2+} and Mn^{2+}. The primary structure of the enzyme was determined in the laboratories of Moore and Stein (Liao et al., 1973), and high-resolution X-ray studies are currently under way in the laboratory of Suck (Suck, 1982). We have determined the stereochemical course of a reaction catalyzed by DNase I so that a mechanism for the enzyme-catalyzed reaction can be formulated when the active site geometry becomes available.

The simplest synthetic substrate for DNase I is NPpTpNP; unlike the specificity found for DNA as substrate, this substrate is hydrolyzed to yield the 3′-nucleotide NPpTp and 4-nitrophenolate (Liao, 1975). This difference in hydrolysis products is presumably caused by differences in binding geometry rather than in the intimate details of catalysis; NPpTpNP was reported to bind weakly to DNase I.

The R_P diastereomer of [^{17}O,^{18}O]NPpTpNP was prepared as described under Materials and Methods, and the configuration at the 3′-phosphorus atom was unambiguously established. The labeled substrate was hydrolyzed by DNase I in the presence of Ca^{2+} and Mn^{2+} (Scheme I), with the progress of the reaction being monitored spectrophotometrically by quantitating the amount of 4-nitrophenolate formed. When the reaction was approximately 70% complete, a mixture of the unreacted substrate and the hydrolysis product, [^{16}O,^{17}O,^{18}O]NPpTp, was recovered following chromatography on DEAE-Sephadex. Hydrolysis of the mixture in the presence of Staphylococcal nuclease produced a mixture of [^{17}O,^{18}O]TpNP and [^{16}O,^{17}O,^{18}O]Tp (Scheme II) which was separated by ion-exchange chromatography. The labeled Tp was chemically cyclized to a mixture of the three types of double-labeled cyclic TMP. Following methylation the configuration of the cyclic [^{16}O,^{18}O]TMP was ascertained by ^{31}P NMR spectroscopy; the ^{31}P NMR spectrum of the mixture of methyl esters is reproduced in Figure 7.

The relative intensities of the resonances associated with species having one and two bonds between ^{18}O and ^{31}P and the known stereochemical course of the chemical cyclization reaction permit the configuration of the chiral monoester in the DNase I hydrolysis product, [^{16}O,^{17}O,^{18}O]NPpTp, to be assigned as R_P. A comparison of the *relative* configurations of the substrate and hydrolysis product allows the conclusion that the DNase I catalyzed reaction proceeds with inversion of configuration at phosphorus (Scheme I).

Such a stereochemical consequence for the DNase I catalyzed reaction is persuasive evidence that a single displacement reaction occurs at phosphorus. While the simplest explanation for a mechanism accompanied by a single displacement is that

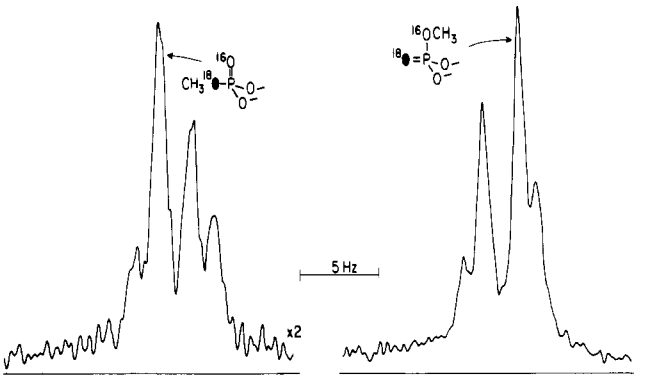


FIGURE 7: Phosphorus-31 NMR spectrum at 81 MHz of the methyl esters of labeled cyclic TMP used to determine the stereochemical course of the reaction catalyzed by DNase I.

the enzyme catalyzes the direct attack of water on the substrate to yield the hydrolysis product, an alternate explanation should be considered. As we have previously discussed (Mehdi & Gerlt, 1982), nucleophilic catalysis by an active site carboxylate would lead to the formation of an acyl phosphate ester intermediate, and hydrolysis of this covalent intermediate would yield the observed 3′-mononucleotide product. If such an intermediate were to undergo hydrolysis by attack of water on the carboxyl carbon, the observed stereochemical course of the reaction would also be inversion of configuration; interestingly, in nonenzymic systems, acyl phosphate esters are hydrolyzed by attack of water on the carboxyl carbon (Kellerman, 1958; DiSabato & Jencks, 1961). The presently available crystallographic data for DNase I show that two asparate residues (Asp-39 and Asp-248) are located in or near the active site of the enzyme (D. Suck, personal communication), and chemical modification studies have shown that carboxyl groups may be essential for catalysis since in the absence of Ca^{2+} the enzyme can be inactivated by glycine ethyl ester and a carbodiimide (Poulos & Price, 1974). Thus, further experiments designed to ascertain the importance of the acyl phosphate ester mechanism are necessary before the present stereochemical study can be unequivocally interpreted.

Acknowledgments

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Solvent Effects on Allosteric Equilibria: Stabilization of T and R Conformations of *Escherichia coli* Aspartate Transcarbamylase by Organic Solvents†

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ABSTRACT: The activity of *Escherichia coli* aspartate transcarbamylase (ATCase) is markedly influenced by the addition of organic solvents to the assay medium. The cosolvents tested, which include simple aliphatic alcohols, amides, and ureas, as well as acetone and dioxane, fall into two different classes: the most polar ones (formamide, acetamide, *N*-methylformamide, and urea) stimulate the enzyme activity for all concentrations tested. In contrast, solvents that are less polar than water inhibit the enzyme at low concentrations but stimulate it at higher concentrations. No comparable effects are observed in the case of the isolated catalytic subunits, a nonregulated form of ATCase. Extensive kinetic studies on ATCase and on two of its Michaelian derivatives, 2-thioU–ATCase and carbamylated ATCase, indicate that solvents modulate the same allosteric transition that is responsible for homotropic interactions between the catalytic sites. The stabilization of the R state of ATCase by comparatively high concentrations of cosolvents is reminiscent of similar findings made on hemoglobin and glycogen phosphorylase, suggesting a common underlying mechanism. Addition of organic cosolvents to water is known to reduce hydrophobic interactions, and we suggest that this effect may preferentially stabilize the more "relaxed" conformations of allosteric proteins, because they have a larger surface exposed to solvent [Chothia, C. (1974) *Nature (London) 248*, 338–339]. On the other hand, we suggest that the stabilization of the T state by low concentrations of all but the most polar cosolvents simply reflects stronger electrostatic interactions in this conformation.

According to the concerted model for allosteric transitions (Monod et al., 1965), allosteric proteins exist in solution as a mixture of conformational states characterized by very different biological activities. Specific ligands can bind preferentially to one conformation or another, thereby shifting the preexisting equilibrium and resulting macroscopically in an increased or decreased activity. Such a conformational equilibrium, like any chemical equilibrium, should be sensitive to physicochemical parameters such as pressure, temperature, or solvent composition: therefore, any model implying an equilibrium between several conformations predicts that a shift in one of these variables eventually results in a change of protein activity, even in the absence of any binding of physiological regulatory ligand. Indeed, early observations made on threonine deaminase (Changeux, 1965) and aspartate transcarbamylase (Weitzman & Wilson, 1966; Chan, 1981) showed that at least some of the effects of positive allosteric effectors could be mimicked by the addition to the assay mixture of molar concentrations of the chaotropic agent urea. More recently, several authors have described the influence of organic solvents on the affinity of human hemoglobin for oxygen (Cordone et al., 1979, 1981; Bulone et al., 1983; Haire & Hedlund, 1983), and the observed effects were interpreted in term of solvent dependence of the T $\rightleftharpoons$ R equilibrium position. Similar but more spectacular solvent effects have been reported in the case of muscle glycogen phosphorylase.

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