DuVal, G., Swaisgood, H. E., & Horton, H. R. (1984) *J. Appl. Biochem. 6*, 240–250.
Engasser, J.-M., & Horvath, C. (1973) *J. Theor. Biol. 42*, 137–155.
Engasser, J.-M., & Horvath, C. (1974a) *Biochemistry 13*, 3845–3849.
Engasser, J.-M., & Horvath, C. (1974b) *Biochemistry 13*, 3849–3854.
Engasser, J.-M., & Horvath, C. (1974c) *Biochemistry 13*, 3855–3859.
Ford, J. R., Lambert, A. H., Cohen, W., & Chambers, R. P. (1972) *Biotechnol. Bioeng. Symp. No. 3*, 267–284.
Goldstein, L. (1976) *Methods Enzymol. 44*, 397–443.
Harada, K., & Wolfe, R. G. (1968a) *J. Biol. Chem. 243*, 4123–4130.
Harada, K., & Wolfe, R. G. (1968b) *J. Biol. Chem. 243*, 4131–4137.
Janolino, V. G., & Swaisgood, H. E. (1982) *Biotechnol. Bioeng. 24*, 1069–1080.
Jurgensen, S. R., Wood, D. C., Mahler, J. C., & Harrison, J. H. (1981) *J. Biol. Chem. 256*, 2383–2388.
Mosbach, K. (1980) *Biochimie 62*, 623–628.
Mullinax, T. R., Mock, J. N., McEvily, A. J., & Harrison, J. H. (1982) *J. Biol. Chem. 257*, 13233–13239.
Ollis, D. F., & Datta, R. (1976) *Methods Enzymol. 44*, 444–450.
Raval, D. N., & Wolfe, R. G. (1962a) *Biochemistry 1*, 263–269.
Raval, D. N., & Wolfe, R. G. (1962b) *Biochemistry 1*, 1118–1123.
Swaisgood, H. E., Horton, H. R., & Mosbach, K. (1976) *Methods Enzymol. 44*, 504–515.
Taylor, J. B., & Swaisgood, H. E. (1980) *Biotechnol. Bioeng. 22*, 2617–2631.
Taylor, J. B., & Swaisgood, H. E. (1981) *Biotechnol. Bioeng. 23*, 1349–1363.
Wood, D. C., Jurgensen, S. R., Geesin, J. C., & Harrison, J. H. (1981) *J. Biol. Chem. 256*, 2377–2382.

Stereochemical Course of Hydrolysis of DNA by Exonuclease I from *Escherichia coli*†

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ABSTRACT: Exonuclease I has been purified from an overproducing strain of *Escherichia coli* K12 [Prasher, D. C., Conarro, L., & Kushner, S. R. (1983) *J. Biol. Chem. 258*, 6340–6343]. The enzyme hydrolyzes deoxyribonucleic acids that contain chiral phosphorothioate diester linkages, and the stereochemical course of the reaction is inversion of configuration at phosphorus. This result is most consistent with hydrolysis occurring via the direct attack of water on a phosphorothioate diester rather than through the intermediacy of a covalent nucleotidyl-enzyme intermediate. This finding represents the first example of a processive exonuclease whose stereochemical pathway has been determined.

Exonuclease I (EC 3.1.4.25) from *Escherichia coli* is a processive 3′→5′ exonuclease that acts specifically on single-stranded DNA. Early studies by Lehman & Nussbaum (1964) showed that the enzyme catalyzes the release of 5′-mononucleotides from the 3′ termini of single-stranded DNA, degrading the chains to 5′-terminal dimers that are inert to further hydrolysis. Thomas & Olivera (1978) subsequently showed that exonuclease I is highly processive in that an enzyme molecule will bind to and degrade a single polydeoxyribonucleotide chain to near completion before reacting with another molecule of nucleic acid. More recently, Kushner and co-workers (Prasher et al., 1983) constructed a strain of *E. coli* that overproduces exonuclease I between 140 and 400 times the level found in wild-type cells. These workers purified the enzyme to near homogeneity and found that the enzyme is a monomer of molecular weight 55 000.

A mechanistic study of exonuclease I must address both the catalytic mechanism of phosphodiester hydrolysis and the protein–nucleic acid interactions that allow the enzyme to hydrolyze successive nucleotides without dissociating from the nucleic acid chain. A necessary initial step in such an investigation is the determination of whether the enzymatic reaction proceeds by direct attack of water on a phosphodiester or via a double displacement mechanism in which a covalent nucleotidyl-enzyme intermediate is formed and subsequently hydrolyzed. The research described in this paper addresses this question via a stereochemical analysis of the reaction of exonuclease I with deoxyribonucleic acids that contain chiral phosphorothioate internucleotide linkages.

Experimental Procedures

Materials. Strain SK 4258 of *E. coli* K12 was a gift of Dr. Sidney Kushner. Poly[d(T_s-A)][1] and pdTp$_s$dApdTp$_s$dA were synthesized as described by Brody & Frey (1981). pdTpdApdTpdA, calf intestine alkaline phosphatase, and T4 polynucleotide kinase were purchased from Pharmacia P-L Biochemicals. [γ-^{32}P]ATP, calf thymus terminal transferase, *E. coli* [^{3}H]DNA, and the Klenow fragment of DNA polymerase I were purchased from New England Nuclear. [^{3}H]-dATP was purchased from Schwarz/Mann. Bio-Gel P-10 was purchased from Bio-Rad. Salmon sperm DNA, adenylate kinase, pyruvate kinase, and yeast inorganic pyrophosphatase were purchased from Sigma.

Enzyme Purification and Assay. Exonuclease I was purified from an overproducing strain of *Escherichia coli* K12 (SK

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4258) according to the procedure of Prasher et al. (1983). Enzyme activity was monitored by a modification of the procedure of Lehman (1966). The assay mixture contained the following in 0.16 mL: 67 mM glycine buffer (pH 9.5), 6.7 mM magnesium chloride, 10 mM 2-mercaptoethanol, and 0.2 μM [^{3}H](dA)$_{130-170}$.[1,2] The specific activity of the substrate was 3.7 mCi/mmol of dAMP. Each assay contained between 0.03 and 0.15 unit of enzyme, and the activity was proportional to the concentration of enzyme under these conditions. After 30 min at 37 °C, 0.1 mL of a solution of salmon sperm DNA (2.5 mg/mL) was added to the assay mixture and the solution mixed on a vortexer. A cold solution of 10% trichloroacetic acid (1 mL) was then added, and, after vortexing, the mixture was cooled in ice for 10 min. The precipitate was then removed by centrifugation for 5 min at 4 °C in a Brinkmann Microfuge, and 0.5 mL of the supernatant was analyzed for radioactivity in a scintillation fluor that is compatible with aqueous samples. The enzyme activity was also determined with bacterial [^{3}H]DNA (specific activity 0.42 mCi/mmol of nucleotide). The bacterial DNA was heated at 100 °C for 10 min in a Tris buffer (0.025 M Tris, pH 7.5, and 0.02 M sodium chloride) and then cooled rapidly in ice water before its use at a total nucleotide concentration of 0.5 mM in the assay previously described.

Protein concentrations were determined with the Bio-Rad protein reagent, and bovine plasma γ-globulin was used as the protein standard. A unit of activity is defined as the amount of enzyme that will cause the release of 10 nmol of acid-soluble nucleotide in 30 min at 37 °C.

Gel Electrophoresis. Gel electrophoresis of protein samples was done on 10% SDS–polyacrylamide slab gels according to the procedure of Laemmli & Favre (1973). The gels were stained with Bio-Rad silver stain.

Gel electrophoresis of nucleic acids was done according to the general procedure of Maniatis et al. (1982) on 6% polyacrylamide gels that contained 7.6 M urea. The gels (32 × 43 × 0.08 cm) were preelectrophoresed for 20 min before the samples were added. The gels were run until the bromophenol blue dye migrated approximately 31 cm. Nucleic acids that contained 5′-phosphate groups were dephosphorylated with alkaline phosphatase and rephosphorylated with [γ-^{32}P]ATP (specific activity ~2900 Ci/mmol) and polynucleotide kinase (Maniatis et al., 1982). After electrophoresis, the location of the ^{32}P on a gel was determined by autoradiography.

The markers used in the determination of nucleic acid size were Bethesda Research Laboratories' 123 base pair ladder and ϕX174 RF DNA *Hae*III fragments.

Synthesis of Homopolymer Substrates. [^{3}H](dA)$_{130-170}$ was synthesized by a modification of the procedure of Chang & Bollum (1971). The reaction mixture contained in 2 mL at 37 °C 0.2 M potassium cacodylate buffer (pH 7.5), 0.1 mg/mL bovine serum albumin, 8 mM magnesium chloride, 0.4 mM zinc chloride, 2 mM [^{3}H]dATP (3.7 mCi/mmol), 0.0065 mM d(pA)$_6$, 10 units of yeast inorganic pyrophosphatase, and 130 units of calf thymus terminal transferase. After 72 h at 37 °C, the reaction mixture was applied to a 1 × 40 cm column of Bio-Gel P-10 and eluted with 0.01 M Tris buffer (pH 7.8). The polymerized nucleotides came off in the column's void volume (60% yield). The small amount of protein associated with the polymer was not removed since aliquots from which protein was removed by a phenol/chloroform extraction (Maniatis et al., 1982) were indistinguishable from the untreated product in their reactions with exonuclease I. The molecular weight distribution of the product was determined by gel electrophoresis on a 6% polyacrylamide gel after the sample had been labeled with ^{32}P in the 5′-position. Most of the radioactivity appeared in a broad band between ~130 and ~170 bases although the gel also showed the presence of smaller oligomers. The product was not purified further.

Kinetics. The V_{max} and K_M values for the reactions of exonuclease I were derived from Lineweaver–Burk plots. Reactions were performed at 37 °C in the buffer used for the exonuclease I assays. At each substrate concentration, reactions were quenched after 6 and 12 min to confirm the linearity of the initial rates. In all cases, less than 10% of the substrate was hydrolyzed at the time of the quench.

Kinetic reactions with [^{3}H](dA)$_{130-170}$ were done at an enzyme concentration of 0.16 unit/mL, and the workup was the same as that for the normal assay procedure.

The kinetic constants for the reaction of exonuclease I with pdTp$_s$dApdTp$_s$dA and pdTpdApdTpdA were determined by a modification of the method used for (dA)$_{130-170}$. The reaction mixtures, which varied in volume from 0.02 mL for concentrated substrate solutions to 0.2 mL for dilute substrate solutions, were quenched by rapid freezing in a dry ice/2-propanol bath. The samples were thawed just prior to analysis by HPLC. Control studies showed that no further reaction occurred during the time that the samples were rapidly thawed and injected into the HPLC. The concentration of exonuclease I was 520 units/mL for the reactions with pdTp$_s$dApdTp$_s$dA and 26 units/mL for the reactions with pdTpdApdTpdA.

HPLC. All HPLC work was done on a Beckman Model 332 gradient liquid chromatograph with an LCD variable-wavelength detector set at 260 nm and a Hewlett Packard 3380A integrator. A Waters μBondapak C$_{18}$ reversed-phase analytical column was used with a guard column packed with the same resin. Gradients were formed with buffer A (0.1 M ammonium acetate, pH 5) and buffer B (methanol/water, 60:40).

Samples from the hydrolyses were analyzed by elution with the following solvent gradient: (a) 15% buffer B (85% buffer A) from 0 to 5 min; (b) a linear gradient of 15–20% buffer B from 5 to 10 min; (c) a linear gradient of 20–40% buffer B from 10 to 35 min. The reaction products eluted at the indicated times: dTMP, 5 min; dAMP, 7 min; dAMPS, 7 min; pdTpdA, 13 min; pdTp$_s$dA, 20 min; pdTpdApdT, 20 min; pdTp$_s$dApdT, 25 min; pdTpdApdTpdA, 24 min; pdTp$_s$dApdTp$_s$dA, 29 min. A strong peak from 2-mercaptoethanol in the reaction mixtures eluted at ~11 min but did not obscure any peaks. In some runs, unidentified impurities obscured a small dTMP peak.

Reaction of Exonuclease I with Poly[d(T$_s$-A)]. The reaction mixture contained the following in 7 mL: 1.5 mM poly[d(T$_s$-A)] (molarity expressed in terms of total nucleotide

[1] Concentrations of deoxyribonucleotide polymers and oligomers are given in terms of intact chains rather than total nucleotide content unless specifically stated otherwise. The concentration of (dA)$_{130-170}$ was calculated from the total nucleotide concentration by assuming a polymer size of 150 base pairs. Routine assays were often done at lower concentrations of substrate.

[2] Abbreviations: dAMPS, 2′-deoxyadenosine 5′-*O*-phosphorothioate; S_p-dATPαS, S_p diastereomer of 2′-deoxyadenosine 5′-*O*-(1-thiotriphosphate); pdTp$_s$dApdTp$_s$dA, oligodeoxyribonucleotide tetramer containing alternating units of 2′-thymidine 5′-phosphate and 2′-deoxyadenosine 5′-*O*-phosphorothioate; poly[d(T$_s$-A)]-alternating copolymer of 2′-thymidine 5′-phosphate and 2′-deoxyadenosine 5′-phosphorothioate; poly[d(A-T)], alternating copolymer of deoxyadenylate and thymidylate; poly(dA), poly(deoxyadenylic acid); GCMS, gas chromatograph interfaced with a mass spectrometer; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

concentration), 33 mM glycine buffer (pH 9.5), 3.3 mM magnesium chloride, 5 mM 2-mercaptoethanol, and 750 units/mL exonuclease I. The reaction was allowed to proceed for 5 h at 37 °C. Thin-layer chromatography on silica gel with a solvent system of 1-propanol/concentrated ammonia/water (6:3:1) showed that the reaction contained dAMPS and dTMP but no poly[d(T_s-A)].

Conversion of dAMPS to dATPαS. The product solution from the poly[d(T_s-A)] hydrolysis was used, without purification, as the source of dAMPS in the synthesis of dATPαS according to procedure B of Brody & Frey (1981). The final yield of dATPαS was 3.8 μmol (72% of the dAMPS in the original poly[d(T_s-A)].

Conversion of dATPαS to dAMPS and Inorganic Pyrophosphate. This conversion was accomplished via a polymerization by DNA polymerase I (Brody & Frey, 1981) followed by a degradation by exonuclease I. The initial reaction mixture contained the following in 2 mL at 37 °C: 0.5 mM dATPαS, 0.5 mM dTTP, 0.003 mM poly[d(A-T)] (total nucleotide content), 60 mM Hepes buffer (pH 7.4), 10 mM magnesium chloride, 1 mM 2-mercaptoethanol, and 40 units/mL Klenow fragment of DNA polymerase I from *E. coli*. The reaction was allowed to proceed for 24 h at which time 0.23 mL of a 1 M solution of magnesium chloride was added and the solution heated at 50 °C for 10 min. After the solution was cooled in ice and the precipitated magnesium pyrophosphate removed by centrifugation, the supernatant was applied to a 1 × 40 cm Bio-Gel P-10 column and the column eluted with 10 mM Tris buffer (pH 8). The product (poly[d(T_s-A)] eluted in the column's void volume and was added to a reaction mixture that contained the following in a total of 12 mL: 67 mM glycine buffer (pH 9.5), 6.7 mM magnesium chloride, 10 mM 2-mercaptoethanol, and 300 units/mL exonuclease I. After 10 h at 37 °C, the reaction mixture was applied to a 1 × 40 cm column of DEAE-Sephadex A-25 (bicarbonate form) and the column eluted at 4 °C with a linear gradient of 300 mL of 0.1 M and 300 mL of 0.4 M triethylammonium bicarbonate buffers (pH 7.5). The fractions that contained dAMPS were pooled and evaporated to dryness. After ethanol was added and the sample again dried, the yield was 0.7 μmol (70%) of dAMPS. The magnesium pyrophosphate was purified by ion-exchange chromatography as described by Brody & Frey (1981).

Mass Spectral Analysis. Pyrophosphate was converted to diethyl methyl phosphate according to the procedure of Brody & Frey (1981) with one modification. After the acid-catalyzed methanolysis reaction, the product solution was not evaporated to dryness but rather was concentrated to ~20 μL by a stream of nitrogen.

dAMPS was converted to triethyl phosphorothioate according to the procedure of Brody & Frey (1981). Mass spectral analysis was performed with a Finnigan 4021 EI mass spectrometer coupled to a gas chromatograph. A 6 ft × 2 mm Altech nickel column containing 10% SE-30 on Chromosorb was used, and the mass region between *m/e* 90 and *m/e* 210 was scanned for each spectrum. Diethyl methyl phosphate came off the column at 4.2 min during an isothermal run at 90 °C. The major peaks at *m/e* 141 and *m/e* 113 were analyzed for the presence of ^{18}O. Triethyl phosphorothioate came off the column at 9.1 min during an isothermal chromatograph at 115 °C. The major peaks at *m/e* 198, 170, and 138 were analyzed for ^{18}O.

RESULTS

Purification of Exonuclease I. The purified enzyme is more than 97% pure as determined by SDS–polyacrylamide gel electrophoresis. Gel electrophoresis also reveals that the enzyme has a molecular weight of 5.5 × 10^4, in agreement with the value obtained by Prasher et al. (1983). The specific activity of the purified enzyme is 6 × 10^5 units/mg when determined with [^{3}H](dA)$_{130-170}$ as the substrate and 3 × 10^5 units/mg when determined with [^{3}H]DNA from *E. coli* as the substrate.

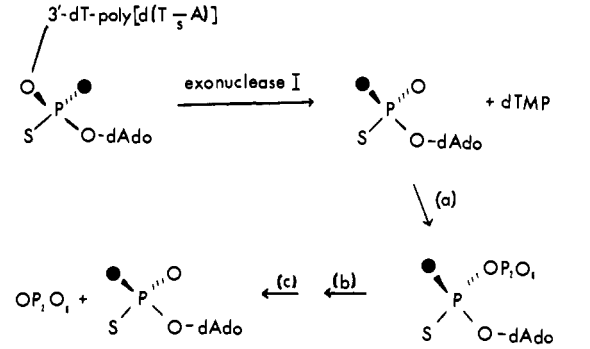

FIGURE 1: Elucidation of the stereochemistry of hydrolysis of poly[d(T_s-A)] by exonuclease I. O is ^{16}O, and shaded O's represent ^{18}O. (a) Adenylate kinase, pyruvate kinase, ATP, and phosphoenolpyruvate; (b) Klenow fragment of DNA polymerase I, dTTP, and poly[d(A-T)]; (c) exonuclease I.

Table I: ^{18}O Enrichment of Products from the Reaction of Exonuclease I with Poly[d(T_s-A)] That Contains ^{18}O-Labeled Phosphorothioates[a]

compd	origin of compd[b]	mass % ^{18}O
dAMPS	exonuclease I hydrolysis of starting poly[d(T_s-A)]	92 ± 1
pyrophosphate	polymerization of [α-^{18}O]dATPαS and dTTP by DNA polymerase I	0 ± 1
dAMPS	exonuclease I hydrolysis of the poly[d(T_s-A)] intermediate in the degradation scheme	93 ± 1

[a] All enrichments are corrected for the natural abundances of ^{34}S and ^{18}O. [b] See Figure 1.

Stereochemistry. The poly[d(T_s-A)] used in this study possesses chiral phosphorothioate linkages with the R_p configuration as a result of the enzymatic synthesis of the polymer from S_p-[α-$^{18}O_2$]dATPαS (Brody & Frey, 1981). Since the nonbridging oxygen of each phosphorothioate diester is labeled with ^{18}O, chirality is preserved in the [^{18}O]dAMPS that is produced when exonuclease I degrades the polymer (Figure 1). The oxygens of [^{18}O]dAMPS are differentiated via a stereospecific phosphorylation catalyzed by adenylate kinase that yields S_p-dADPαS (Sheu & Frey, 1977; Jaffe & Cohn, 1978). The S_p-[α-^{18}O]dADPαS is phosphorylated by pyruvate kinase, and the resulting [α-^{18}O]dATPαS is incorporated into poly[d(T_s-S)] by DNA polymerase I. The polymerization reaction catalyzed by DNA polymerase I releases into solution inorganic pyrophosphate that contains the oxygen that bridged the α- and β-phosphorus atoms of [α-^{18}O]dATPαS. The nonbridging oxygen remains with the phosphorothioate during polymerization and subsequent degradation to dAMPS. The inorganic pyrophosphate and dAMPS are then analyzed, after the appropriate chemistry is done to obtain volatile phosphate and phosphorothioate derivatives, by GCMS to determine the location of the ^{18}O. The data in Table I clearly show that the stereochemical course of the reaction is inversion (Figure 1); the configuration of the phosphorothioate is inverted in the exonuclease I reaction so that the ^{16}O is phosphorylated by adenylated kinase and incorporated into inorganic pyrophosphate while the ^{18}O is not phosphorylated but rather remains with dAMPS.

Table II: Kinetic Constants for the Reaction of Exonuclease I with Phosphate and Phosphorothioate Substrates[a]

substrate	V_{max}[b]	K_M (M)
$(dA)_{130-170}$	1	$(3 \pm 1) \times 10^{-6}$
pdTpdApdTpdA	0.12	$(4 \pm 2) \times 10^{-5}$
$pdTp_sdApdTp_sdA$	0.006	$(9 \pm 2) \times 10^{-5}$
poly[d(A-T)]	0.004[c]	
poly[d(T_s-A)]	0.008[c]	

[a] Reaction conditions are given in the text. [b] The V_{max} values are relative to that of $(dA)_{130-170}$ which is assigned a value of 1. The actual V_{max} of $(dA)_{130-170}$ is 0.2 mmol/(mg·min). [c] The reactions of poly[d(A-T)] and poly[d(T_s-A)] were done at a single concentration of substrate, 5×10^{-4} M.

Kinetics. Exonuclease I was found by Lehman & Nussbaum (1964) to degrade poly[d(A-T)] by attacking single-stranded frayed ends of the mainly duplex polymer. We find that the enzyme hydrolyzes poly[d(A-T)] 5 times faster than it hydrolyzes poly[d(T_s-A)] (Table II). This rate difference, however, does not accurately reflect the relative rates for hydrolyses of phosphorothioate and phosphate diesters since poly[d(T_s-A)] has a lower T_m than poly[d(A-T)] and hence the thiolated polymer should have a greater concentration of unpaired ends (Eckstein & Joven, 1983). A better comparison employs the tetramers pdTpdApdTpdA and $pdTp_sdApdTp_sdA$ as these molecules are single stranded at 37 °C (Kornberg et al., 1964). Exonuclease I hydrolyzes the terminal residue from each of these molecules, and the production of dAMP or dAMPS is monitored via HPLC.[3] As seen in Table II, the K_M's for the tetramers differ by approximately a factor of 2 while the V_{max} for pdTpdApdTpdA is 20 times that of its thiolated analogue. The reduced rate of phosphorothioate hydrolysis is consistent with phosphorothioate diester bond cleavage, which is chemically slower than phosphate diester cleavage, being at least partially rate limiting in the exonuclease I reaction (Eckstein, 1975, 1983; Frey, 1982). Exonuclease I hydrolyzes $(dA)_{130-170}$ with a V_{max} that is ~8-fold greater and a K_M that is ~1000-fold lower than the values for pdTpdApdTpdA (Table II).

Discussion

We have shown in this study that exonuclease I catalyzes the hydrolysis of chiral phosphorothioate diesters with inversion of configuration about phosphorus. This is the first example of a processive exonuclease whose stereochemical pathway has been determined.

The stereochemical pathways of over 40 enzymatic phosphoryl and nucleotidyl transfer reactions have now been investigated, and the accumulated evidence indicates that each cleavage of a phosphate bond in an enzymatic reaction occurs with inversion of configuration. Thus, a reaction that requires a single displacement at phosphorus will result in inversion, and a reaction that proceeds via two displacements, and hence involves the formation and breakdown of a covalent enzyme intermediate, will yield a product that has retained the starting configuration (Westheimer, 1980; Knowles, 1980; Frey, 1982; Gerlt et al., 1983; Eckstein, 1983). These conclusions are not entirely unambiguous as other mechanisms could yield the stereochemical result of inversion. For example, an active-site carboxylate might attack the phosphodiester to form an acyl phosphate ester. Were such a covalent intermediate to undergo hydrolysis by attack of water on the carboxyl carbon, the stereochemical result would be inversion (Gerlt et al., 1983). Another mechanism that would yield inversion is a triple displacement at phosphorus that proceeds through two covalent enzyme intermediates. There is at present, however, no evidence to compel the consideration of these more complicated mechanisms in any enzyme system. The most likely conclusion, therefore, from the stereochemistry of the exonuclease I reaction is that the enzyme catalyzes the direct attack of water on phosphate diester bonds of deoxyribonucleic acids.

We have performed this study with chiral phosphorothioate diesters rather than phosphate diesters that are chiral by virtue of oxygen isotope substitution at phosphorus (Gerlt et al., 1983; Potter et al., 1983). In the instances where chiral phosphorothioates and phosphates have been used as substrates for the same enzyme, however, the stereochemical courses of the reactions have been identical (Gerlt et al., 1983).

Our configurational analysis involves enzymatic and chemical modification of $[\alpha\text{-}^{18}O]dATP\alpha S$ to determine if ^{18}O or ^{16}O is bridging. The final result is obtained via a GCMS analysis of volatile phosphate and phosphorothioate derivatives. Other methods exist that could have been applied directly to $[\alpha\text{-}^{18}O]dATP\alpha S$ in order to determine the bridging oxygen. These methods are ^{31}P NMR spectroscopy (Frey, 1982; Gerlt et al., 1983) and negative-ion fast atom mass spectrometry (Connally et al., 1984).

The stereochemical results may be considered in light of the processive nature of the exonuclease I reaction (Thomas & Olivera, 1978) in which the enzyme remains bound to a nucleic acid chain during successive hydrolyses of nucleotides. Processive behavior is frequently found in enzymes that react with nucleic acids (Kornberg, 1980), but in no instance is the mechanism understood. Although a determination of the stereochemical pathway of exonuclease I does not elucidate the protein–nucleic acid interactions that enable the enzyme to be processive, the stereochemistry does limit certain mechanistic alternatives. The single displacement mechanism allows for the possibility that the enzyme's active site translocates to the newly exposed 3′-terminal phosphodiester simultaneous with or immediately following diester bond cleavage. A double displacement reaction, on the other hand, would have required both cleavage of the initial diester and hydrolysis of the nucleotidyl-enzyme intermediate before the new 3′-terminal diester could have occupied the active site. Prior to hydrolysis of the intermediate, the new 3′ terminus of the polymer would have had to move away from the catalytic site to allow a water molecule to occupy its position with respect to the phosphorus atom of the intermediate. This additional movement would have been necessary since the stereochemistry of enzymatic reactions at phosphorus (Westheimer, 1980) requires that the water molecule attack the phosphorus atom of the nucleotidyl intermediate at the face that was previously occupied by the leaving 3′ terminus. A double displacement mechanism would thus require several complex steps, and, at least in retrospect, it appears reasonable that exonuclease I reacts via the more direct single displacement mechanism.

Although we lack sufficient data to comment on the generality of the link between processive behavior and a single displacement mechanism for exonucleases, it is instructive to examine the four exonucleases whose stereochemical courses

[3] When the tetramer pdTpdApdTpdA is partially hydrolyzed by exonuclease I, the major reaction product is the trimer pdTpdApdT. Exhaustive hydrolysis produces the 5′-terminal dimer which is inert to further hydrolysis by exonuclease I. In the reaction of exonuclease I with $pdTp_sdApdTp_sdA$, the concentration of trimer is greater than that of dimer during the initial 5% of the reaction. These results are interesting in light of the high processivity shown by exonuclease I in its reaction with polydeoxyribonucleotides (Thomas & Olivera, 1978; R. S. Brody, K. G. Doherty, and P. D. Zimmerman, unpublished results). Studies on the effect of oligodeoxyribonucleotide chain length on the kinetics and processivity of the exonuclease I reaction are in progress.

have been determined (Gupta et al., 1982; Gerlt et al., 1983; Eckstein, 1983; this paper). Two of these enzymes, snake venom phosphodiesterase and bovine spleen phosphodiesterase, yield retention of configuration at phosphorus and hence react via a covalent nucleotidyl-enzyme intermediate. Both these enzymes are nonprocessive (Nossal & Singer, 1968; Thomas & Olivera, 1978). T4 DNA polymerase possesses an exonuclease activity that hydrolyzes phosphodiesters with inversion and is also nonprocessive (Thomas & Olivera, 1978) while exonuclease I is a processive enzyme that reacts with inversion. Thus, single displacement reactions have been found to occur with processive and nonprocessive exonucleases while double displacements have, so far, only been associated with nonprocessive behavior.

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REFERENCES

Brody, R. S., & Frey, P. A. (1981) *Biochemistry 20*, 1245–1250.

Chang, L. M. S., & Bollum, F. J. (1971) *Biochemistry 10*, 536–542.

Connolly, B. A., Eckstein, F., & Grotjahn, L. (1984) *Biochemistry 23*, 2026–2031.

Eckstein, F. (1975) *Angew. Chem., Int. Ed. Engl. 14*, 160–166.

Eckstein, F. (1983) *Angew. Chem., Int. Ed. Engl. 22*, 423–439.

Eckstein, F., & Jovin, T. M. (1983) *Biochemistry 22*, 4546–4550.

Frey, P. A. (1982) *Tetrahedron 38*, 1541–1567.

Gerlt, J. A., Coderre, J. A., & Mehdi, S. (1983) *Adv. Enzymol. Relat. Areas Mol. Biol. 55*, 291–380.

Gupta, A., DeBrosse, C., & Benkovic, S. J. (1982) *J. Biol. Chem. 257*, 7689–7692.

Jaffe, E. K., & Cohn, M. (1978) *Biochemistry 17*, 652–657.

Knowles, J. R. (1980) *Annu. Rev. Biochem. 49*, 877–919.

Kornberg, A. (1980) *DNA Replication*, W. H. Freeman, San Francisco, CA.

Kornberg, A., Bertsch, L. L., Jackson, J. F., & Khorana, H. G. (1964) *Proc. Natl. Acad. Sci. U.S.A. 51*, 315–323.

Laemmli, U. K., & Favre, M. (1973) *J. Mol. Biol. 80*, 575–599.

Lehman, I. R. (1966) *Procedures in Nucleic Acid Research* (Cantoni, G. L., & Davies, D. R., Eds.) pp 203–211, Harper and Row, New York.

Lehman, I. R., & Nussbaum, A. L. (1964) *J. Biol. Chem. 239*, 2628–2636.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Nossal, N. G., & Singer, M. F. (1968) *J. Biol. Chem. 243*, 913–922.

Potter, B. V. L., Connolly, B. A., & Eckstein, F. (1983) *Biochemistry 22*, 1369–1377.

Prasher, D. C., Conaro, L., & Kushner, S. R. (1983) *J. Biol. Chem. 258*, 6340–6343.

Sheu, K.F. R., & Frey, P. A. (1977) *J. Biol. Chem. 252*, 4445–4448.

Thomas, K. R., & Olivera, B. M. (1978) *J. Biol. Chem. 253*, 424–429.

Westheimer, F. H. (1980) *Rearrangements in Ground and Excited States* (deMayo, P., Ed.) Vol. 2, pp 229–271, Academic Press, New York.