reagents such as **2** will be of use in preparing and elucidating the chemistry of sulfenic acids.

Finally, the possibility that the nucleophilic catalytic function of certain sulfhydryl enzymes actually involve transient sulfenic acids needs to be seriously considered. The acyl phosphatase activity of glyceraldehyde-3-phosphate dehydrogenase has, for example, been suggested by Allison[4,5a] and others[5b-d] to involve a stable sulfenic acid.

Acknowledgment. We thank Professor John L. Kice, Texas Tech University, for valuable discussions and Steven Yocklovich for preliminary studies. It is a pleasure to acknowledge support of this investigation by the donors of the Petroleum Research Fund, administered by the American Chemical Society, and the National Science Foundation through Grant CHE-78-19890.

Oxygen Chiral Phosphodiesters. 5. Stereochemical Course of the Hydrolysis of Thymidine 3′-[(4-Nitrophenyl)[^{17}O,^{18}O]phosphate] in $H_2{}^{16}O$ Catalyzed by the Exonuclease from Bovine Spleen

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Determination of the stereochemical course of nucleophilic displacement reactions at phosphorus catalyzed by enzymes is now considered to be the most direct experimental method for determining whether the enzyme-catalyzed reaction involves the formation of a covalent adduct between the enzyme and substrate. Although the use of chiral phosphorothioate substrate analogues to solve these stereochemical problems is often the experimentally easiest approach,[1,2] fears that the results obtained from this approach are mechanistically ambiguous due to the low rates at which these analogues are processed by enzymes has led to the development of methodology for the synthesis and configurational analysis of phosphate mono- and diesters which are chiral by virtue of the three stable isotopes of oxygen.[3-6] The stereochemical consequences of the reactions catalyzed by seven enzymes have now been determined with both oxygen chiral and phosphorothioate substrates;[7] in each case, the stereochemical outcomes obtained from the two approaches were identical, suggesting that sulfur substitution is not expected to alter the stereochemical course of enzyme-catalyzed displacement reactions at phosphorus. In this communication we report the stereochemical course of the hydrolysis reaction catalyzed by the exonuclease from bovine spleen using one of the diastereomers of thymidine 3′-[(4-nitrophenyl)[^{17}O,^{18}O]phosphate]([^{17}O,^{18}O]-TpNP) as substrate. With this enzyme, stereochemical studies of the hydrolysis reaction using the phosphorothioate analogue of TpNP, Tp(S)NP, do not appear to be feasible, since the enzyme does not readily catalyze the hydrolysis of either diastereomer of Tp(S)NP; instead, the enzyme catalyzes a transphosphorylation reaction to yield oligonucleotides as products.[8] Both the retention of the configuration observed for the hydrolysis of [^{17}O,^{18}O]-TpNP and the transphosphorylation reaction observed with the separate diastereomers of Tp(S)NP are in accord with the formation of a nucleotidylated enzyme intermediate during the course of the reaction.

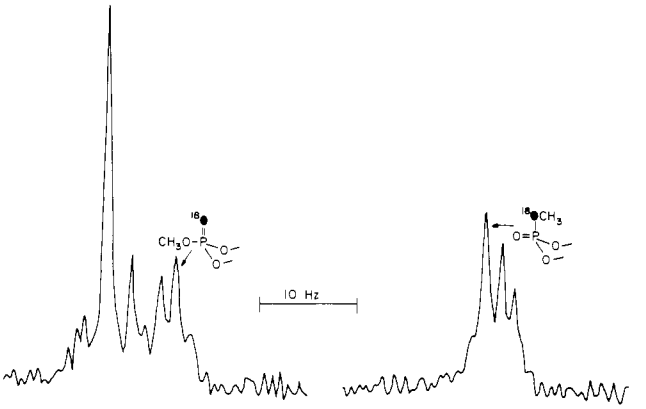
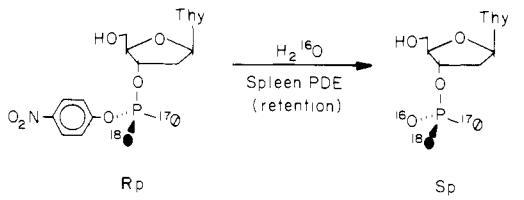


Figure 1. Proton-decoupled ^{31}P NMR spectrum at 81 MHz of the methyl esters of isotopically labeled cTMP derived from enzymatic hydrolysis of the R_P diastereomer of [^{17}O,^{18}O]TpNP in $H_2{}^{16}O$ followed by chemical activation and cyclization. The spectrum was obtained with a 1600-Hz sweep width and a 2-s acquisition time; 400 transients were obtained prior to application of a 0.1-Hz line broadening and Fourier transformation. The approximate chemical shift of the diester and the equatorial methyl ester is −2.7 ppm and that of the axial ester is −3.5 ppm (upfield, relative to an external capillary of 85% H_3PO_4).

Scheme I



The diastereomers of [^{17}O,^{18}O]-TpNP were prepared by reaction of the sodium salts of the diastereomerically pure [^{17}O]-enriched *P*-anilidates of 5′-(monomethoxytrityl)thymidine 3′-[(4-nitrophenyl)phosphate] with $C^{18}O_2$.[9,10] After removal of the trityl groups, the ^{17}O,^{18}O-chiral diesters were purified by chromatography on Amberlite XAD-2. The products which were obtained were identical with an authentic sample of TpNP (Sigma) using the criteria of TLC and ^{1}H NMR spectroscopy at 270 MHz; the ^{31}P NMR spectra at 32 and 81 MHz revealed the expected ratio of ^{16}O,^{18}O and ^{18}O,^{18}O resonances.[11] Instead of assuming that the stereochemical outcome of the reaction of the acyclic

† NIH Research Career Development Awardee (CA-00499), 1978-1983; Fellow of the Alfred P. Sloan Foundation, 1981-1983.

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(11) Since the carbon dioxide used to prepare the chiral diesters was 99% enriched we only observe resonances arising from the ^{16}O and ^{18}O present in the ^{17}O-enriched position.

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P-anilidates with $C^{18}O_2$ would be the expected retention of configuration as verified for the reaction of cyclic *P*-anilidates with [^{18}O]carbonyl compounds,[5,6] we independently determined the configurations of the diastereomers of [^{17}O,^{18}O]-TpNP. Hydrogenolysis of [^{17}O,^{18}O]-TpNP yielded a sample of chiral [^{16}O,^{17}O,^{18}O]-Tp.[12] The configuration of this sample was determined by chemical activation using diphenylphosphorochloridate followed by chemical cyclization to yield a mixture of the three types of oxygen chiral cyclic thymidine 3′,5′-monophosphate (cTMP);[13] following methylation with diazomethane,[14] measurement of the ^{18}O perturbations on the ^{31}P NMR resonances of the axial and equatorial methyl esters of the labeled cTMP[5,7f] revealed that the reaction of the acyclic *P*-anilidates with $C^{18}O_2$ had proceeded with predominant retention of configuration at phosphorus. Methylation of [^{17}O,^{18}O]-TpNP with diazomethane afforded a mixture of diastereomeric methyl esters, and measurement of the ^{18}O perturbations on the ^{31}P NMR resonances of the esters revealed that the optical purity of the [^{17}O,^{18}O]-TpNP is approximately 80%. Stec and co-workers have reported that the optical purity of oxygen chiral cyclic diesters is approximately 95%.[6]

The R_p diastereomer of [^{17}O,^{18}O]-TpNP was hydrolyzed in $H_2{}^{16}O$ by using the exonuclease from bovine spleen (Boehringer) as catalyst;[15] a low substrate concentration (2.7 mM) was selected to minimize the transphosphorylation reaction which this enzyme is known to catalyze.[16] The reaction was allowed to proceed until the amount of 4-nitrophenol liberated was about 50% that expected for complete reaction so that enzyme-catalyzed exchange of product phosphoryl oxygens with solvent oxygen would be minimized. A ^{31}P NMR spectrum of the reaction mixture revealed resonances for only the starting material and Tp; the product was purified by chromatography on DEAE-Sephadex A-25.

The configuration of the ^{16}O,^{17}O,^{18}O-chiral Tp was determined by using the procedure described previously. The ^{31}P NMR spectrum obtained is presented in Figure 1. Examination of this spectrum reveals that the ^{16}O,^{18}O-chiral cTMP formed in the cyclization has ^{18}O located in the axial exocyclic position, leading to the conclusion that the enzyme-catalyzed hydrolysis reaction proceeded with *retention* of configuration at phosphorus (Scheme I). This result can be most easily explained by postulating the necessary formation of a thymidylated enzyme intermediate during the enzyme-catalyzed reaction.

Nucleotidylated enzyme intermediates have not been isolated for the reactions catalyzed by the spleen exonuclease and the snake venom phosphodiesterase. An alternative explanation for the retentions of configuration found for these enzymes[7j] is the formation of a 3′,5′-cyclic nucleotide intermediate, since these enzymes require a free 5′- or 3′-hydroxyl, respectively, for activity; it is well established that the reaction catalyzed by pancreatic ribonuclease proceeds with overall retention of configuration as the result of a two-step reaction involving the formation of a 2′,3′-cyclic nucleotide intermediate.[1] Neither the spleen nor the snake venom enzyme appears to utilize this type of mechanism, since we have observed that neither enzyme will catalyze the hydrolysis of cTMP.

The finding that the hydrolysis reaction catalyzed by the spleen enzyme occurs with retention of configuration and previous reports that this enzyme can catalyze transphosphorylation reactions as well as hydrolysis reactions[16,17] suggest an explanation for recent observations made in the laboratories of Stec and Benkovic[18] regarding the processing of the diastereomers of Tp(S)NP by the enzyme. Both groups observed that the products obtained did not have the ^{31}P NMR chemical shifts expected for Tp(S) (δ_P +43.27 ppm, pH 5.7); resonances downfield of the starting materials [δ_P +51.57 ppm (R_P) and δ_P +51.63 ppm (S_P)] were found. To establish an explanation for these observations, we reacted the separated diastereomers of Tp(S)NP[9] in the presence of the spleen enzyme and purified the reaction products by chromatography on DEAE-Sephadex A-25; from each reaction one product was recovered in addition to unreacted starting material. The ^{1}H-decoupled ^{31}P NMR spectrum of each of these products revealed two phosphorus resonances [δ_P +55.45 and +51.76 ppm (R_P) and δ_P + 55.57 ppm and +51.63 ppm (S_P)] of equal intensity; without ^{1}H decoupling, the downfield resonances are multiplets whereas the upfield resonances are doublets. The ^{1}H NMR spectrum at 270 MHz of each product revealed the presence of one 4-nitrophenyl group and two thymidine moieties. The chemical shifts of the downfield ^{31}P NMR resonances are similar to those reported for the diastereomers of Ap(S)A and of Up(S)A by Benkovic[19] and Eckstein,[20] respectively. Taken together, the ^{31}P and ^{1}H NMR data allow the identification of the products obtained from the reactions catalyzed by the spleen enzyme as isomers of Tp(S)-Tp(S)NP, the products expected from transphosphorylation using the 5′-hydroxyl group of Tp(S)NP as the acceptor. Since only a single resonance is observed in each spectrum for the internucleotide phosphorothioate linkage, the transphosphorylation reaction appears to be stereospecific and may be predicted to occur with retention of configuration at phosphorus. Exclusive transphosphorylation was observed using both 3 and 20 mM concentrations of Tp(S)NP. As indicated previously, no transphosphorylation was observed with 3 mM TpNP at 50% reaction; approximately 20% transphosphorylation was observed with 20 mM TpNP at 50% reaction.

The exclusive formation of transphosphorylation product with the phosphorothioate diesters and the apparent stability of these products in the presence of enzyme[21] suggest that an investigation

(12) One hundred micromoles of diester was dissolved in 25 mL of absolute ethanol, and 100 μL of concentrated HCl and 25 mg of Adam's catalyst were added. The hydrogenolysis was conducted in a Parr apparatus using 50–60 psi of H_2 pressure. The reaction was followed by TLC and was judged to be complete after about 10 h.

(13) One hundred micromoles of Tp (mono-tri-*n*-octylammonium, mono-tri-*n*-butylammonium salt) was dried by repeated evaporation from dry dioxane. The residue was dissolved in 1.5 mL of dry dioxane, a few 4-Å molecular sieves were added, and the solution was allowed to stand for 30 min. Ninety micromoles of diphenylphosphorochloridate was added, and after 30 min the solvent was evaporated. A suspension of approximately 0.5 g of potassium *tert*-butoxide in 20 mL of dry DMF was added with a syringe while flushing the reaction flask with dry N_2. After stirring at room temperature for 10 min, the reaction mixture was poured into approximately 25 mL of Amberlite IR-120 (pyridinium). The resin was removed by filtration and washed with water; the filtrate and washings were combined and evaporated. The residue was redissolved in water, washed with ether, and the aqueous layer was applied to a column of DEAE-Sephadex A-25 (HCO_3^-); the product was eluted with a linear gradient of triethylammonium bicarbonate, pH 7.5. Recent work published by Lowe's laboratory[7g] and this laboratory[7f] has established that the stereochemical course of the cyclization reaction is inversion of configuration at phosphorus.

(14) The triethylammonium salt of cTMP (30–50 μmol) was dissolved in 5 mL of methanol. Five microliters of concentrated HCl was added followed by 2–3 mL of an ethereal solution of diazomethane (0.5 M). Acidification and addition of diazomethane was repeated twice, and the solvent was evaporated after the yellow solution was allowed to stand for 30 min. The sample was prepared for ^{31}P NMR spectroscopy by dissolution in 1.8 mL of 20% D_2O and percolation through a small column of Chelex-100 into an acid-washed 10-mm NMR tube; 0.2 mL of 0.1 M EGTA, pH 7.0, was then added to the NMR tube.

(15) The reaction mixture (76 mL) contained the R_P diastereomer of [^{17}O,^{18}O]-TpNP, ammonium acetate, pH 5.7 (0.167 M), EDTA (0.67 mM), and enzyme (5 μg/mL). The reaction was monitored by diluting 20-μL aliquots of the reaction into 0.98-mL aliquots of 0.1 M Tris–HCl, pH 8.0, and measuring the absorbance at 400 nm. An additional 0.2 mg of enzyme was added after 4.2 h. After 54% hydrolysis (6.9 h), 4-nitrophenol was extracted with ether. The aqueous layer was adjusted to pH 8.0 with ammonium hydroxide and applied to a column of DEAE-Sephadex A-25 (HCO_3^-). The product was eluted with a linear gradient of triethylammonium bicarbonate, pH 7.5. The Tp was further purified by chromatography on Dowex-1 (formate) using ammonium formate as the eluting buffer.

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of the stereochemical course of the hydrolysis reaction catalyzed by the spleen exonuclease would by extremely difficult with phosphorothioate methodology; thus, the results we report in this communication demonstrate an unanticipated advantage in using oxygen chiral phosphate esters to determine the stereochemical course of an enzyme-catalyzed reaction, i.e., the ratio of potential reaction products need not be the same when phosphorothioates are used. This problem can occur only with enzymic reactions which involve the formation of an intermediate which can partition to different acceptors.

In addition, the ability of the enzyme to catalyze an efficient transphosphorylation reaction with phosphorothioate substrates may provide a useful synthetic approach to the synthesis of oligonucleotides which have internucleotide phosphorothioate linkages: with low concentrations of nucleoside 3'-[(4-nitrophenyl)phosphorothioates] and high concentrations of nucleoside or nucleotide acceptors, good yields of a wide variety of oligonucleotide analogues can be anticipated.

Acknowledgment. We thank Professors Wojciech J. Stec and Stephen J. Benkovic for providing us with details of their unpublished observations regarding the behavior of Tp(S)NP in the presence of the spleen enzyme. The high-field ^{31}P NMR spectra essential to this research were obtained at Wesleyan University through the generous cooperation of Professor Philip Bolton and at the NSF Northeast NMR Facility at Yale University (CHE-7916120) with the assistance of Peter Demou. This research was supported by a grant from the National Institutes of Health (GM-22350).

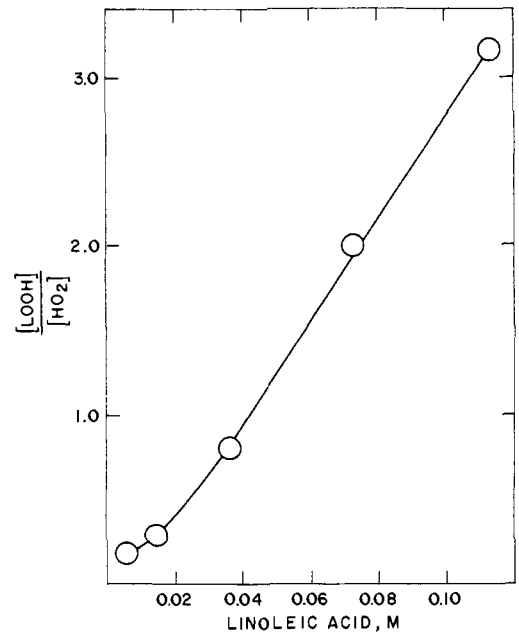

Figure 1. Effect of linoleic acid concentration upon the yield of linoleic hydroperoxide (LOOH) per HO_2 added. 11.5 mL of a photolyzed ethanol solution (70% ethanol, 1.25 mM O_2, 0.01 M KOH) containing 88.15 μM O_2^- were added dropwise to 15 mL of a rapidly stirred 70% ethanol solution containing 0.09 M H_2SO_4 and varying amounts of linoleic acid. The yields monitored at 233 nm (absorption maximum of LOOH) were computed on the basis of the final volume.

Comparison of the Capacities of the Perhydroxyl and the Superoxide Radicals To Initiate Chain Oxidation of Linoleic Acid

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Received June 26, 1981

The discovery that tissues of aerobic organisms contain an enzyme whose specific role appears to be the removal of superoxide radicals[1] has led to the suggestion that O_2^- is a potent agent of biological damage.[2] Many studies support this view. Conditions which lead to the generation of this radical have been associated with some significant pathological disorders[3,4] that can often be prevented by the addition or enhancement of naturally occurring levels of superoxide dismutase.[2] It has been shown that few biochemicals react directly with O_2^-;[5,6] those which do react undergo electron-transfer reactions that occur under normal metabolic conditions and are therefore unlikely to cause cell injury.

It was then proposed[6] that a derivative of O_2^-, an "active oxygen" species such as OH or $^1O_2$ (singlet molecular oxygen), could be the agent of damage. The notion that OH is formed in the so-called Haber-Weiss[7] reaction

$$O_2^- + H_2O_2 \rightarrow OH + OH^- + O_2 \qquad (1)$$

had to be abandoned when its rate constant k_1 was shown to lie between 0.5 and 0.1 M^{-1} s^{-1}.[8] On the other hand, the very short lifetime in water (about 2 μs) and high specificity of its reactions make $^1O_2$ an unlikely candidate for the role of an important biological intermediate. Also, there is evidence accumulating which negates the formation of singlet molecular oxygen from O_2^-.[9] Little can be said at present about an alternative scheme which involves the participation of transition-metal complexes in reactions of O_2^- and H_2O_2. It has long been known that OH can be generated in Fenton-type processes,[10] but the chemical complexity of tissues has so far not allowed the unequivocal demonstration of their occurrence in vivo.

A transient species always formed in presence of O_2^- in water whose possible role in biology has been almost completely ignored is its conjugate acid, the perhydroxyl radical (HO_2). From systematic studies of the rates of reaction of HO_2/O_2^- with a number of biochemicals as a function of pH (Table I) a picture emerges that suggests that HO_2 may be responsible for some of the biological effects presently attributed to O_2^-. Not only does HO_2 react faster than O_2^- with the compounds listed, but its

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