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Inhibition of Micrococcal Nuclease with 5′-Deoxythymidine-5′-phosphonic Acid Containing Oligomers*,†

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ABSTRACT: Micrococcal nuclease catalyzes the hydrolysis of both DNA and RNA. On these substances its action is both endo- and exonucleolytic. This diversity in specificity has made this enzyme an interesting model on which to study the mechanism of nucleic acid hydrolysis. Thymidine nucleotides have been useful in these mechanistic studies; however, thymidine oligonucleotides, because they can be hydrolyzed, have not been used. This report describes a new class of thymidine oligonucleotide analogs composed of 5′-deoxythymidine-5′-phosphonic acid. These oligomers cannot be hydrolyzed by Micrococcal nuclease because they lack the carbon–oxygen–phosphorus bond at $C_{5'}$ of the analog nucleotide. The following substances were tested for their capacity to inhibit the hydrolysis of DNA by Micrococcal nuclease: 5′-deoxythymidine-5′-phosphonic acid (pT*); 5′-deoxythymidylyl-(3′→5′)-5′-deoxythymidine-5′-phosphonic acid (pT*pT*); 5′-deoxythymidylyl(3′→5′)-5′-deoxythymidylyl-(3′→5′)-5′-deoxythymidine-5′-phosphonic acid (pT*pT*pT*); (3′→5′)-cyclic pT*pT* and (3′→5′)-cyclic pT*pT*pT*. All of these substances inhibit; however, the degree of inhibition varies considerably. Thus, on a molar basis, pT* and pT*pT* are about 50% as effective as their thymidine counterparts and pT*pT*pT* is 85% as active. The trimer, pT*pT*pT*, is about 9 times more active than pT* and 3 times more active than pT*pT*. The cyclic analogs were tested because they do not contain a free $C_{5'}$ phosphonic acid end. These substances are less effective than their linear counterparts.

The extracellular phosphodiesterase of *Micrococcus pyrogenes* var. *aureus* hydrolyzes both DNA and RNA yielding dinucleotides and 3′-mononucleotides (Cunningham *et al.*, 1956; Reddi, 1958). Its hydrolytic activity is both exo- and endonucleolytic with phosphodiester bond cleavage occurring preferentially but not absolutely between the dXp-Tp and dXp-dAp bond in DNA and Xp-Ap and Xp-Up in RNA. The position of cleavage is somewhat size dependent with endonucleolytic activity occurring primarily with large polynucleotides (see Cuatrecasas *et al.*, 1968, for summarizing references). In addition to its value as a phosphodiesterase for both DNA (Dirksen and Dekker, 1960; von Hippel and Felsenfeld, 1964) and RNA (Reddi, 1967; Roberts *et al.*, 1962), this enzyme, because of its multiple specificity, provides an interesting model for studies on the mechanism of nucleic acid hydrolysis. Its utility in this regard is further enhanced because it can be readily obtained pure and in quantity and its primary structure has been determined (Taniuchi and Anfinsen, 1966). Certain features of its catalytic site are already known. Their delineation has been greatly assisted by the use of deoxynucleoside 5′-phosphates and deoxynucleoside 3′,5′-diphosphates which are specific inhibitors. Among the most effective nucleotide inhibitors are thymidine 3′,5′-diphosphate and thymidine 5′-phosphate which have been shown to bind the enzyme in a 1:1 ratio in a region which appears to be similar to that which binds both DNA and RNA (Cuatrecasas *et al.*, 1967).

Studies on how the size of polynucleotides effect where the enzyme cleaves phosphodiester bonds is complicated because natural polynucleotides used in such a study can be hydrolyzed by the nuclease. Thus, in this type of study a single polynucleotide species of known size cannot be assured.

In this laboratory we have been concerned with the preparation and use of nucleoside phosphonates in biological systems (Rammler *et al.*, 1967). These substances have the advantage of not being susceptible to hydrolytic cleavage at the $C_{5'}$ position. Their polymerization would thus afford polynucleotides which would be resistant to Micrococcal nuclease action. To this end linear and cyclic homopolymers of 5′-deoxythymidine-5′-phosphonic acid were prepared (Bax and Rammler, 1971[1]). They are schematically represented in Figure 1. Because these substances are unique, no information was at hand concerning their effect on biological systems. This report documents the first example of a specific biochemical effect of this type of polymer, its ability to inhibit Micrococcal nuclease activity.

Materials and Methods

Oligonucleotides. 5′-Deoxythymidine-5′-phosphonic acid containing oligonucleotides were prepared as described by Bax and Rammler (1971).[1] The thymidine-containing oligonucleo-

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† In this publication, the nucleoside phosphonic acids have been described in keeping with the traditional names used for nucleic acid derivatives. The more exact nomenclature used by the American Chemical Society's Organic Indexing Department for the principle monomer reported here is 5′-deoxy-5′-phosphonothymidine. Using the traditional manner for naming nucleotides this substance was called 5′-deoxythymidine-5′-phosphonic acid, and its dinucleotide was named 5′-deoxythymidylyl-(3′→5′)-5′-deoxythymidine-5′-phosphonic acid. These substances were abbreviated as pT* and pT*pT* with an asterisk to indicate the nucleoside phosphonic acid.

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[1] To be published.

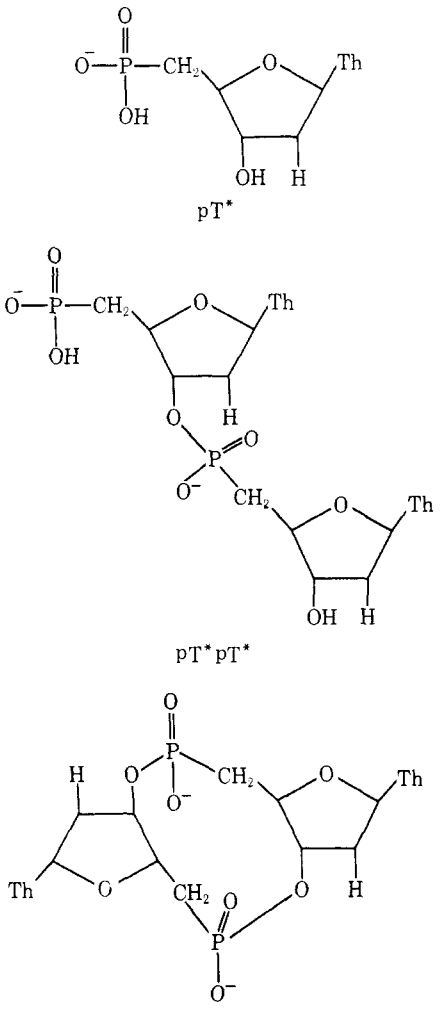

FIGURE 1: 5′-Deoxythymidine-5′-phosphonic acid (pT*); 5′-deoxythymidylyl-(3′→5′)-5′-deoxythymidine-5′-phosphonic acid (pT*-pT*) and cyclic 5′-deoxythymidylyl-(3′→5′)-5′-deoxythymidine-5′-phosphonic acid (pT*pT*).

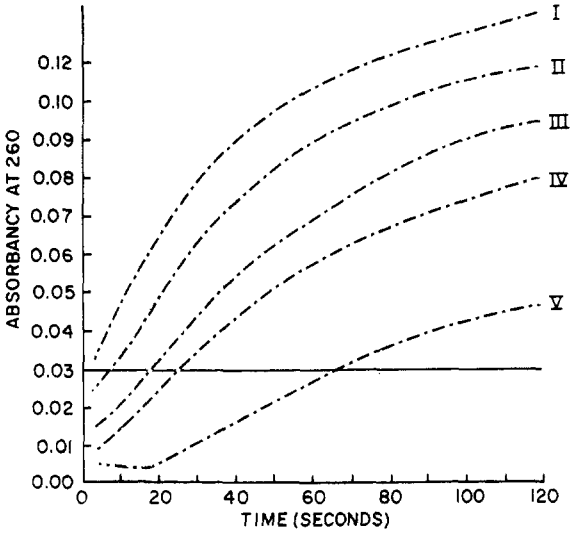

FIGURE 2: Inhibition of Micrococcal nuclease. Curve I is the rate of hyperchromic increase in the absence of oligonucleotide inhibitor. Curve II represents the rate in the presence of pT*; curve III, with pT; curve IV, with pT*pT*; and curve V, with pTpT. The reaction solution was 0.06 mM in inhibitor and was prepared as described in Methods.

tides were a gift from Dr. J. G. Moffatt of this Institute. The concentration of both types of oligonucleotides was determined using a molar extinction value of 9.6×10^3 at 267 nm. No corrections were made for the possible hypochromicity of the oligonucleotides. Enzyme Micrococcal nuclease was obtained from Worthington Biochemicals and salmon sperm DNA from Schwarz Biochemical Co. The DNA was heated to 100° and quick cooled in Dry Ice–acetone for denaturation. The assay procedure was essentially as described by Cuatrecasas *et al.* (1967). The system measures the hyperchromicity at 260 nm which accompanies the hydrolysis of DNA. The reaction solution contained heat-denatured DNA (25 μg), Tris-chloride (0.025 M, pH 8.8), calcium chloride (0.01 M), and Micrococcal nuclease (0.032 μg) all in a total volume of 1 ml. The hydrolysis was carried out at 37° and was initiated by the addition of the enzyme.

Results and Discussion

Inhibitor Properties of Analog Oligomers. Oligomers containing 5′-deoxythymidine-5′-phosphonic acid (pT*) are resistant to cleaveage by Micrococcal nuclease because they do not contain a hydrolyzable phosphorus–oxygen bond at the $C_{5'}$ of the nucleoside as shown in Figure 1. In addition to preventing nuclease hydrolysis at $C_{5'}$, the carbon–phosphorus bond significantly alters the structure of the analog compared with thymidine 5′-phosphate. The most immediate effect of the structural change is to make pT* and oligomers containing it nonisosteric with thymidine 5′-phosphate (pT) and its oligonucleotides; to alter the bond angles between the phosphorus and the $C_{5'}$ carbon atom of the nucleoside and to increase the base strength of the ionizable groups on the phosphorus (Rammler *et al.*, 1967).

In this study the differences in the pK_a's of pT* and pT should not be important because at the pH of the nuclease assay, 8.8, both substances will have the same ionic form. If there is a specific conformation requirement for maximum oligonucleotide binding to the enzyme, this requirement cannot be separately assessed from the effect of the chemical change at $C_{5'}$ because the actual conformational changes induced by the chemical modification at $C_{5'}$ have not as yet been determined in the oligomers. Nevertheless, an approximation of the conformational requirements can be had by comparing the inhibitory capacity of the monomers pT* and pT and relating this to the effectiveness of their oligomers.

The results of the comparison of pT and pT* indicate that the shortened dimensions and/or missing oxygen of the analog has an effect on its inhibitory properties as shown in Figure 2. In this graph, concentration of the inhibitors is 0.06 mM. Curve I is the rate of hydrolysis with no inhibitor and curve II is the rate with pT*. Although not substantial, pT*'s capacity to inhibit is significant; it is 50% of pT shown by curve III. Thus, despite a major structural change, this type of analog appears to retain sufficient nucleotide character to suggest that its oligomers will be useful inhibitors.

The most convincing comparison of the two types of oligomers should be obtained with their dimers because both are resistant to enzymic hydrolysis while larger thymidine oligonucleotides can undergo some hydrolysis. A comparison of the inhibitory action of the dimers is also shown in Figure 2. Curve IV is the rate of DNA hydrolysis with pT*pT* and

curve V, that with pTpT. It is evident that both dimers are substantially more active than their monomers on a molar basis; yet, their relative activity remains similar to that found for their monomer, that is, pTpT is about 50% more active than pT*pT*.

The inhibition caused by the dimers has two interesting characteristics, namely the apparent constancy in the difference of activity between them and between their monomers and their increased activity over the monomers. With the monomers this 50% difference in activity must stem from the structural change in pT* and since this feature is present in the dimer, pT*pT*, a similar reduction in relative activity is reasonable. On the other hand, the increased activity of both types of dimers must result from a structural feature common to both. The most obvious similarity is an esterified $C_{3'}$ hydroxyl, that is, both contain a carbon–oxygen–phosphorus bond at $C_{3'}$. This notion is consistent with the finding of Cuatrecasas *et al.* (1967) who showed thymidine 3′,5′-diphosphate to be about 3 times more effective an inhibitor than pT. It is apparent from the structures of the dimers that they can be analogized with thymidine 3′,5′-diphosphate. For example, pTpT can be considered to be a thymidine ester of thymidine 3′,5′-diphosphate. In addition, the substantial enhancement of activity found for pT*pT* suggests that the binding of the dinucleotide with the enzyme is not severely influenced by the nature of the phosphate ester at $C_{3'}$. This suggestion was tested using the trinucleotide pTpTpT and its analog pT*pT*pT*. If the enhanced inhibition found for the dinucleotide results solely from its "diphosphate end," the activity of the trimers should be similar to that found for dimers because this feature is common to both. The results of this comparison, given in Table I, show that this is not the case and demonstrate that the trinucleotide is considerably more effective than the dinucleotide. Because pTpTpT can be hydrolyzed by the nuclease, a more reliable comparison between homologs is obtained with the analog oligomers. In this comparison, pT*pT*pT* is almost three times more effective than pT*pT* suggesting that more is involved in the inhibitory reaction than just the "diphosphate end." Interestingly, the 50% difference in activity between the analog and its natural counterpart found for the monomers and dinucleotides has been substantially reduced with this higher homolog.

Among the deoxynucleosides tested, only those which have a phosphomonoester group at $C_{3'}$ and $C_{5'}$ show substantial inhibitory activity (Cuatrecasas *et al.*, 1967). In this report, we have shown that the nature of the phosphate ester at $C_{3'}$ is not critical. However, the effect of a phosphodiester at both $C_{3'}$ and $C_{5'}$ is unknown. This type diester, of course, more closely resembles the enzyme's natural substrate than pT or thymidine 3′,5′-diphosphate. The problem is that it cannot be prepared in the linear oligonucleotide series. These latter substances must have ends and unless ended by a nucleoside cannot be prepared without a phosphomonoester termini. Thus, the inhibitory reaction associated with a 5′-phosphomonoester group cannot be excluded with linear oligonucleotides. One type of oligonucleotide which is completely bonded *via* phosphodiesters is cyclic as shown in Figure 1. The use of this type of small oligonucleotide is important in mechanistic studies of phosphodiesterases because they present to the catalytic site an oligonucleotide of infinite length, that is, one with no ends. We, therefore, measured the inhibitory capacity of these substances and these data are given in Table I. As indicated, a free 5′-phosphomonoester group is not an absolute requirement for inhibition and in all cases, the activity of the cyclic oligonucleotides is reduced compared with their linear counterparts. Again, because the cyclic trinucleotide is hydrolyzed by the enzyme, the most reliable comparison is found with the analogs. Interestingly, no change in activity is found between the two types of analog dimers but a significant reduction is found for cyclic pT*pT*pT* compared with pT*pT*pT*. The substantial increase in the inhibition with the trimers, both linear and cyclic, over the dimers suggests an additional binding site for maximum effectiveness. Thus, in the case of the linear trimer, this requirement is more fulfilled than with its shorter cyclic homolog and less than with the longer pTpTpT. This supposition is not unusual and is consistent with the observation that this enzyme can distinguish between long polynucleotides on which its action is endonucleolytic and oligonucleotides on which its action is primarily exonucleolytic.

TABLE I: A Comparison of Thymine-Containing Inhibitors.[a]

5′-Deoxythymidine 5′-phosphonic and Oligomers	% Inhibition	Thymidine 5′-Phosphate and Oligomers	% Inhibition
pT*	8	pT	16
pT*pT*	22	pTpT	38
pT*pT*pT*	69	pTpTpT	79
pT*pT* 	20	pTpT 	21
pT*pT*pT* 	53	pTpTpT 	65

[a] The concentration of the oligonucleotides was 0.029 mM. The reaction was carried out as described in the section on Methods. The inhibitory activity of the dimers pTpT and pT*pT* was shown to be proportional to their concentration to at least 0.03 mM.

One observation found in all of the kinetic analyses with inhibitors deserves comment. It is the hypochromicity always found immediately after beginning the assay. This observation is shown in Figure 1. In these experiments, the initial optical density of the reaction solution, less enzyme, was set at 0.03 unit. Addition of the enzyme to the reaction less the inhibitor, provided the rate of hyperchromicity given by curve I. Addition of enzyme to the pT*pT* reaction not only reduced the rate of hyperchromicity as shown by curve VI, but also significantly affected its initial absorbancy. In this case, the initial absorbancy was reduced to about 0.01 unit and then proceeded to increase at a rate of about 20% that of the control. The addition of enzyme to the reaction containing pTpT reduced the initial absorbancy even further and delayed the onset of hyperchromicity by about 20 sec. The cause of the initially reduced absorbancy with the inhibitors is unknown. It apparently does not have a trivial explanation such as the order of mixing, temperature, source of DNA, or enzyme and it was found for all of the oligonucleotides and analogs tested.

The purpose of this report has not been to make definitive proposals concerning the mode of action of this enzyme. This is apparent from the extent and scope of the kinetic data presented. Rather, the study has been carried out to demonstrate a first impression of the utility of a completely new class of oligonucleotide analogs in enzymological studies.

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Specificity of Rat Liver Lysine Transfer Ribonucleic Acid for Codon Recognition*

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ABSTRACT: Rat liver lysine tRNA was separated into two well-defined isoaccepting species by reversed-phase Freon chromatography, and the coding properties of each peak were determined in an *Escherichia coli* ribosomal-binding assay. Lys-tRNA$_{I}$ binding was specific for ApApG, whereas Lys-tRNA$_{II}$ was specific for ApApA. In a tRNA-dependent, cell-free, protein-synthesizing system from rat liver, the addition of tRNA$_{II}^{Lys}$ gave a 2- to 4-fold stimulation of lysine incorporation with a poly(A) messenger, whereas tRNA$_{I}^{Lys}$ was only slightly active. When precharged samples of Lys-tRNA$_{I}$ and Lys-tRNA$_{II}$ were added to the rat liver protein-synthesizing system, Lys-tRNA$_{II}$ readily incorporated lysine with poly(A), whereas Lys-tRNA$_{I}$ was inactive. Both Lys-tRNAs were active with a poly(AG) (3:2) message and the ratio of incorporation of Lys-tRNA$_{II}$:Lys-tRNA$_{I}$ approximated the ratio of AAA:AAG in the poly(AG) random copolymer. Both peaks were also active in a rabbit reticulocyte system with Lys-tRNA$_{I}$ giving a 2-fold greater incorporation of lysine into hemoglobin. Therefore, tRNA$_{I}^{Lys}$ and tRNA$_{II}^{Lys}$ in the rat liver appear to be specific for translating the codons AAG and AAA, respectively. The unusual specificity of tRNA$_{II}^{Lys}$ for AAA may be due to a thiolated base in the anticodon region since I_2 oxidation of Lys-tRNA$_{II}$ resulted in a loss of ribosomal-binding activity.

Fractionation of tRNA from a tissue frequently results in multiple peaks of acceptance activity for the same amino acid (Hatfield and Portugal, 1970; Nishimura and Weinstein, 1969; Söll and RajBhandary, 1967; Caskey *et al.*, 1968). Lysine tRNA may be fractionated into two isoaccepting species from *Escherichia coli* (Söll and RajBhandary, 1967), guinea pig liver (Caskey *et al.*, 1968), and from rat liver (Nishimura and Weinstein, 1969). The coding specificity of these two lysine tRNA species for the condons AAA and AAG vary in the literature. With *E. coli*, Söll and RajBhandary (1967) reported that both peaks synthesized protein in response to poly(A) and poly(A-A-G) messages. With guinea pig liver, Caskey *et al.* (1968) concluded that one Lys-tRNA peak binds with ApApG and the other binds with both ApApA and ApApG in an *E. coli* ribosomal-binding assay. Nishimura and Weinstein (1969) however, reported that both peaks of rat liver Lys-tRNA[1] responded equally well to poly(A) and poly(AG) (3:1) in a ribosomal-binding assay. Carbon *et al.* (1965) showed that Lys-tRNA$_{I}$ binds with ApApG and poly(AG), whereas a mixture of Lys-tRNA$_{I}$ and Lys-tRNA$_{II}$ binds with poly(A) as well. We wish to report in this communication that rat liver tRNA$_{I}^{Lys}$ and tRNA$_{II}^{Lys}$ recognize ApApG and ApApA, respectively, in an *E. coli* ribosomal-binding assay. Furthermore, that the specificity observed in the ribosomal-binding assay is also reflected in a poly(A)- and poly(AG)-dependent protein-synthesizing system. A preliminary account of this work has been presented (Liu and Ortwerth, 1971).

Material and Methods

Preparation of Rat Liver tRNA. tRNA was prepared from livers of Wistar rats by phenol extraction and ethanol precipitation. Livers were homogenized in 2 volumes of buffer containing 0.15 M KCl, 0.01 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, 0.001 M Na_2EDTA, and 0.02% polyvinyl sulfate. The homogenate was centrifuged for 20 min at 30,000*g*. The supernatant fraction was extracted with an equal volume of water-saturated phenol at 4° for 60 min. The aqueous layer, after centrifugation, was precipitated with 2.5 volumes of 95% ethanol and stored overnight at −18°. The precipitated nucleic acids were dissolved in a minimum amount of buffer C (0.05 M NaCl, 0.01 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, and 0.001 M Na_2EDTA). The tRNA was further purified by applying it to a 2.0 × 30 cm DEAE-cellulose column which

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[1] Abbreviations used are: tRNA$_{I}^{Lys}$, the first major peak of lysine tRNA to elute from a reversed-phase Freon column; tRNA$_{II}^{Lys}$, the second major peak of lysine tRNA to elute from a reversed-phase Freon column; [^{14}C]Lys-tRNA, tRNA aminoacylated with [^{14}C]lysine; poly(AG) (3:2), a random copolymer containing adenine and guanine residues in a ratio of 3:2.