Studies on Polynucleotides

IV. ENZYMIC DEGRADATION. THE STEPWISE ACTION OF VENOM PHOSPHODIESTERASE ON DEOXYRIBO-OLIGONUCLEOTIDES*

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(Received for publication, March 9, 1959)

In the preceding paper (1) detailed studies of the properties and specificity of the venom diesterase with a variety of phosphodiesterases as substrates were reported. Extension of these studies to several homologous series of synthetic deoxyribonucleic acids has permitted further insight into the mode of action of this diesterase on substrates containing multiple phosphodiester bonds. It has been found that attack by the venom diesterase occurs stepwise from the end of the oligonucleotide chain bearing a 3'-hydroxyl group and results in the successive liberation of nucleoside 5'-phosphate units. The present communication contains a detailed report of these findings as well as a discussion of the use of this enzyme in the problem of sequential analysis of polynucleotides. Preliminary reports of this work have been published (2-4).

EXPERIMENTAL

Enzymes—Venom phosphodiesterase was purified by acetone precipitation followed by chromatography as described previously (1). These preparations were stable at 2° for several weeks. However, the same preparation was not used throughout. Prostate phosphomonoesterase was a preparation kindly given by Dr. R. Markham (5). This preparation was virtually free from dies- terase activity as tested against deoxyribo-oligonucleotides.

Oligonucleotides—TpT, TpTpT, and higher homologues, TpT, TpTpT, and higher homologues, and the corresponding cyclic-oligonucleotides (cyclo-pTpT and homologues) were all synthesized chemically in this laboratory (4, 6, 7); pTpTpC and pTpTpTpC were also synthesized chemically.

Chromatography—Paper chromatographic assay described in the preceding paper (1) was used to measure the rate of disappearance of the starting material. Chromatography is the only valid method presently available to follow the hydrolysis of polymeric compounds and to separate the products. The Rf's of cyclic oligonucleotides in Solvent A (1), relative to pT (1.0), are: cyclo-pTpT, 2.5; cyclo-pTpTpT, 0.75; cyclo-pTpTpTpT, 0.35.

RESULTS

Degradation of Thymidine Oligonucleotides Lacking 5'-Phosphate End Groups—Results of a kinetic study of the hydrolysis of TpTpTpTpT are shown in Fig. 1. In Fig. 2 are plotted the results of a second experiment in which chromatography was prolonged to improve separation of the larger intermediates, with loss of thymidine and TpT. The amount of enzyme used and the timing for these experiments (11 µg. for 0.27 µmole of initial substrate and 12 µg. for 0.85 µmole, respectively) were based on the rate of degradation of TpT (1) (Vmax for this as previously determined, 300 µmoles per hour per mg. of protein).

Thus all the lower homologues appear successively; mononucleotide accumulates and thymidine appears only towards the completion of degradation. The mononucleotide is, as expected, thymidine 5'-phosphate, since it was rapidly dephosphorylated by the 5'-nucleotidase in crude venom.

Individual experiments with lower homologues, TpTpT and TpTpT, confirmed the above pattern of stepwise hydrolysis. Furthermore with TpTpC, the first mononucleotide to be liberated was pC.

These results point to a stepwise attack by the enzyme from the end of the oligonucleotide chain bearing the 3'-hydroxyl group (Diagram I).

Hydrolysis of Oligonucleotides Bearing 5'-Phosphate End Groups—Results of kinetic study of the degradation of pTpTpTpTpT are shown in Fig. 3. Again, all the lower homologues are detected as intermediates and it is concluded that the degradation is stepwise. It may be noted that in a separate experiment, the addition to the reaction mixture of 0.02 M Mg++ did not affect the pattern of degradation.

An interpretation of these results, which is consistent with those described in the preceding section, is that in this series as well, the degradation proceeds from the end of the chain bearing the 3'-hydroxyl group. The suggestion to the contrary has, however, been made previously by Privat de Garilhe and Las-kowski (8); namely, that the enzyme may cleave successively.


Vol. 234, No. 8, August 1959
Printed in U.S.A.
nucleoside 5'-phosphate units from polynucleotide ends bearing 5'-phosphate groups. In order to decide between the two possibilities, experiments were carried out on (a) thymidine tetranucleotide in which the 3'-hydroxyl group was acetylated (Diagram 2, I) and (b), on the trinucleotide, pTpTpC (Diagram 2, II).

Results of degradation of 3'-O-acetyl thymidine tetranucleotide (I) are shown in Fig. 4. This hydrolysis was carried out at pH 8, in view of the lability of the acetyl group in more alkaline solution. Although substitution of the 3'-hydroxyl group by acetyl slows down the attack by the enzyme (1), 3'-O-acetylthymidine 5'-phosphate was again found to be the first mononucleotide product of the action of the enzyme. Since the second product of initial attack, pTpTpT, was expected to be attacked more rapidly than the acetylated tetranucleotide, pTpTp and pT were

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**Figure 1.** Reproduction of a chromatogram illustrating a kinetic study of hydrolysis of TpTpTpTpT by diesterase. System, in 37 µL: 10 µmoles of Tris, pH 8.9; 13 OD units (267 nm) of TpTpTpTpT (0.27 µmole); 11 µg. of diesterase. Aliquots of 6 µL removed at times shown, mixed with 1 µL of glacial acetic acid and chromatographed in Solvent A, overnight. In the figure the origin is at the left. Products are 1, TpTpTpTpT; 2, TpTpTpT; 3, TpTpT; 4, pT; 5, TpT; 6, thymidine. Numbers within spots are the percentage of the thymidine residues (OD at 267 nm) present in the aliquot.

**Figure 2.** Kinetic study of degradation of TpTpTpTpT by diesterase. System as in Fig. 1 except 41 OD units (0.85 µmole) of substrate, 12 µg. of diesterase, and chromatography continued 3 days (TpT and thymidine have run off the paper). Products are Curve 1, pT; Curve 2, TpTpT; Curve 3, TpTpT; Curve 4, TpTpTpT; Curve 5, TpTpTpTpT. The numbers of the curves correspond to the number of nucleosides in the products.

**Figure 3.** Kinetic study of degradation of pTpTpTpTpT by diesterase. System contained 20 OD units (0.53 µmole) of pTpTpTpT, 10 µmoles of Tris, pH 8.9, 17 µg. of diesterase (paper chromatography on pTpT indicated 520 µmoles per hour per mg. of protein), in 54 µL. Aliquots of 16 µL chromatographed 4 days in Solvent A. Numbers within spots are the percentage of the thymidine residues (OD at 267 nm) present in the aliquot.

**Diagram 1**

Schematic representation of the action of the diesterase on TpTpTpTpT. Dashed arrows refer to the points at which hydrolysis occurs.
also to be expected in the short time aliquot. pTpTpT, the tri-nucleotide formed first, coincided with the starting material in the solvent system used (Solvent B) (1), and its amount was calculated by hydrolysis of the eluted material with ammonia and subsequent chromatography in Solvent A (see legend to Fig. 4). The results thus again indicated that degradation began from the end bearing the 3'-acetyl group. Had it begun from the end bearing the 5'-phosphate group, pT would have accumulated far in advance of 3'-acetyl-pT, and intermediates of the type 3'-O-acetyl-pTpTpT and lower homologues should have resulted. None of these products was detected. The possibility that such products might have been superimposed on the spots corresponding to the unacetylated lower homologs was eliminated by eluting the spots, treating the eluate with ammonia, and rechromatographing in Solvent A.

Confirmatory results, obtained on the hydrolysis of pTpTpC are shown in Table I. Thus the mononucleotide formed first was pC, the dinucleotide pTpT was a major intermediate, and no pTpC was detected.

Hydrolysis of Cyclic Oligonucleotides—Intermediates in the hydrolysis of cyclic-pTpT, cyclic-pTpTpT, and so forth, have not been observed. Incubation of these compounds with diesterase results in a slow liberation of pT (1), the rate of which appears independent of the size of the oligonucleotide. Thus, at a time when 39 per cent of the cyclic-pTpT had been cleaved to pT, 43 per cent of the cyclic-pTpTpT, and 35 per cent of the cyclo-pTpTpTpT were hydrolyzed. Although the initial product of enzymic attack must be the corresponding linear oligonucleotide, failure to detect it is probably due both to its greater rate of hydrolysis and to tighter binding to the enzyme of the linear form and subsequent linear intermediates (1).

**DISCUSSION**

The present work has elucidated the mode of action of the venom phosphodiesterase. Thus, hydrolysis occurs stepwise from the end of the chain bearing the 3'-hydroxyl group on the terminal nucleoside. Work carried out by Singer et al. (9) and our own more recent studies on polyribonucleotides confirm this mode of action of the enzyme.

The above "exopolynucleotidase" type of action by the enzyme is not absolute, since cyclic oligonucleotides, which lack terminal groups, were also slowly hydrolyzed. In addition, pre-

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**TABLE I**

**Hydrolysis of pTpTpC**

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>pTpTpC (0.22)</th>
<th>pTpTpT (0.56)</th>
<th>pC (0.70)</th>
<th>pT (1.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.08</td>
<td>0.018</td>
<td>0.02</td>
<td>0.002</td>
</tr>
<tr>
<td>6</td>
<td>0.06</td>
<td>0.027</td>
<td>0.04</td>
<td>0.022</td>
</tr>
<tr>
<td>15</td>
<td>0.04</td>
<td>0.036</td>
<td>0.05</td>
<td>0.034</td>
</tr>
</tbody>
</table>

* Figures in parentheses are Rf values.
vious studies with oligonucleotides bearing 3'-phosphomonoester groups (10) as well as recent experiments in this laboratory (11) have shown that these compounds, which are hydrolyzed even more slowly, are attacked rather randomly at points within the oligonucleotide chain. From the competitive inhibition studies and the constant ratio of activities toward linear and cyclic oligonucleotides during purification (1) it appears that only one enzyme is involved in the exo- as well as endopolyribonucleotidase action.

The exopolynucleotidase action of the enzyme offers promise for the sequential analysis of polynucleotides bearing 3'-hydroxyl (deoxyribopolynucleotides) or 2'- and 3'-hydroxyl groups (ribopolynucleotides). From the present results it is clear that the endopolynucleotidase action is insignificant for oligonucleotides of this type and it is possible that it would be reduced further due to the hydrogen bonded internal structure of larger polynucleotides. Earlier studies (12) had indicated that deoxyribonucleic acids are attacked in an exopolynucleotidase fashion and a recent study by Adler et al. (13) on venom phosphodiesterase degradation of deoxyribonucleic acids in which a limited number of labeled deoxyribonucleotide units were added to the 3'-hydroxyl end of the pre-existing polynucleotide chains, have confirmed this view and furthermore provided confirmation of the mode of action of the enzyme as established here.

Concurrent work with spleen phosphodiesterase (14), which already has been briefly reported (3), shows that this enzyme also preferentially attacks polynucleotides in a stepwise and exopolynucleotidase fashion. Its action is complementary to that of the venom diesterase in that it begins from the end of the chain bearing the 5'-hydroxyl group, liberating nucleoside 3'-phosphate residues. The mode of action shown for these enzymes appears to be common to others, for example, polynucleotide phosphorylase (15).

**SUMMARY**

Kinetic studies of the degradation of several series of deoxyribonucleotides by venom phosphodiesterase have been carried out. The compounds studied were thymidine oligonucleotides lacking terminal phosphomonoester groups, thymidine oligonucleotides bearing 5'-phosphomonoester groups, thymidine tetranucleotide bearing a 3'-O-acetyl group at one end, and a 5'-phosphomonoester group at the other end, and deoxycytidylyl-(5'-3')-thymidylyl-(5'-3')-thymidylic- (5') acid. The general mode of action of the phosphodiesterase has been shown to be stepwise from the end of the oligonucleotide chain bearing a 3'-hydroxyl group, resulting in the successive liberation of nucleoside 3'-phosphate units.

Cyclic oligonucleotides which do not have any terminal group were hydrolyzed slowly by the phosphodiesterase; no intermediates could be detected during their degradation.

**REFERENCES**

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