Deoxythymidine 3',5'-Di-p-nitrophenyl Phosphate as a Synthetic Substrate for Bovine Pancreatic Deoxyribonuclease*

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SUMMARY

Bovine pancreatic deoxyribonuclease liberates p-nitrophenol from the 3'-group of deoxythymidine 3',5'-di-p-nitrophenyl phosphate. A similar hydrolysis occurs with deoxythymidine 3'-p-nitrophenyl phosphate and with deoxythymidine 3'-p-nitrophenyl phosphate 5'-phosphate, but the rate is less than 2% of that with the di-p-nitrophenyl ester. The rate of formation of the p-nitrophenol, measured spectrophotometrically at 400 nm, varies linearly with DNase concentration. The binding of the substrate is not strong (Km(app) in the 10 mM range), but the hydrolysis is rapid; 1 µg of DNase (free from other phosphodiesterases) can be assayed in 3 min after addition to a 10 mM substrate solution at pH 7.2, 10 mM in MnCl₂, and 1 mM in CaCl₂.

All four bovine pancreatic DNases (A, B, C, and D) show the same relative activities toward DNA and toward the di-p-nitrophenyl ester; both activities are lost when DNase is inactivated by iodoacetate or by trypsin.

The specificity of DNase toward the di-p-nitrophenyl substrate is different from that which has been established for the enzyme's predominant action on DNA or synthetic oligonucleotides, where a monoesterified phosphate group is formed at the 5'-position.

Pancreatic deoxyribonuclease is an enzyme for which there has been no small synthetic substrate that is hydrolyzed rapidly (1). p-Nitrophenyl esters have proved to be convenient substrates for use in studies on the kinetics and mechanisms of action of a number of phosphodiesterases (2, 3). Razzell and Khorana (4) have shown that snake venom phosphodiesterase hydrolyzed the p-nitrophenyl ester of deoxythymidine 5'-phosphate about 1000 times more rapidly than it hydrolyzed di-p-nitrophenyl phosphate. Bernardi and Bernardi (5) have found that preparations of spleen acid deoxyribonuclease released p-nitrophenol at a slow rate from the p-nitrophenyl ester of deoxythymidine 3'-phosphate. Cuatrecasas et al. (6), in an extensive study of the susceptibility of synthetic substrates to hydrolysis by staphylococcal nuclease, found the strongest binding (Km(app) = 9.6 × 10⁻⁶) and rapid hydrolysis with the di-p-nitrophenyl ester of deoxythymidine 3',5'-diphosphate. The present experiments were undertaken to ascertain whether this compound, or one of its analogs, might be a suitable substrate for pancreatic deoxyribonuclease.

EXPERIMENTAL PROCEDURE

Materials—NO₂Ph-pdTp, pdTp-NO₂Ph, and NO₂Ph-pdPp-NO₂Ph (Ba salts) were obtained from Aldrich. NO₂Ph-p-NO₂Ph, NO₂Ph-p-dTp-NO₂Ph, NO₂Ph-pdT-NO₂Ph, NO₂Ph-pdT, and p-nitrophenol were from Sigma; pdTp was from P-L Biochemicals. All of these chemicals were homogeneous by electrophoresis at pH 8.0. Snake venom phosphodiesterase (from Crotales atrox) was obtained from Sigma; the powder was dissolved in buffer immediately before use. Trypsin was from Worthington.

Assays—The hydrolysis of the synthetic substrates was followed by measuring the liberated p-nitrophenol at 400 nm on a Zeiss PMQ II spectrophotometer equipped with a linear scale-expander (0 to 0.2 A, full scale) and connected to a recorder with a variable chart speed (usually 1 inch per min). The temperature for all assays was maintained at 25°C. In order to conserve samples of the p-nitrophenyl esters, the assay was conducted in a small volume (100 µl). The cells had a cross-sectional area 2 x 10 mm (Scientific Cells, series 460); the light entrance aperture in the photometer

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A slit width of 0.2 mm was employed. When the cell and the light beam were well aligned the instrument could be readily brought to zero absorbance by amplification.

In a routine assay with DNase, 10 \( \mu l \) of a solution of DNase in the assay buffer (representing 1 to 5 \( \mu g \) of DNase) were added to 100 \( \mu l \) of the substrate dissolved in 0.025 \( M \) Tris HCl, pH 7.2, 10 mM in MnCl2, and 1 mM in CaCl2. The slope was measured in the first 1 to 5 min, before the absorbance reached 0.1. A blank, without the enzyme, gives no measurable p-nitrophenol. The \( k_a \) for p-nitrophenol at pH 7.2 was 11,000. One unit of activity was equal to the release of one micromole of p-nitrophenol per min. Specific activities were based upon concentrations of DNase determined with calf thymus DNA as substrate (8); in that assay a unit of activity was equal to the release of one micromole of p-nitrophenol per min per mg, the pH optimum for the substrate is at about pH 6.4.

The conditions adopted for the assay represent a compromise to conserve substrate. With 10 \( \mu M \) NDPh-pdTp-pDOPh, a result. Shack and Bynum (11), in assays with DNA as substrate, have shown that variations in the bivalent metal and substrate concentrations can affect the rate in several ways. Product inhibition is another possible factor. We have not undertaken to study the kinetic parameters in detail with NDPh-pdTp-pDOPh as substrate. However, the essentiality of bivalent metals and the increased activity in the presence of Ca\(^{2+}\) are illustrated in Fig. 3. The synergistic effect of Ca\(^{2+}\) is similar to that first shown by Wiberg (12) when DNA was used as the substrate. NDPh-pdTp-pDOPh was used as the Ba salt in our experiments; the presence of Ba\(^{2+}\) does not have an effect in the assay, the results of which were unchanged if the Ba salt was removed by passage of the substrate solution over Dowex 50 in the Na\(^{+}\)form.

**Fig. 1 (left).** Specific activity of DNase A in the presence of varying amounts of NDPh-pdTp-pDOPh and different concentrations of MnCl2. The assay solution was 0.025 \( M \) Tris HCl, pH 7.2, 1 mM in CaCl2, and contained the indicated concentrations of MnCl2. The concentration of NDPh was 6 \( \mu M \) in 110 \( \mu l \).

**Fig. 2 (right).** The initial rate of hydrolysis of NDPh-pdTp-pDOPh with different amounts of DNase A per 110 \( \mu l \) of assay medium. The hydrolyses were performed in 0.025 \( M \) Tris HCl, pH 7.2, 1 mM in CaCl2, and 10 mM in MnCl2.

**RESULTS**

**Hydrolysis of NDPh-pdTp-pDOPh by DNase A**—Preliminary experiments showed that with 6 \( \mu M \) of DNase A the amounts of p-nitrophenol liberated in 1 min from the di-p-nitrophenyl ester were readily measurable when the substrate concentration was 10 to 20 mM. This type of result, shown in Fig. 1 for different concentrations of MnCl2, indicated that the binding of the substrate was not strong, but that the hydrolysis rate was rapid. At a given substrate concentration the initial rate of hydrolysis varies linearly with enzyme concentration (Fig. 2). The most practical pH for the assay is about pH 7.2. The \( k_a \) of p-nitrophenol is about 6.8. and only the unprotonated form has the absorbance at 400 nm; measurements of p-nitrophenol are therefore most sensitive if the pH of the assay solution is above pH 7. In terms of the absorbance readings, the maximum response is obtained at pH 7.2. When each absorbance reading is corrected for the change in the \( k_a \) of p-nitrophenol with pH in order to give the specific activity of the enzyme in terms of micromoles of p-nitrophenol liberated per min per mg, the pH optimum for the hydrolysis is at about pH 6.4.

A reciprocal plot, of the Michaelis-Menten type, of the data used for Fig. 1 does not give a straight line. There are a number of characteristics of DNase action which may contribute to such
The assays were performed in 0.025 M Tris HCl, pH 7.2. The concentration of substrate was 5 mM; 5 μg of DNase A were present in 110 μl. Concentrations of all ions were 10 mM and the chlorides were used.

<table>
<thead>
<tr>
<th>Divalent metal ion</th>
<th>Without CaCl₂</th>
<th>1 mM in CaCl₂</th>
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</thead>
<tbody>
<tr>
<td>Cd²⁺</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>8.3</td>
<td>4.6</td>
</tr>
<tr>
<td>Mg²⁺</td>
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<tr>
<td>Mn²⁺</td>
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<td>109.4</td>
</tr>
<tr>
<td>Ni²⁺</td>
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<td>0.0</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>7.5</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Fig. 4. Electrophoretic separation of the products formed by the action of DNase A and snake venom phosphodiesterase on various p-nitrophenyl esters of deoxythymidine phosphates. The conditions of hydrolysis are defined in Table II, except that the time was extended to 16 hours. Electrophoresis was carried out on a flat plate at 45 volts per cm for 1 hour in 0.06 M sodium phosphate, pH 7.0, on Whatman No. 3MM paper. The abbreviations are defined in Footnote 1, except SVPase for snake venom phosphodiesterase. The yellow p-nitrophenol spots are shaded.

1 μg of DNase A can be measured with a reproducibility of about 5%.

Identification of Products Formed by Action of DNase A on NO₂Ph-pdT-NO₂Ph—In order to determine the specificity of DNase A toward this substrate, which contains four different phosphoester bonds, the products of the hydrolysis were analyzed qualitatively by paper electrophoresis (Fig. 4). Line 6 in the figure shows that the only detectable nucleotide product is NO₂Ph-dTp (line 2, Fig. 4, and Table II). Thus, with this type of synthetic substrate for DNase, the major product has a phospho group in the 3'-position.

This specificity can be compared with that of other phosphodiesterases that have been tested against NO₂Ph-pdT-NO₂Ph. Rassell and Khorana (4) have shown that snake venom phosphodiesterase liberates p-nitrophenol from the 5'-phospho group. We have repeated such tests with snake venom phosphodiesterase with our series of substrates and have included the data, for comparison, in Fig. 5 and Table II. Even though our preparation of snake venom phosphodiesterase was not a purified enzyme, the specificity which we have observed was clear-cut and in full agreement with that found by Rassell and Khorana (4). The comparison with DNase A is not a simple one, however, since DNase A (1) and the snake venom enzyme (4) both hydrolyze DNA to give measurable amounts of 5'-nucleotides, yet against the p-nitrophenyl esters they show opposite specificities.

Comparison of activities of DNase A and snake venom phosphodiesterase against p-nitrophenyl esters of deoxythymidine phosphates

The substrate concentrations were 10 mM. For the DNase A assays, the buffer was 0.025 M Tris HCl, pH 7.2, 1 mM in CaCl₂, and 10 mM in MnCl₂; the DNase concentration was 5 μg per 110 μl. For the snake venom phosphodiesterase assays, the buffer was 0.1 M Tris HCl, pH 8.8, 10 mM in MgCl₂; 10 μg of the crude enzyme were used per 110 μl.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>dTp-NO₂Ph</td>
<td>1.0</td>
</tr>
<tr>
<td>NO₂Ph-pdT</td>
<td>0.0</td>
</tr>
<tr>
<td>dTp-NO₂Ph</td>
<td>1.6</td>
</tr>
<tr>
<td>NO₂Ph-pdT</td>
<td>0.0</td>
</tr>
</tbody>
</table>
| NO₂Ph-pdT-NO₂Ph | 100 -

* In each instance the activity of the enzyme against NO₂Ph-pdT-NO₂Ph was taken as 100.

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Cautrecasas et al. (6) have shown that staphylococcal nuclease hydrolyzes NO₂Ph-pdT-NO₂Ph predominantly to give p-nitrophenyl phosphate plus dTp-NO₂Ph. With DNase there is no electrophoretic evidence for the formation of p-nitrophenyl phosphate (Fig. 4) and when analysis for the presence of the compound was performed spectrophotometrically at 320 to 350 nm (0), no change in the absorbance was observed. Thus, these three enzymes, DNase A, snake venom phosphodiesterase, and staphylococcal nuclease hydrolyze NO₂Ph-pdT-NO₂Ph in three different ways.

**Proof that Hydrolysis of NO₂Ph-pdT-NO₂Ph is an Intrinsic Property of DNase**—The preceding conclusions on the action of DNase on this synthetic substrate depend upon the absence of any contaminating phosphodiesterase that might have a specificity different from that of DNase. A pancreatic phosphodiesterase studied by Terao and Ukita (14) and by Hurst and Findley (15) hydrolyzes only p-nitrophenyl esters of 5'-phospho groups, and could not account for the present results. Further evidence can be obtained by checking chromatographically purified DNases A, B, C, and D (7, 8, 16-18) against NO₂Ph-pdT-NO₂Ph; the four enzymes have similar activities against DNA and if the action against the synthetic substrate is an inherent property of the chemical structure, the specific activities against DNA (measured by hyperchromicity), and the p-nitrophenyl ester substrate should run parallel. The ratios (× 10⁻⁵) of the specific activities against DNA and NO₂Ph-pdT-NO₂Ph were...
2.88, 2.36, 2.52, and 2.85 for DNases A, B, C, and D, respectively. Thus, all four of the enzymes possess close to the same relative activity toward the two substrates. In addition, the two activities were lost in parallel when we inactivated DNase A by iodoacetate under the conditions defined by Price et al. (19) or exposed the enzyme to inactivation by trypsin in Ca²⁺-free solution (20, 21).

**DISCUSSION**

NO₂Ph-pdTp-NO₂Ph is a synthetic substrate for pancreatic DNase which provides a rapid, highly sensitive assay for the enzyme. The assay is potentially useful only with purified DNase since many phosphodiesterases have the capability of hydrolyzing p-nitrophenyl esters. The hydrolysis by DNase occurs in minutes compared to hours with low molecular weight synthetic oligonucleotides (22). A single bond is cleaved, which should facilitate kinetic analysis.

The specificity of the cleavage, which yields a 3'-monophosphate group, does not correlate with the specificity of the hydrolysis of polynucleotides by the same enzyme. With DNA (reviewed in Ref. 1) and with oligonucleotides (22) as substrates, pancreatic DNase A has been characterized as a producer of 5'-mononucleotides. The two types of substrates are thus hydrolyzed at differently placed ester bonds by pancreatic DNase. On the other hand, snake venom phosphodiesterase (4), spleen acid DNase (5), and staphylococcal nuclease (6) all show consistent specificity toward oligonucleotides and the p-nitrophenyl analogs.

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**REFERENCES**

Deoxythymidine 3', 5'-di-p-nitrophenyl phosphate as a synthetic substrate for bovine pancreatic deoxyribonuclease.

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