# The Action of Staphylococcal Nuclease on Synthetic Substrates*

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ABSTRACT: A number of *p*-nitrophenyl ester derivatives of deoxythymidine 5′-phosphate can be hydrolyzed by staphylococcal nuclease, with concomitant release of *p*-nitrophenyl phosphate. The appearance of free *p*-nitrophenyl phosphate can be continuously monitored spectrophotometrically by following the increase in absorbance at any wavelength in the range 310–350 mμ. The shift of the ultraviolet spectrum of *p*-nitrophenyl phosphate (to higher wavelengths) accompanies conversion of the mono- into the dianionic phosphate species. Consideration of the patterns of cleavage of the synthetic substrates, the structures of various 5′-deoxythymidyl inhibitors, and the relation of these to the observed kinetic constants, permit a partial formulation of the mode of action and specificity of this enzyme. The basic structural unit necessary for recognition as substrate appears to be R-pdT-R′, hydrolysis resulting in the release of R-phosphate. The enzyme appears to be essentially unspecific with respect to R. In the absence of an R substitution, there is strong competitive inhibition of activity. The R′ substituent can be small since the maximal rates of hydrolysis, $K_{cat}$, are achieved with a 3′-OH or 3′-acetyl substitution. The nature of the R′ group, however, contributes very significantly to the strength of binding of the substrate (or inhibitor) to enzyme. There is a 200-fold increase in affinity (of substrate and of inhibitor) if R′ is a phosphate rather than a hydroxyl or acetyl group, emphasizing the importance of the phosphate substituents.

Micrococcal nuclease is an extracellular phosphodiesterase of *Staphylococcus aureus* that can hydrolyze either RNA or DNA to produce 3′-phosphomononucleosides and dinucleotides. Considerable data have been obtained on a variety of physicochemical properties of this protein (for review, see Cuatrecasas *et al.*, 1969a). Studies of catalytic mechanisms and substrate specificity, however, have been hampered by the unavailability of substrates of low molecular weight. Studies of substrate specificity have hitherto been performed with nucleic acids and oligonucleotides, and the only satisfactory substrates available for the assay of enzymatic activity have been DNA and RNA (Cuatrecasas *et al.*, 1967a, 1969a).

The present paper presents studies on various synthetic substrates that can be utilized for convenient and sensitive measurement of the activity of this and other phosphodiesterase enzymes. The substrates described are, principally, simple *p*-nitrophenyl esters of derivatives of deoxythymidine 5′-phosphate. Many *p*-nitrophenyl compounds have been used successfully in the past as synthetic substrates for measuring the activity of a variety of esterases including the phosphodiesterases from spleen and liver (Khorana, 1961; Razzell, 1963), since upon hydrolysis they release a chromophore, *p-nitrophenol*. The present studies demonstrate that, above a pH of 6, the release of *p-nitrophenyl phosphate* by staphylococcal nuclease from a diester results in a shift in the ultraviolet spectrum of the chromophore which may be followed spectrophotometrically. Consideration of the sites of cleavage of the substrates described here, as well as the related kinetic constants, permits specific formulation of the mode of action of staphylococcal nuclease.

## Materials and Methods

*Enzymes.* Staphylococcal (Foggi strain) nuclease, which is purified in the final step by passage through a phosphorylated cellulose column (Fuchs *et al.*, 1967), was purchased from Worthington.

Spleen phosphodiesterase (bovine) and venom phosphodiesterase were purchased from Worthington.

*Determination of Extinction Coefficients.* The molar extinction coefficients, $E_M$, of those compounds containing *p*-nitrophenyl ester groups were determined on the basis of the amount of *p*-nitrophenol release after heating (100°) the compound in 1 N NaOH. Ultraviolet and visible spectra were obtained at pH 7, after adjustment with NaOH to 1 N, and periodically during heating until completion of hydrolysis. Hydolysis was complete between 2 and 4 hr with all the compounds used in this study.

The $E_M$ of *p*-nitrophenol (at 405 mμ) was 17,200 (1 N NaOH). The extinction coefficients of those compounds lacking *p*-nitrophenyl ester groups were determined on the basis of weights obtained after drying in a desiccator over phosphorus pentoxide.

*Chromatography.* The solvent system used for paper chromatography was *n*-butyl alcohol–acetic acid–water (4:1:5, v/v). For thin-layer chromatography the solvents used were (A) isopropyl alcohol–$NH_4OH$–water (7:1:2, v/v) and (B) 1-butanol–acetic acid–water (4:1:5, v/v).

*Ultraviolet Spectra and Kinetics.* Ultraviolet spectra and difference spectra were obtained at room temperature by means of a Cary Model 15 spectrophotometer. Two sets of cells in tandem were used for the difference spectra, as described previously (Cuatrecasas *et al.*, 1967b). The Michaelis constant, $K_{M_{app}}$, and the catalytic rate constant, $K_{cat}$, were obtained from Lineweaver–Burk plots (Lineweaver and Burk, 1934). Dissociation constants, $K_i$, were obtained from Dixon plots (Dixon, 1953), from the inhibition of enzymatic release of *p*-nitrophenyl phosphate from nitrophenyl-pdTp-nitro-

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phenyl[1] (Cuatrecasas *et al.*, 1969b). At least two substrate concentrations were used for each study, and the results were confirmed by independent measurements of $V_{max}$. All measurements of enzymatic activity were obtained by measuring absorbance changes continuously with a Gilford Model 2000 multiple-sample absorbance recorder equipped with a Beckman DU monochromator. Four assays can be done simultaneously. The temperature of the cuvet chamber was maintained at 24°. In all instances, initial velocities were obtained from the early linear stages of substrate hydrolysis.

Nuclease concentration was determined by measurements of absorbance at 277 or 280 mμ with the use of $E^{1\%}_{1\,cm}$ of 9.70 and 9.30, respectively (Fuchs *et al.*, 1967). The molecular weight of the enzyme is 16,800 (Taniuchi *et al.*, 1967).

*Substrates and Inhibitors.* dTp-nitrophenyl and nitrophenyl-pdT were obtained from Raylo Chemicals Ltd., Alberta, Canada; both were chromatographically pure on thin-layer chromatography. Nitrophenyl-pdT also was purchased from Calbiochem; the compound was separated from several impurities, which represented about 30–40% of the total material based on absorption at 270 mμ, by chromatography for 20 hr on Whatman No. 3MM paper using *n*-butyl alcohol–acetic acid–water (4:1:5, v/v). The dried chromatograms were sprayed with 1 N NaOH and heated at 80° to detect any nitrophenyl-containing compound. Two such yellow substances were found, the principal one being nitrophenyl-pdT ($R_F$ 0.95). Elution was performed with distilled water. Prolonged incubation of dTp-nitrophenyl and nitrophenyl-pdT with staphylococcal nuclease resulted in the release of *p*-nitrophenol and deoxythymidine 3′-phosphate, and *p*-nitrophenyl phosphate and deoxythymidine, respectively, as determined by thin-layer chromatography with solvents A and B.

3′-*O*-Acetylnitrophenyl-pdT and 5′-*O*-acetylnitrophenyl-dTp were prepared by treating the corresponding *p*-nitrophenyl derivatives with acetic anhydride in anhydrous pyridine according to the methods described by Razzell and Khorana (1959).

Nitrophenyl-pdTpdT was found as the major contaminant of the nitrophenyl-pdT purchased from Calbiochem. Of the seven ultraviolet spots detected on the paper chromatograms, it was the fourth from the origin, with an $R_F$ of 0.35. It was detected by spraying with 1 N NaOH, and it was eluted in pure form with distilled water. This compound accounted for about 15% of the contaminating material having absorbancy at 270 mμ. Incubation of this compound with staphylococcal nuclease (0.05 M borate buffer (pH 8.8) and 10 mM $CaCl_2$) resulted in rapid release of *p*-nitrophenyl phosphate. It was determined, by thin-layer chromatography, that after prolonged incubation with nuclease the starting compound was completely converted into *p*-nitrophenyl phosphate, deoxythymidine 3′-phosphate, and deoxythymidine. Release of *p*-nitrophenyl phosphate by staphylococcal nuclease was complete before hydrolysis of dTpdT could be detected. Incubation of the substrate with snake venom diesterase resulted in disappearance of the starting compound and in the appearance of *p*-nitrophenol and deoxythymidine 5′-phosphate. Ultraviolet spectral studies of this compound after complete hydrolysis in base (1 N NaOH, 3 hr, 100°) revealed that the concentration of *p*-nitrophenol (405 mμ) was half that of the thymidine derivatives (265 mμ) released during hydrolysis.

The following compounds were kindly provided by Dr. Michael Sporn, National Cancer Institute: nitrophenyl-pdTp, nitrophenyl-pdTpdTp-nitrophenyl, 5′-sulfate-dTp-nitrophenyl, nitrophenyl-pdTp-methyl, methyl-pdTp-nitrophenyl, fluoro-pdT, dTp-fluoro, 5′-chloromethyl-pdTp-nitrophenyl, and sulfate-dT-sulfate. These compounds were synthesized by Dr. Ronald P. Glinski of Ash Stevens Inc., Detroit, Mich., in Contract pH-43-66-929 with the National Cancer Institute. All the compounds are pure by thin-layer chromatography and elemental analysis.

*Deoxythymidine 3′,5′-Diphosphate.* This compound was purchased from Calbiochem. It has also been synthesized in this laboratory by procedures similar to those used by Tener (1961) for the synthesis of pdCp; 1 mmole of thymidine (242 mg) was dissolved in 10 ml of pyridine, and 6 ml of a molar solution of cyanoethyl phosphate (pyridinium salt) was added. The solution was concentrated to dryness *in vacuo*, dissolved in 2 ml of pyridine, and concentrated again to dryness. It was then dissolved in 10 ml of pyridine, 3.6 g of dicyclohexylcarbodiimide was added, and the mixture was left for 2 days at room temperature; 2 ml of water was then added. After 1 hr, 100 ml of water was added and the precipitate was removed by filtration; 1.5 ml of concentrated $NH_4OH$ was added and the solution was heated to boil and allowed to concentrate to about 40 ml. It was then boiled under reflux for 45 min. After cooling, the material was passed through a column of Dowex 50 (in the hydrogen form) to remove ammonia and excess pyridine. A saturated solution of barium hydroxide was added, and the mixture was neutralized with carbon dioxide and centrifuged. The solution was concentrated *in vacuo* to 30 ml and centrifuged to remove excess inorganic barium salt. It was then concentrated again to about 10 ml and boiled for a few minutes. The granular precipitate that formed was collected. The yield of deoxythymidine 3′,5′-diphosphate $Ba_2$ was 300 mg. The material was pure and identical with authentic pdTp by thin-layer chromatography on solvents A and B, ultraviolet spectrum, and inhibition of staphylococcal nuclease activity.

*Nitrophenyl-pdTp-nitrophenyl.*[1] Deoxythymidine 3′,5′-diphosphate sodium salt (586 mg, 1 mmole) was desalted by passing through a Dowex 50 (hydrogen form) column (10 × 1 cm). After lyophilization, the residue was suspended in dry pyridine and concentrated to dryness *in vacuo* at room temper-

[1] The abbreviations used are: nitrophenyl-pdT and nitrophenyl-pdTpdT, *p*-nitrophenyl esters of deoxythymidine 5′-phosphate and deoxythymidyl-(5′→3′)-deoxythymidylic (5′-) acid, respectively; nitrophenyl-pdTp-nitrophenyl, thymidine 3′,5′-di-*p*-nitrophenyl phosphate; nitrophenyl-pdTpdTp-nitrophenyl, di-*p*-nitrophenyl phosphate ester of dithymidine; dTp-nitrophenyl, *p*-nitrophenyl ester of deoxythymidine 3′-phosphate; nitrophenyl-pdTp, deoxythymidine 5′-*p*-nitrophenyl phosphate 3′-phosphate; pdTp-nitrophenyl, deoxythymidine 3′-*p*-nitrophenyl phosphate 5′-phosphate; pdTp-aminophenyl, deoxythymidine 3′-*p*-aminophenyl phosphate 5′-phosphate; nitrophenyl-pdTp-methyl, deoxythymidine 3′-methyl phosphate 5′-*p*-nitrophenyl phosphate; methyl-pdTp-nitrophenyl, deoxythymidine 3′-*p*-nitrophenyl phosphate 5′-methyl phosphate; aminophenyl-pdT, *p*-aminophenyl ester of deoxythymidine 5′-phosphate; 5′-sulfate-Tp-nitrophenyl, thymidine 3′-*p*-nitrophenyl phosphate 5′-sulfate; 3′-*O*-acetylnitrophenyl-pdT, 3′-*O*-acetyldeoxythymidine 5′-nitrophenyl phosphate; 5′-*O*-acetyl-dTp-nitrophenyl-5′-*O*-acetyldeoxythymidine 3′-*p*-nitrophenyl phosphate; fluoro-pdT and dTp-fluoro, deoxythymidine 5′-fluorophosphate and deoxythymine 3′-fluorophosphate, respectively; 5′-chloromethyl-pdTp-nitrophenyl, deoxythymidine 5′-chloromethylphosphonate 3′-*p*-nitrophenyl phosphate; sulfate-dT-sulfate, deoxythymidine 3′,5′-disulfate. Nitrophenyl-pdTp-nitrophenyl is now available from Ash Stevens Inc., Detroit, Mich.

TABLE I: Synthetic Substrates and Inhibitors of Staphylococcal Nuclease.[a]

| Compound | $K_{Mapp}$ (M) | $K_{cat}$ ($min^{-1}$) | $K_i$[b] (M) | Group Released |
|---|---|---|---|---|
| Nitrophenyl-pdT | $2.2 \times 10^{-3}$ | 9.1 | | Nitrophenyl phosphate |
| 3′-*O*-Acetylnitrophenyl-pdT | $2.1 \times 10^{-3}$ | 9.2 | | Nitrophenyl phosphate |
| Nitrophenyl-pdTp | $1.0 \times 10^{-5}$ | 10.6 | | Nitrophenyl phosphate |
| Nitrophenyl-pdTp-nitrophenyl | $4.2 \times 10^{-5}$ | 10.5 | | Nitrophenyl phosphate |
| | $6.4 \times 10^{-3}$ | 0.1 | | Nitrophenol[c] |
| Nitrophenyl-pdTpdT | $1.7 \times 10^{-5}$ | 9.3 | | Nitrophenyl phosphate[d] |
| Nitrophenyl-pdTpdTp-nitrophenyl | $9.6 \times 10^{-6}$ | 10.2 | | Nitrophenyl phosphate |
| | $4.6 \times 10^{-2}$ | 0.03 | | Nitrophenol[c] |
| Nitrophenyl-pdTp-methyl | $5.9 \times 10^{-5}$ | 10.9 | | Nitrophenyl phosphate[e] |
| Aminophenyl-pdT | | | | Aminophenyl phosphate |
| Methyl-pdTp-nitrophenyl | | | | Methyl phosphate[f] |
| | $1.3 \times 10^{-2}$ | 0.08 | | Nitrophenol |
| Fluoro-pdT | | | | Fluorophosphate |
| dTp-nitrophenyl | $2.2 \times 10^{-2}$ | 0.7 | | Nitrophenol |
| 5′-Sulfate-dTp-nitrophenyl | $5.9 \times 10^{-3}$ | 1.9 | | Nitrophenol |
| 5′-*O*-Acetyl-dTp-nitrophenyl | $2.1 \times 10^{-3}$ | 0.7 | | Nitrophenol |
| 5′-Chloromethyl-pdTp-nitrophenyl | $1.7 \times 10^{-2}$ | 0.4 | | Nitrophenol |
| dTp-fluoro | | | $10^{-3}$[g] | |
| pdT | | | $1.9 \times 10^{-4}$[h] | |
| pdTp | | | $2.0 \times 10^{-7}$[h] | |
| Sulfate-dT-sulfate | | | $10^{-3}$[g] | |
| pdTp-nitrophenyl | | | $1.1 \times 10^{-6}$ | |
| pdTp-aminophenyl | | | $1.0 \times 10^{-6}$ | |
| pdTpdT | | | $6.3 \times 10^{-7}$[h] | |

[a] All the values were determined in 0.05 M borate buffer (pH 8.8) and 10 mM $Ca^{2+}$, according to the procedures described in the text. [b] Inhibition was competitive in all cases. [c] From initial velocities determined simultaneously at 330 and 405 mμ with a dual-wavelength recorder (see text). [d] Slow cleavage of dTpdT occurs, after complete release of *p*-nitrophenyl phosphate (see text). [e] There was no cleavage of the methyl group (see text). [f] Release of methyl phosphate was much more rapid than *p*-nitrophenol release, as determined by thin-layer chromatography. [g] No detectable inhibition of enzymatic activity. [h] From Cuatrecasas *et al.* (1969b).

ature. It was resuspended in dry pyridine, 2.8 g (20 mmoles) of *p*-nitrophenol was added, and it was concentrated to dryness. The mixture was then dissolved in 10 ml of dry pyridine and 4 g (20 mmoles) of dicyclohexylcarbodiimide was added. The mixture was left for 3 days at room temperature, with stirring. The solvent was then removed *in vacuo* at 25° and the residue was suspended in 10 ml of water and washed with ether. The aqueous solution contained all the unreacted pdTp as well as about 20% of the product; 2 equiv of NaOH was added to the ether solution which was then extracted twice with 25 ml of water. The aqueous filtrate was extracted three times with ether and passed through a column of Dowex 50 (hydrogen form). The acid effluent was brought to pH 3.5 with NaOH and extracted with ether repeatedly until the extracts no longer formed a yellow color on shaking with $NH_4OH$. It was then evaporated to dryness, and the residue was dissolved in 5 ml of methanol. After removal of a small amount of insoluble material by centrifugation, 50 ml of ether was added. The white, flocculent precipitate that formed was filtered and washed with ether. The compound obtained was chromatographically pure on thin-layer chromatography on solvent A ($R_F$ 0.8). The yield was 490 mg (65%). After incubation at 100° for 4 hr in 1.0 N NaOH, 2 equiv of *p*-nitrophenol was released per equiv of pdTp, as measured by ultraviolet spectra ($E_M$ of pdTp, 9600; $E_M$ of *p*-nitrophenol, 17,200). *Anal.* Calcd for $C_{22}H_{20}N_4Na_2O_{15}P_2 \cdot 4H_2O$: C, 34.7; H, 3.6; N, 7.3; P, 8.2. Found: C, 34.1; H, 3.3; N, 7.1; P, 8.5.

*pdTp-nitrophenyl.* Nitrophenyl-pdTp-nitrophenyl (200 mg) was dissolved in 30 ml of 0.2 M $NH_4HCO_3$ (pH 8.2) containing 5 mM $MnCl_2$ and 2 mg of snake venom diesterase. The solution was left at room temperature until there was no further release of *p*-nitrophenol (overnight). The solution, after adjusting the pH to 4.5 with 1 N HCl, was extracted with ether until addition of NaOH no longer produced a yellow color in the ether extracts. The aqueous solution was then lyophilized, dissolved in 4 ml of 0.05 M ammonium acetate (pH 5.5), and passed through a 3.5 × 45 cm column of Sephadex G-15. This was passed through a column (1 × 5 cm) of Dowex 50 in the hydrogen form; the product eluted well after the protein. After lyophilization, the residue was dissolved in a small volume of water and converted into the sodium salt by bringing the pH of the solution to 3.5 with 1 N NaOH. This solution was lyophilized, then dissolved in methanol, and precipitated with ether. The yield was 120 mg. The $\lambda_{max}$ of the compound was 272 mμ, and the $E_M$, 15,500. The material was pure on thin-layer chromatography with solvents A and B. *Anal.* Calcd for $C_{16}H_{16}N_3O_{13}P_2 \cdot 3H_2O$: C, 29.86; H, 3.42; N, 6.53; P, 9.60. Found: C, 30.31; H, 3.48; N, 6.83; P, 9.56.

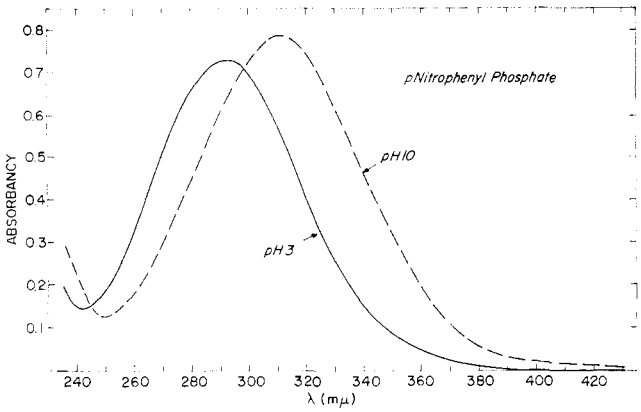


FIGURE 1: Shift of the ultraviolet spectrum of *p*-nitrophenyl phosphate (86 μM) which occurs upon complete ionization of the phosphate group. The $\Delta E_M$ values at 320, 330, and 340 mμ are 4020, 4200, and 3580, respectively.

*pdTp-aminophenyl.* This compound, previously employed for affinity chromatography as a Sepharose conjugate (Cuatrecasas *et al.*, 1968), was prepared by reducing pdTp-nitrophenyl by catalytic hydrogenation. Palladium on charcoal (10%, w/w) was added to a methanolic solution of pdTp-nitrophenyl (about 0.5 g/30 ml), and reduction was carried out at room temperature in a Parr hydrogenation apparatus for 1.5 hr at 35 psi (Boyland and Manson, 1957). The palladium was removed by filtration and the solution was concentrated to dryness. The residue was redissolved in a small volume of methanol and precipitated by addition of ether. It was recrystallized from aqueous ethanol. The yield was quantitative. It was chromatographically pure on thin-layer chromatography on solvent A ($R_F$ 0.37). The $\lambda_{max}$ of this compound was 268 mμ, and the $E_M$, 8800, at pH 7.0.

## Results

*Substrates, Inhibitors, and Specificity of Action of Nuclease.* A number of synthetic deoxythymidine 5′-phosphate esters of the general class, R′-pdT-R, are readily hydrolyzed to R′-phosphate and dT-R by staphylococcal nuclease (Table I). The chromophoric *p*-nitrophenyl esters have been the most extensively studied. However, the nature of the ester substituent is not a major determinant of substrate specificity. Aminophenyl-pdT, as well as an alkyl phosphate ester, methyl-pdT-R, are also hydrolyzed. The substrate, methyl-pdTp-nitrophenyl, is rapidly hydrolyzed by staphylococcal nuclease into methyl phosphate and dTp-nitrophenyl, the latter identified by thin-layer chromatography. *p*-Nitrophenol and dTp are released upon incubation with spleen diesterase if the substrate is previously digested with the staphylococcus enzyme. pdTp-nitrophenyl is formed on incubation of this substrate with venom diesterase, but no hydrolysis is detected if the substrate has first been digested with staphylococcal nuclease.

It is interesting that the requirement for a phosphodiester linkage is not absolute, since deoxythymidine 5′-fluorophosphate is cleaved slowly to deoxythymidine (by thin-layer chromatography) and fluorophosphate. However, the 5′-ester bond of the phosphonium derivative, 5′-chloromethyl-pdTp-nitrophenyl, is not cleaved. Furthermore, a 5′-carboxylic acid ester, 5′-*O*-acetyl-dTp-nitrophenyl, is not hydrolyzed by the enzyme into acetic acid and dTp-nitrophenyl.

Although it is not known whether the base is an essential feature of a substrate, the nucleoside moiety appears to be necessary since bis- and tris-*p*-nitrophenyl phosphate esters are not detectably hydrolyzed by the nuclease. The nature of the nucleoside moiety, or of the R′ substitution, clearly affects the affinity of the substrate (or inhibitor) for the enzyme, even though it does not affect the catalytic rate constant (Table I). For example, the presence of a phosphate on the 3′ position of nitrophenyl-pdT results in a 200-fold increase in affinity. The important role of the 5′- and 3′-phosphate groups in determining the affinity of inhibitors has been emphasized previously (Cuatrecasas *et al.*, 1967a, 1969a), and their contribution to substrate affinity, found in the present study, is in harmony with those observations (Table I). It is clear, furthermore, that these effects are due to the phosphoryl groups themselves, rather than to alteration or elimination of the free hydroxyl groups, since *O*-acetyl substitution (of nitrophenyl-pdT and dTp-nitrophenyl) does not substantially affect the kinetic constants obtained with the substituted derivatives (Table I).

The effects of the phosphate groups on affinity can only be very weakly mimicked by sulfate groups (Table I). For example, a threefold fall in $K_M$ results by placing a sulfate on the 5′ position of dTp-nitrophenyl; placement of a phosphate in the same position causes a 20,000-fold decrease in $K_M$. The $K_i$ of pdTp is $2 \times 10^{-7}$ M, whereas with sulfate-pdTp-sulfate, inhibition of activity could not be detected.

It appears that the basic structural element for substrate specificity is R-pdT, with release of Rp upon enzymatic hydrolysis. The nature of the R group is essentially unimportant with regard to specificity. Further, nucleotide units added to the right, or 3′ side, of this substrate increase the affinity (Table I). Derivatives having a free 5′-phosphate are strong competitive inhibitors of DNA and synthetic substrate hydrolysis. The dissociation constants of a series of 5′-phosphoryl oligothymidyl derivatives decrease with increasing chain length until a trinucleotide is reached (Cuatrecasas *et al.*, 1969b).

These observations are all consistent with the view that substrate binding "subsites" exist in the enzyme which interact primarily with the substrate structure to the right (or 3′ side) of the diester bond which is to be hydrolyzed, or to the right of the free 5′-phosphoryl moiety in the case of inhibitors.

It is noteworthy that, in the absence of a free 5′-phosphate group, nucleosides having a 3′-*p*-nitrophenyl ester group, such as dTp-nitrophenyl, can be cleaved, albeit poorly, resulting in the release of 3′-nucleoside and *p*-nitrophenol. Staphylococcal nuclease also causes release of both *p*-nitrophenyl phosphate and *p*-nitrophenol from substrates that contain both types of diester substituents, such as nitrophenyl-pdTp-nitrophenyl. The rate of release of both products can be followed simultaneously in a reaction mixture by continuous spectrophotometric measurements with an alternating dual-wavelength (330 and 410 mμ) device, as will be described shortly. During the early phases of the reaction (always less than 10 min), used to compute initial velocities, the rate of release of *p*-nitrophenol is less than 5% the rate of release of *p*-nitrophenyl phosphate.[2] In addition, *p*-nitrophenol contributes very little

[2] Under varying conditions of pH and $Ca^{2+}$ concentration there is at least a 20-fold difference in the $K_M$ and $K_{cat}$ values measured by the release of *p*-nitrophenyl phosphate and *p*-nitrophenol.

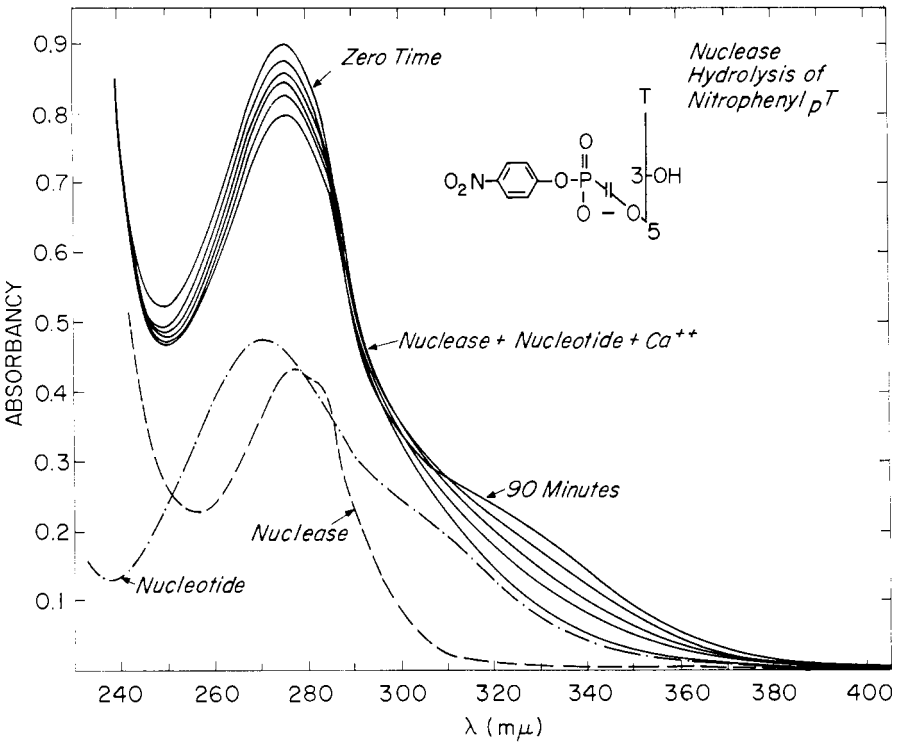


FIGURE 2: Ultraviolet spectral changes resulting from hydrolysis of nitrophenyl-pdT (28 μM) by staphylococcal nuclease (27 μM). The spectra of the enzyme and of the substrate alone are shown, as well as that of the mixture of the two in the presence of $Ca^{2+}$ (10 mM). The spectrum of the mixture was taken periodically until the reaction was complete (90 min). In the absence of added $Ca^{2+}$ there were no time-dependent spectral changes. The buffer used in these studies was 0.1 M borate (pH 8.8). The increase in absorbance above 300 mμ and the fall at lower wavelengths are due to formation of the unprotonated species of *p*-nitrophenyl phosphate.

absorbance at the wavelength (330 mμ) used to measure *p*-nitrophenyl phosphate. Thus, despite the biheaded nature of nitrophenyl-pdTp-nitrophenyl, it is a good substrate for measurement of staphylococcal nuclease activity if the criterion used is the rate of release of *p*-nitrophenyl phosphate.

It is not clear at the moment how recognition and hydrolysis of *p*-nitrophenol from such substrates can be reconciled with the observations and interpretations presented in this report. It is possible that a weakly hydrolytic region exists adjacent to the principal hydrolytic site of the enzyme. This hypothesis is most unlikely since there are very large differences in the $K_M$ values obtained from the release of *p*-nitrophenyl phosphate and *p*-nitrophenol from single substrates, such as nitrophenyl-pdTp-nitrophenyl and nitrophenyl-pdTpdTp-nitrophenyl (Table I). In view of the highly unfavorable affinities and catalytic rate constants for all the substrates which release *p*-nitrophenol, it is likely that the apparent hydrolysis (release of *p*-nitrophenol) of such compounds is relatively nonspecific and unrelated to the principal cleavage patterns discussed above.

This nonspecific cleavage may be related to the chemical nature of the leaving group (*p*-nitrophenol), since no enzymatic hydrolysis of dTp-aminophenyl could be detected. Furthermore, incubation of nitrophenyl-pdTp-methyl with large amounts of staphylococcal nuclease caused rapid release of *p*-nitrophenyl phosphate but little or no methyl ester hydrolysis, since no dTp could be detected by thin-layer chromatography. On addition of spleen diesterase, however, there was complete conversion into dTp, but only if the substrate had first been digested with staphylococcal nuclease. Another reason for suspecting that the release of *p*-nitrophenol from such substrates is not a true substrate effect is the absence of competitive inhibition by pdTp on the release of *p*-nitrophenol from nitrophenyl-pdTp-nitrophenol. The inhibition obtained on the release of *p*-nitrophenyl phosphate is, on the other hand, competitive, and the $K_i$ ($3.2 \times 10^{-6}$ M) is identical with that calculated by other techniques (Cuatrecasas *et al.*, 1967b).

None of the substrates or inhibitors of Table I, in large excess, caused irreversible inhibition of staphylococcal nuclease activity after prolonged incubation (2–3 days) at pH 7 or 8.8.

*The Basis of the Spectrophotometric Assays.* A number of compounds (Table I) that on reaction with staphylococcal nuclease discharge *p*-nitrophenyl phosphate can be used as substrates in the spectrophotometric assay for this enzyme. The assay is based on the fact that the ultraviolet spectrum of *p*-nitrophenyl phosphate shifts to higher wavelengths on dissociation of the secondary hydrogen of the phosphate (Figure 1). The p*K* of this ionization, measured by spectrophotometric titration at 330 mμ in 0.05 N NaCl, is 5.1. At pH 6.0, 91% of the maximal spectral shift has occurred. The isosbestic point occurs at 299 mμ.

If the reaction of staphylococcal nuclease with nitrophenyl-pdT (Figure 2) or nitrophenyl-pdTp-nitrophenyl (Figure 3) is performed at pH values greater than 6, the *p*-nitrophenyl phosphate released during hydrolysis will be instantly ionized. Thus, a spectral change will occur at wavelengths (320–350 mμ) where the absorption of the unhydrolyzed substrate is minimal. Since thymidine does not contribute to the absorbancy in the 320–350-mμ region of the spectrum, the quantity of *p*-nitrophenyl phosphate formed can easily be calculated

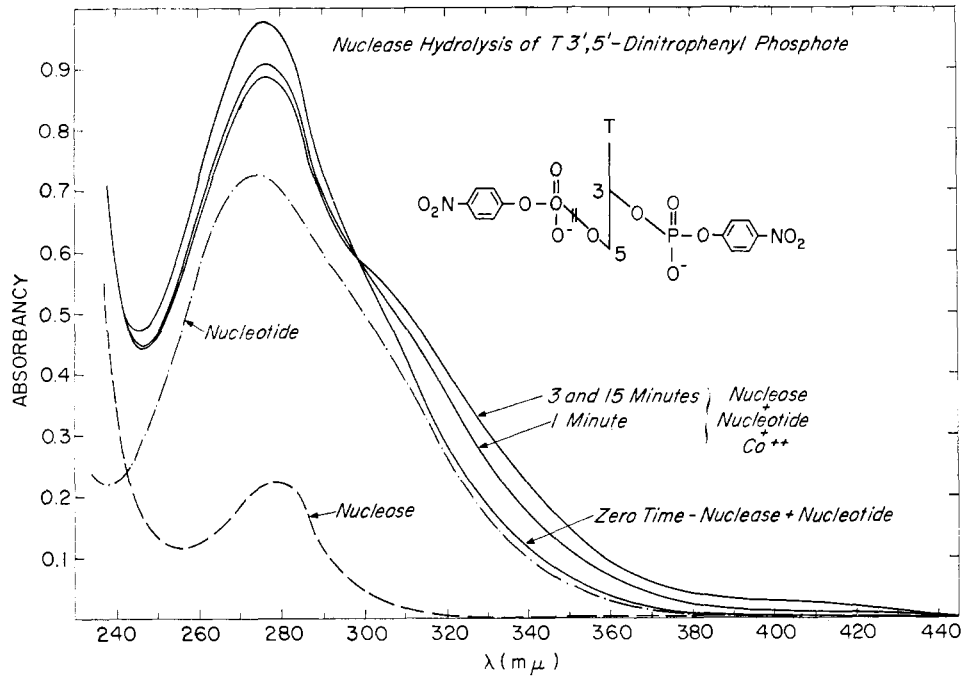


FIGURE 3: Ultraviolet spectral changes resulting from hydrolysis of nitrophenyl-pdTp-nitrophenyl (32 μM) by staphylococcal nuclease (14 μM). The spectrum at zero time is that of the mixture of enzyme and substrate before addition of $Ca^{2+}$. The reaction was started by addition of $Ca^{2+}$ (10 mM); by 3 min the reaction was complete. Other conditions are as described in Figure 2.

from the change in the absorbancy occurring in this spectral region, provided that the difference in molar extinction coefficient of the ionized and un-ionized forms of p-nitrophenyl phosphate, $\Delta E_M$, is known. The $\Delta E_M$ values at 320, 330, and 340 mμ are 4020, 4260, and 3580, respectively.

*Kinetic Measurements with Synthetic Substrates.* The ultraviolet absorption coefficients of various useful synthetic substrates are given in Table II. With an automatic recording spectrophotometer (see Methods) the rate of absorbance change at an appropriate wavelength (320–350 mμ) can be monitored continuously beginning within 10 sec of addition of enzyme or substrate to the reaction mixture. Steady-state kinetics usually prevail over a sufficient length of time to allow accurate measurements of initial velocities. The absorbance at 330 mμ, at pH 8.8, is directly proportional to the concentration of *p*-nitrophenyl phosphate over a range of 2.5 optical density units if the slit width is properly adjusted. Accurate measurements of activity can therefore be performed with high initial concentrations of substrate, and the course of reactions can be followed without dilution for considerable periods of time. If very high substrate concentrations are to be used, activity measurements can be made at 350 mμ rather than at 330 mμ.

TABLE II: Wavelengths of Maximal Absorption and Molar Extinction Coefficients of Synthetic Substrates of Staphylococcal Nuclease.

| Substrate | $\lambda_{max}$ (mμ) | $E_M$ ($\lambda_{max}$) |
|---|---|---|
| Nitrophenyl-pdT | 271 | 16,250 |
| Nitrophenyl-pdTp | 270 | 15,400 |
| Nitrophenyl-pdTp-nitrophenyl | 275 | 22,500 |
| Nitrophenyl-pdTpdT | 270 | 24,000 |
| Nitrophenyl-pdTpdTp-nitrophenyl | 271 | 31,500 |
| Nitrophenyl-pdTp-methyl | 270 | 15,200 |

The spontaneous rate of hydrolysis of the synthetic substrates (Table I) is negligible if the temperature is not higher than 24°, if the pH is below 10, if the $Ca^{2+}$ concentration is below 0.1 M, and if the substrate concentration is not too high, *i.e.*, if the absorbance at 330 mμ is not greater than 2.0. When the spontaneous rate of hydrolysis is measurable, it is subtracted from the measured enzymatic rate of hydrolysis.

The initial velocities obtained with the substrates listed in Table I are proportional to the concentration of protein over at least a 30-fold range. A typical assay mixture, using the substrate nitrophenyl-pdTp-nitrophenyl, contained 1 ml of 0.05 M borate buffer (pH 8.8–9.4), 0.1 mM substrate, and 10 mM $CaCl_2$. The initial velocities obtained on addition of from 1 to 40 μg of nuclease (in 5–20 μl) were directly proportional to the amount of enzyme.

Perhaps the substrate best suited for routine assay of staphylococcal nuclease activity is nitrophenyl-pdTp, since it has highly favorable kinetic constants and there are no possible complications resulting from release of *p*-nitrophenol. Saturating concentrations can be easily achieved with minimal basal absorbancy at 330 mμ, there is virtually no spontaneous hydrolysis at 24°, and the rate of change in absorbance is linear for many minutes with enzyme concentrations as high as 80 μg/ml.

Table I lists the kinetic constants of the various synthetic substrates of staphylococcal nuclease. The dependence of

these constants upon the concentration of $Ca^{2+}$, pH, and ionic strength is not the same for all the substrates. These detailed kinetic studies will be considered in a separate communication.[3] In general, however, with all these substrates the maximal velocities are achieved between pH 9 and 10, maximal affinity for substrate occurs between pH 7.5 and 8.5, and inhibition of enzymatic activity is observed with NaCl concentrations greater than 0.1 N.[3] A similar dependence upon these parameters is seen when activities are measured using DNA and RNA as substrates.

*Cleavage of P–O Bond.* Studies were performed to determine if release of *p*-nitrophenyl phosphate from the corresponding deoxythymidine 5′-ester substrates results from cleavage of the P–O or C–O bond; 4 μmoles of nitrophenyl-pdT was dissolved in 350 μl of a solution of 0.05 M borate buffer, containing 25% $H_2{}^{18}O$ (pH 8.8), 0.07 M $CaCl_2$, and 30 mμmoles of staphylococcal nuclease. A separate incubation mixture was obtained which differed only in having 0.001 M $CaCl_2$. At 4 hr, when the hydrolysis of the substrate was complete, the reaction mixture was passed through a 0.5 × 30 cm column of Sephadex G-25 equilibrated with 1 mM ammonium acetate (pH 5.0). Deoxythymidine was collected and identified by thin-layer chromatography and ultraviolet spectra. It was then lyophilized and analyzed by mass spectroscopy.[4] Under conditions of low and high $Ca^{2+}$ concentration the lack of any exchange with $H_2{}^{18}O$ was clearly demonstrated by the absence of peaks in the mass spectrum of thymidine at *m/e* 244 (M + 2) and at *m/e* 119 (S + 2). Staphylococcal nuclease therefore must have cleaved the P–O bond of the diester. P–O bond cleavage is also found with the phosphodiesterases from spleen and pancreas (Hilmoe *et al.*, 1961).

*Difference Spectrum Due to Nuclease-pdTp-nitrophenyl Interaction.* The difference spectrum which results from the interaction of staphylococcal nuclease with the strong competitive inhibitor, pdTp, shows a large red shift in tyrosyl absorption as well as changes indicating significant perturbation of the nucleotide chromophore (Cuatrecasas *et al.*, 1967b). Similar studies were performed with the inhibitor pdTp-nitrophenyl to determine if strong interactions with the nitrophenyl group might be detected by an alteration in its ultraviolet or visible spectrum. The resulting difference spectrum, which occurred only on addition of $Ca^{2+}$, was identical with that previously reported for pdTp. There was no component which could be attributed to perturbation of the nitrophenyl group.

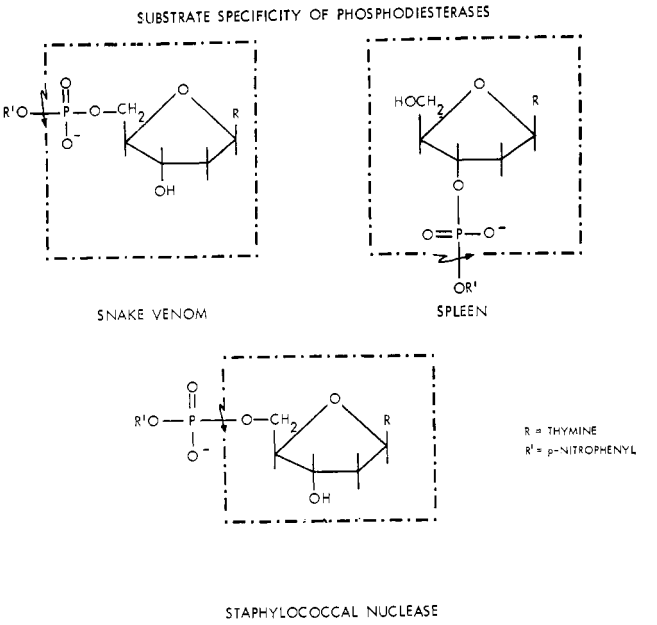


FIGURE 4: Proposed structural requirements for substrates of phosphodiesterases that hydrolyze DNA and RNA, those from venom, spleen, and staphylococcus. The structures indicated for the venom and spleen enzymes are those suggested by Khorana (1961).

## Discussion

Although the principal and preferred action of staphylococcal nuclease is endonucleolytic (Alexander *et al.*, 1961; Sulkowski and Laskowski, 1962; Cuatrecasas *et al.*, 1969a), it is clear from the present studies that the basic structural element required for recognition as substrate (R-pdT-R′) need not be part of a polynucleotide chain. Hydrolysis occurs irrespective of the size of the substrate, since it appears that the nature of the R group is unimportant so long as a diester bond is present, and since substitution of the 3′-OH group is not required to achieve the *maximal* rate of catalysis.

The *affinity* for substrates and inhibitors is clearly affected by the size and charge of the R′ substituent. Study of the dissociation constants of a progressively larger series of 5′-phosphoryl oligothymidyl inhibitors, $(pdT)_x$, previously demonstrated that the optimal stability of the enzyme–nucleotide complex occurs with a trinucleotide sequence (Cuatrecasas *et al.*, 1969b). It was postulated that three unequal phosphate binding "subsites," principally electrostatic in nature, existed in the substrate binding region of the enzyme. The most important site is thought to coincide with the hydrolytic site, and the other two are presumed to exist to the "right" of this hydrolytic site. In the case of inhibitors, the free 5′-phosphoryl group, due to its strong anionic charge and properly placed adjacent nucleoside, is strongly recognized by the hydrolytic region. In this case ($(pdT)_x$), nucleotide units other than that bearing the 5′-phosphoryl group are considered to the "right" of the hydrolytic site and would be expected to contribute principally to the affinity constant. The rationalization can then be made in the case of substrates, so that R-pdT-R′ derivatives, unlike the inhibitors, possess a cleavable phosphodiester bond on the 5′ position and will thus release $R_p$ upon hydrolysis. The R′ group, as in the case of the 5′-phosphoryl group of inhibitors, should contribute solely in terms of affinity. Inspection of Table I suggests that this is

[3] P. Cuatrecasas, M. Wilchek, and C. B. Anfinsen, unpublished data. These kinetic studies indicate that the $K_M$ values measured with the synthetic substrates are true dissociation constants, $K_s$. Furthermore, the $K_i$ constants of these substrates, obtained from their inhibition of DNase activity, are identical with the $K_{M_{app}}$ values derived from their hydrolysis; this suggests that these substrates are specifically bound to the same region of enzyme which binds and hydrolyzes DNA. Substrate affinity depends upon the $Ca^{2+}$ concentration; the lowest $K_{M_{app}}$ for nitrophenyl-pdTp-nitrophenyl occurs at 20 mM $Ca^{2+}$, but for nitrophenyl-pdTpT it is at 2.5 mM $Ca^{2+}$. Apart from its effect on $K_M$, $Ca^{2+}$ increases the $K_{cat}$ directly; maximal effects are observed with about 0.1 M $Ca^{2+}$. With all the substrates, concentrations of $Ca^{2+}$ higher than 0.05 M result in an apparent inhibition of activity, an effect which is due solely to an increase in $K_M$.

[4] These studies were kindly performed by Dr. Kenneth Kirk and Dr. Louis Cohen, National Institutes of Health. Mass spectra were obtained with an Hitachi Perkin-Elmer Model RMU GE mass spectrometer.

indeed the case. As with the $K_i$ values of inhibitors, the $K_M$ values of substrates are very sensitive to the addition of anionic phosphate groups to the "right" of the hydrolyzable bond. Furthermore, in contrast to the $K_M$ constants, the $K_{cat}$ constants of the various deoxythymidyl 5′-phosphate ester substrates are essentially identical.

In the above discussion no mention is made of how the base may contribute in the process of substrate recognition. Although no direct proof is available, it is considered probable that the contribution by the base is minor compared with the effects exerted by the sugar and phosphate groups (Cuatrecasas *et al.*, 1969a). In this respect staphylococcal nuclease may be like other known phosphodiesterases (Khorana, 1961; Razzell, 1963).

Apart from important differences in the endo- and exonucleolytic properties of staphylococcal nuclease and other well-studied phosphodiesterases, those from snake venom and spleen, the basic structural substrate elements for these enzymes appear to be quite different (Figure 4). The staphylococcal enzyme may appear to be more akin in its mode of action to the spleen enzyme because they both hydrolyze DNA and RNA to 3′-nucleotides, whereas the venom enzyme releases 5′-nucleotides. However, their mode of action and specificity are quite different, and the structural requirements of the staphylococcal enzyme substrates are perhaps more nearly similar to those of the venom enzyme. The principal difference is that the staphylococcal enzyme cleaves the diester bond between the phosphate and the 5′-carbon of the sugar, whereas the venom enzyme cleaves on the other side of the phosphate, that is, between the phosphate and the nonspecific hydroxylic component of the diester bond. In contrast to both spleen and venom diesterases, the primary product released by staphylococcal nuclease hydrolysis is a derivative bearing a hydroxyl group (on the 5′ position) rather than a phosphoryl group. The 3′-phosphoryl product formed from polynucleotide hydrolysis is a secondary consequence of such cleavage, and it is an unnecessary feature of staphylococcal nuclease action, as demonstrated by the present studies with synthetic substrates.

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