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16. Supported by NIH grant No. AM 05851 and by contract No. AT(30-1)-3242 from the AEC Biology Division. Part of the work was carried out during the tenure by R.S. of a Fulbright Fellowship at the Weizmann Institute of Science, Rehovoth, Israel. We thank Dr. R. F. Kibler for instrumental facilities, and Mr. A. Lanier for assistance.

16 September 1968; revised 29 October 1968

## Staphylococcal Nuclease: Size and Specificity of the Active Site

Abstract. *The dissociation constants and standard free energies of complex formation determined with staphylococcal nuclease and a series of 5′-phosphoryloligothymidyl derivatives of increasing chain length suggest that maximum stability is reached with an oligonucleotide containing three nucleotide units. A proposed model of the active site that contains other knowledge of the specificity and the catalytic mechanism of this enzyme postulates the existence of three nonequivalent phosphate binding subsites and a closely related phosphodiester hydrolytic subsite.*

The affinities of enzymes for substrates or inhibitory analogs reflect the number, strength, and spatial distribution of interacting sites in the enzymically active centers. By studying the effects of a number of peptides on affinity and rate constants, it was suggested that the active site of papain extends for approximately 25 Å and accommodates seven amino acids of the substrate molecule (*1*). On the same basis, the active site of carboxypeptidase A covers five "subsites" over 18 Å (*2*). The activity of the exonuclease of Ehrlich ascites tumor cells is progressively more strongly inhibited by a series of 3′-phosphoryloligoadenyl derivatives, $(Ap)_x$, of increasing chain length up to $x = 6$, suggesting that a hexanucleotide sequence is the substrate unit best accommodated in the active site of this enzyme (*3*). Using a similar approach, we have examined the length and orientation of oligonucleotide sequences recognized by the active site of staphylococcal nuclease.

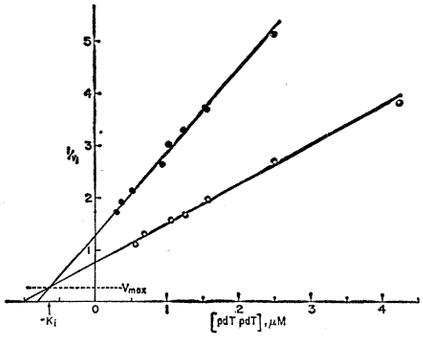


Fig. 1. Dixon plot (*11*) of inhibition of staphylococcal nuclease activity by $(pdT)_2$. Enzyme (20 μg) was added to a solution containing 0.05*M* borate buffer (*p*H 8.8), 0.01*M* $CaCl_2$, 10 μ*M* (●) or 20 μ*M* (○) synthetic substrate, deoxythymidine 3′,5′-di-*p*-nitrophenylphosphate (*5*, *10*), and the amount of oligonucleotide indicated.

Staphylococcal nuclease, which hydrolyzes DNA and RNA to yield 3′-phosphomononucleosides, is inhibited competitively by 5′-nucleotides and derivatives bearing a 5′-terminal phosphate (*4*, *5*). Oligonucleotides bearing 5′-phosphomonoester end groups are very resistant to hydrolysis (*6*, *7*). We have therefore determined the dissociation constants ($K_i$) of a series of 5′-phosphoryloligothymidyl derivatives, $(pdT)_x$, ranging in length from one to six nucleotide residues.

Staphylococcal nuclease (Foggi strain) was obtained by a modification (*8*) of techniques described by Fuchs *et al.* (*9*); thymidine-3′,5′-diphosphate (pdTp) and pdT were purchased from Calbiochem, and $(pdT)_4$ was a gift of Dr. H. G. Khorana; the other $(pdT)_x$ derivatives were provided by Dr. M. Nirenberg. Deoxythymidine 3′,5′-di-*p*-nitrophenylphosphate (NP-pdTp-NP) was synthesized from pdTp, and deoxythymidine 5′-phosphate-3′-*p*-nitrophenylphosphate (pdTp-NP) was obtained from the latter by hydrolysis with snake venom diesterase (*10*).

Dissociation constants ($K_i$) were obtained from Dixon plots (*11*) of the inhibition of the enzymatic release of *p*-nitrophenylphosphate from NP-pdTp-NP (*5*, *10*) (Fig. 1). At least two substrate concentrations were used for each study, and the results were confirmed by independent measurements of $V_{max}$ (Fig. 1). The oligonucleotide concentration was determined spectrophotometrically after hydrolysis in 0.3*M* NaOH for 18 hours at 37°C with $E_{1l}$ (267 nm) $= 9.6 \times 10^{-3}$ (*12*).

The dissociation constants ($K_i$) and standard free energies ($\Delta F°$) of $(pdT)_x$-nuclease complex formation indicate that maximum stability of the complex is reached with an oligonucleotide containing three nucleotide units (Table 1 and Fig. 2). As the chain length increases to six, there is progressively less inhibition. This is probably due to the slow hydrolysis that can occur with oligonucleotides of chain length greater than three (*6*), although some steric interference with binding cannot be excluded. As the nucleotide chain increases in length, regions of its sequence that are susceptible to the principal, endonucleolytic action of the enzyme may compete with the inhibitory 5′-phosphoryl end, until very large oligonucleotides become excellent substrates.

Table 1. Binding constants of oligonucleotide-nuclease complexes.

| Nucleotide | Dissociation constant* (molar) | $\Delta F°$† (cal/mole) |
|---|---|---|
| pdT | $1.9 \times 10^{-4}$ | −4,400 |
| $(pdT)_2$ | $6.3 \times 10^{-7}$ | −8,500 |
| $(pdT)_3$ | $9.3 \times 10^{-8}$ | −10,000 |
| $(pdT)_4$ | $1.1 \times 10^{-7}$ | −9,500 |
| $(pdT)_5$ | $1.4 \times 10^{-7}$ | −9,400 |
| $(pdT)_6$ | $6.0 \times 10^{-7}$ | −8,500 |
| pdTp | $2.0 \times 10^{-7}$ | −9,100 |
| pdTp-NP ‡ | $1.1 \times 10^{-6}$ | −8,100 |
| NP-pdTp-NP ‡ | $1.7 \times 10^{-5}$ | −6,500 |

* $K_i$, obtained by Dixon plots (Fig. 1); the value for NP-pdTp-NP is $K_m$ (approx.), obtained from Lineweaver-Burk plots under identical experimental conditions. † $\Delta F° = RT \ln K_i$. ‡ *p*-Nitrophenyl phosphate (NP) esters.

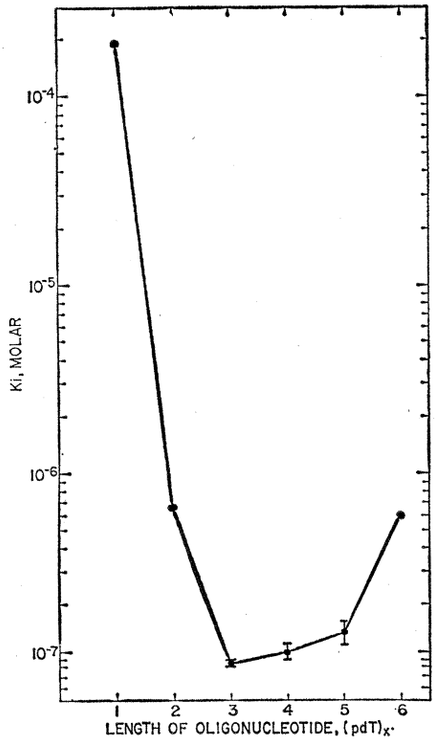


Fig. 2. Semilogarithmic plot of the dissociation constant ($K_i$) of $(pdT)_x$ as a function of the length of the oligonucleotide ($x$). The range of experimental values observed in the case of the tetra- and pentanucleotides is indicated.

A diagrammatic representation of these results is suggested in a scheme (Fig. 3) that also integrates other available information concerning the specificity and catalytic mechanism of this enzyme. The active site must involve a large portion of the surface of the protein molecule, whose estimated dimensions are shown (*13*); it probably exists as a groove that can accommodate a trinucleotide sequence (as much as 20 Å in length).

The contribution of the purine and pyrimidine bases to the overall strength of complex formation is minor compared to the electrostatic effects of the phosphate groups (*5*). This is illustrated by the 1000-fold greater affinity of pdTp as compared to pdT and by the weaker binding of the monoanionic *p*-nitrophenyl derivatives (Table 1). Therefore, the predominant forces involved in binding seem to be ionic interactions with three phosphate binding "subsites" (P1, P2, P3). The P "subsites" probably contribute unequally to complex formation, with P1 > P2 > P3. This is suggested by the data in Table 1 and Fig. 2, by the tenfold greater affinity of 5′-adenosine monophosphate as compared to 3′-adenosine monophosphate (*14*), and by the great susceptibility of 5′-phosphodiester deoxythymidyl substrates (*5*, *10*). Oligonucleotides with 5′-phosphomonoester termini will thus bind in a manner that does not properly expose a phosphodiester bond to the hydrolytic site (Fig. 3B).

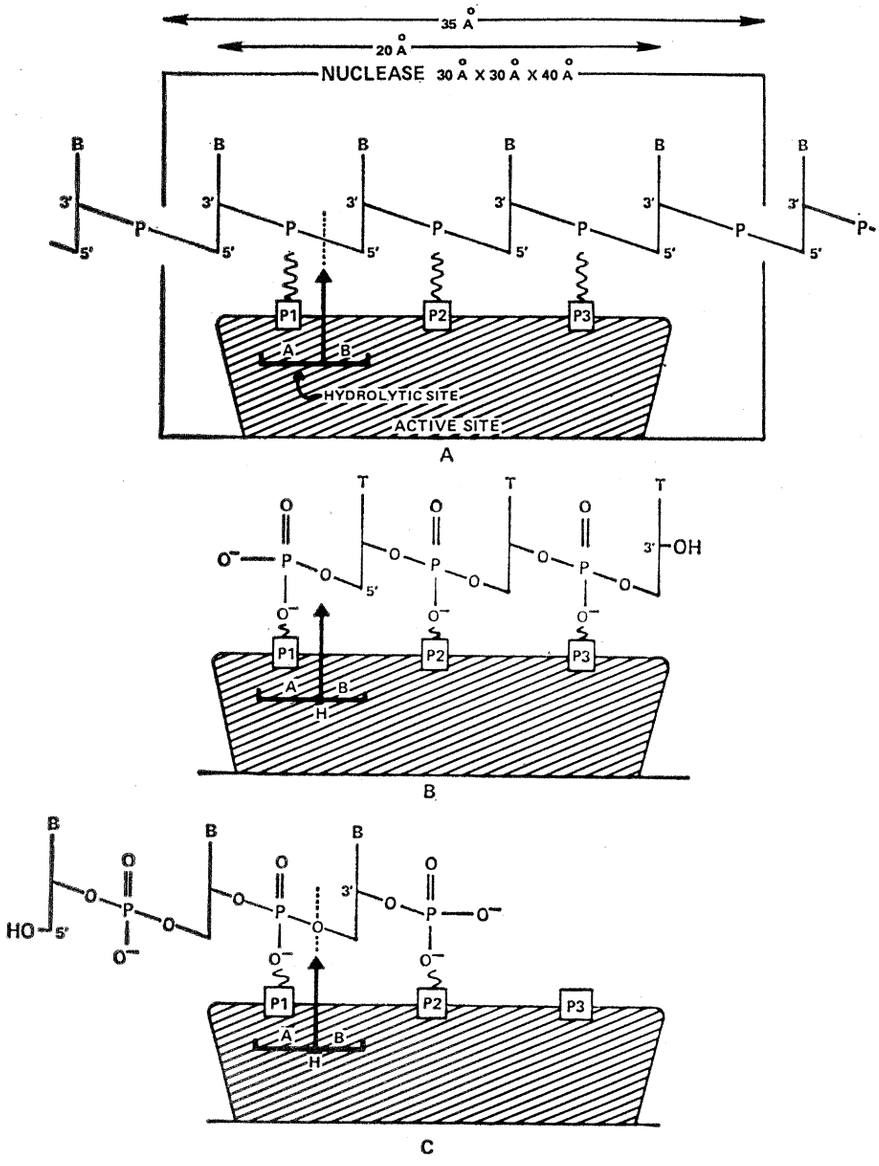


Fig. 3. Schematic representation of (A) complex formation between staphylococcal nuclease and polynucleotide substrates; (B) inhibitory oligonucleotides bearing 5′-phosphoryl termini; and (C) a slowly hydrolyzed 3′-phosphoryloligonucleotide, XpYpZp. The major substrate binding regions of the enzyme, or "phosphate subsites," are indicated as P1, P2, and P3. The hydrolytic site (*H*) consists of a region closely related to P1 which recognizes the phosphodiester bond (*A*), and a region which recognizes the sugar-base moiety whose 5′-OH is linked to the phosphate group (*B*).

The hydrolytic site (*H*) is visualized as an integral part of P1 (Fig. 3A). For hydrolysis to occur, this region of the enzyme must recognize in the substrate (i) the phosphodiester nature of the bond, and (ii) the 5′-OH substituted sugar-base moiety. Studies with synthetic substrates indicate that it is the nucleoside on the 5′-side of the phosphodiester bond, rather than any substituents on the 3′-side, that dictates the specificity of cleavage (*5*, *10*), in agreement with the scheme proposed in Fig. 3.

The principal and preferred action of this enzyme is endonucleolytic, with cleavage of large polynucleotides to segments three to nine mononucleotide units in length (*6*, *7*). At this stage of hydrolysis, the terminal 3′-phosphate groups of oligonucleotides begin to interfere with the endonucleolytic action, apparently by strong interactions with the P2 "subsite," with the result that the 3′-monophosphate end group nucleotides are preferentially released (*7*, *15*). Thus, the trinucleotide XpYpZp (Fig. 3C) is preferentially cleaved to XpYp and Zp (*7*, *16*).

It is likely that no binding "subsites" are located to the left of P1 (Fig. 3) since, as pointed out earlier, the corresponding portion of synthetic substrates appears not to affect binding or catalysis significantly (*5*, *10*). Furthermore, the substrate NP-pdTpdT is cleaved completely to *p*-nitrophenylphosphate and dTpdT before significant hydrolysis of dTpdT can be detected (*10*).

For simplification, the scheme shown in Fig. 3 does not take into account the essential role of calcium ions in binding and hydrolysis of substrates and inhibitors (presumably in interactions with phosphate groups) (*5*). The substitution of strontium ions for calcium ions in the case of DNA hydrolysis but not RNA hydrolysis also has suggested a specific role for the 2′-OH group of the substrate in the catalytic mechanism (*5*).


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30 September 1968; revised 14 November 1968 ■


## Warfarin Treatment of Mice Bearing Autochthonous Tumors: Effect on Spontaneous Metastases


Abstract. *Long-term oral administration of sodium warfarin significantly reduced the incidence of spontaneous metastases in the lungs from 83 percent in controls to 8 percent in treated C57/BL/6N mice. The size and weight of primary tumors in mice treated with warfarin were less than in control mice. Length of survival was unaffected.*


The mechanism of blood coagulation, and in particular of fibrin deposition, is important in the growth of a primary tumor and its metastatic spread. O'Meara and Jackson (*1*) have noted deposition of fibrin in a wide variety of tumors, particularly at the invading periphery, and they felt that fibrin was an important lattice for tumor growth. Human cancer cells contain an agent [cancer coagulative factor (*2*)] that induces fibrin formation and is thought to resemble thromboplastins (*3*). The action of this factor can be blocked in humans and rabbits by dicoumarol and similar coumarin anticoagulants (*3*). The formation of an enveloping microthrombus around embolic tumor cells is a decisive part of the mechanism of endothelial adherence and penetration (*4*). Adequate anticoagulation or fibrinolysis with numerous agents significantly reduces the incidence of metastases (*4–7*), whereas agents that increase the ability of blood to coagulate increase this incidence (*4, 5*). A clinical study by Michaels suggested that treatment with anticoagulants might similarly affect the metastatic spread of human tumors (*8*). In recent evaluations of protamine (*9*), plasmin (*10*), and protease (*11*) in patients with cancer, investigators attempted to impair tumor growth by altering the mechanisms of coagulation or fibrinolysis, but the results have been inconclusive.

Most studies from which a relationship between coagulation, fibrin, and the growth and spread of tumors has been established have been performed with transplanted tumors in inbred animals. There have been valid questions regarding the degree to which the interaction of host and transplanted tumor resembles the relation of patient and autochthonous tumor (*12*). For this reason we have studied the effect of long-term treatment with sodium warfarin on the incidence of spontaneous metastatic spread from autochthonous tumors to see if the suppressive effect found with transplanted tumors would persist.

Autochthonous tumors were induced in 50 female C57/BL/6N mice, 6 to 8 weeks old, by the injection intramuscularly in the right thigh of 0.1 ml of 3-methylcholanthrene (1 percent) in sesame seed oil. Those mice (25) in which the injected thigh enlarged two to three times between the 8th and 10th weeks after injection were alternately placed in the control or treated group. Although nearly all 50 mice would, over several more months, develop tumors, those with tumors arising in this early period were selected for study because growth and metastatic behavior of these tumors is more uniform. Sodium warfarin (9.10 mg/liter) was added to the drinking water of treated mice. In mice with tumors, this dosage is adequate to maintain a prothrombin time two to three times the normal average value over many weeks (*7*). The warfarin solution was changed every 3rd or 4th day. All mice were kept under observation until death, and their lungs were examined macroscopically for metastases. India ink, used as the stain, differentially colors normal lung and tumor tissue (*13*). Because the tumor frequently was larger than the mouse at death and because it could not be cleanly dissected free, the entire carcass was weighed.

All mice had progressive enlargement of the injected thigh to beyond five times the normal size. Histologic examination revealed that the tumors were poorly differentiated sarcomas, quite uniform in pattern. Frequently, the tumor was larger than the host mouse at the time of death. In our laboratory, the average weight of 6-month old C57/BL/6N mice, the age at which the tumor-bearing mice died, is 18.4 g. The average weight of the tumor-bearing carcass of the control mice was 34.5 g (ranging from 25 to 45 g); the average weight of treated mice was 24.5 g (ranging from 21 to 30 g). The primary tumors of mice treated with warfarin, although large, never reached the huge proportions of tumors in controls.

The mean survival time of control mice after injection was 130 days (range, 123 to 148 days); that of the mice treated with warfarin was 138 days (range, 124 to 158 days). Examination of warfarin treated mice shortly after death frequently showed hemorrhage within the large primary tumors.

At death, ten of the twelve control mice (83 percent) had pulmonary metastatic lesions, and in six of the ten the lesions were too numerous and confluent to permit accurate counting and sizing. Three of these six mice had metastases to inguinal and pelvic lymph nodes as well. Only one of 13 mice treated with warfarin (8 percent) had metastases at death and this was a single lesion. This difference in incidence is significant statistically at $P$ less than .005 by the $\chi^2$ test.

The relation between host and tumor is similar in autochthonous tumors in highly inbred strains of mice and in tumors in man. Transplanted tumors, however, have many possible factors of variance, as discussed by Klein (*12*), making the extrapolation of results from such studies to the clinical situation more hazardous than the obvious host differences alone would imply. The interactions of host and tumor, of decisive importance, may be different. Yet, because transplanted tumors are so much easier to use, studies with