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Streptococcal Nucleases. V. Specificities of Deoxyribonuclease Action of the A, B, C, and D Enzymes*

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ABSTRACT: The specificity of the deoxyribonuclease (DNase) activity of the streptococcal nucleases A–D was investigated using the deoxyhomopolynucleotides poly dA, poly dC, poly dT, and poly dG (where A, C, T, and G are adenine, cytosine, thymine, and guanine, respectively). Nuclease C showed little substrate preference while nucleases A, B, and D showed preferences for the degradation of poly dA, poly dC, and poly dA, respectively. When tested on deoxyadenylate oligonucleotides of chain length 2–4 to determine the smallest fragment degradable by these enzymes, nuclease A degraded $d(pA)_4$ but not $d(pA)_2$ and $d(pA)_3$, whereas nucleases B–D degraded all three oligonucleotides to varying extents. The presence of a 5′-terminal phosphate group does not appear essential for the activity of the four enzymes on adenylate dinucleotide.

Group A streptococci elaborate four immunologically distinct nucleases in their extracellular medium (nucleases A, B, C, and D) (Wannamaker, 1958, 1962; Winter and Bernheimer, 1964). All four enzymes are endonucleases producing fragments ending in 5′-phosphate. The B and D enzymes also possess RNase as well as DNase activity with both activities believed to reside in single proteins. In the previous paper (Gray and Yasmineh, 1968) on the specificities of nucleases B and D using ribohomopolynucleotides it was shown that the RNase activity of the B enzyme possesses a 6-amino base specificity, *i.e.*, it degraded poly A and poly C but not poly U or poly I. Nuclease D showed a high preference for poly C although it could degrade two of the other three polynucleotides at a slow rate. With the urgent need for base-specific deoxyribonucleases for use in DNA base sequence analysis it was of interest to determine whether these specificities are also exhibited by the DNase activity of the B and D enzymes. The base preferences of action of these enzymes as reflected in exhaustive digests of DNA have been examined by Winter and Bernheimer (1964). The present study seeks to establish the specificities at the initial stages of the reaction. This can most readily be accomplished by the use of deoxyhomopolymer substrates.

Experimental Section

Methods

The deoxyribonucleoside triphosphates dATP,[1] dCTP, dGTP, and dTTP were purchased from Pabst Research Biochemicals, Milwaukee, Wis. Tritiated dATP was purchased from Schwarz Bioresearch Inc., Orangeburg, N. Y. The deoxyhomopolynucleotides poly dA, poly dC, poly dT, and poly dG were obtained by enzymatic synthesis (Bollum, 1966) utiliz-

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[1] Abbreviations used: dATP, dCTP, dGTP, and dTTP, deoxyribosyladenosine, -cytidine, -guanosine, and -thymidine triphosphates.

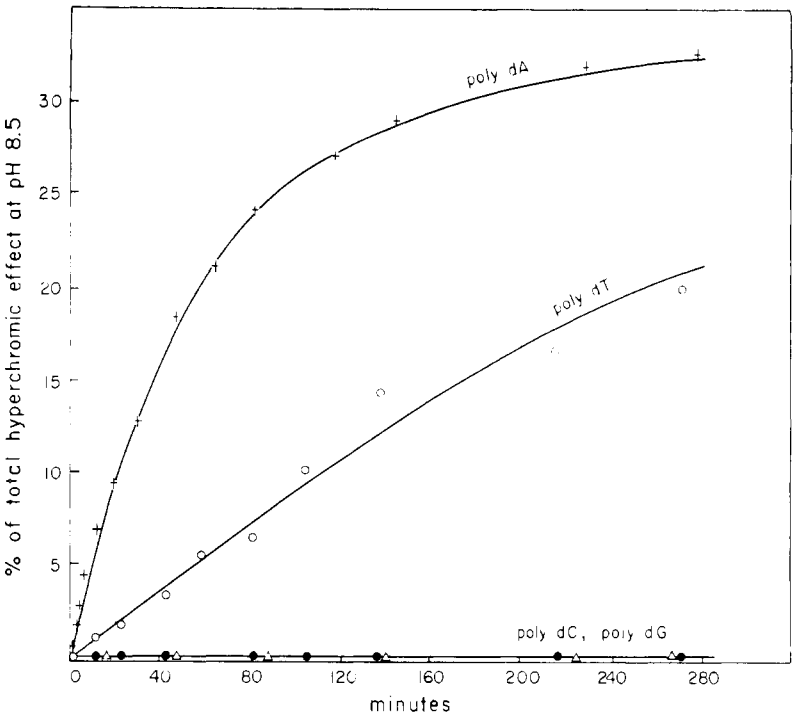

FIGURE 1: Degradation of deoxyhomopolynucleotides by nuclease A as measured by the hyperchromic effect assay. The reaction mixtures (total volume, 0.4 ml) at 30° contained 0.1–0.3 ODU of deoxyhomopolynucleotide, 10 μmoles of Tris buffer (pH 8.5), 0.20 μmole of $CaCl_2$ and $MgCl_2$, and 3 units of enzyme.

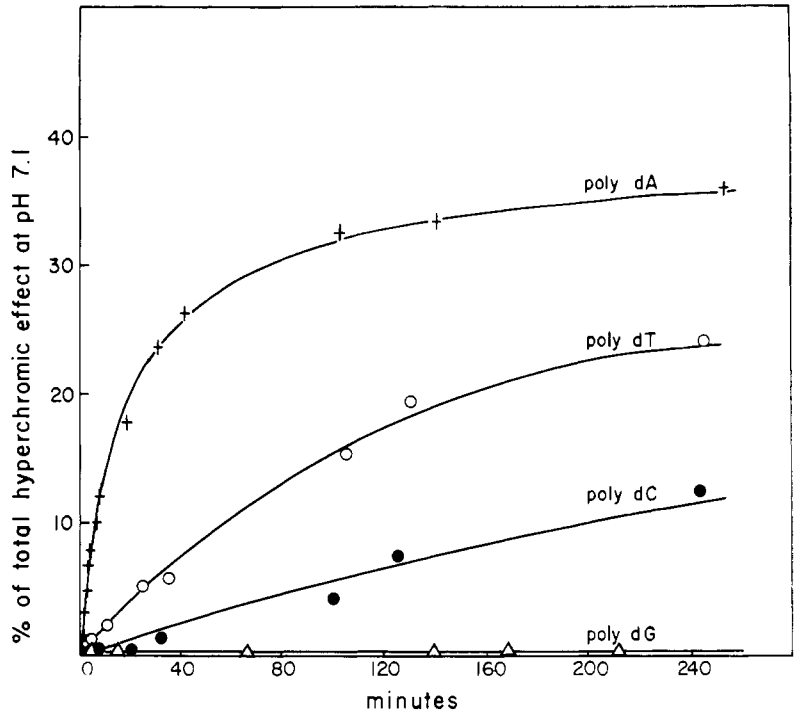

FIGURE 2: Degradation of deoxyhomopolynucleotides by nuclease A as measured by the hyperchromic effect assay. The reaction mixtures were made up as in Figure 1, except that the pH was 7.1.

ing the respective deoxyribonucleoside triphosphates, terminal deoxyribonucleotidyl transferase and the appropriate primers.

The starter primers $d(pA)_2$, $d(pA)_3$, $d(pA)_4$, and $d(pT)_4$ were graciously provided by Dr. F. Bollum. $d(pT)_4$ was prepared by organic synthesis while the deoxyadenylate oligomers were prepared by partial digestion of poly dA with pancreatic DNase, followed by DEAE-cellulose chromatography (Bollum, 1965). *Crotalus adamanteus* venom phosphodiesterase was obtained from Worthington Biochemical Corp., Freehold, N. J.

Streptococcal nuclease A was prepared by the method of Wannamaker (1958) in which the final separation of the enzyme was achieved by starch block electrophoresis. Nucleases B, C, and D were prepared essentially by the same method with the exception that the final separation of the enzymes was achieved by DEAE-cellulose by a modification (E. D. Gray, unpublished data) of the method described by Dillon and Wannamaker (1965). The concentration of these nucleases will be designated in terms of units of activity. The unit is defined as the amount of enzyme that degrades thymus DNA, releasing acid at an initial rate of 10^{-5} mequiv/min, at 30°, pH 6.5, and under the condition of substrate excess (Yasmineh *et al.*, 1968).

Nuclease activity was assayed by the hyperchromic effect technique. The reaction mixture containing nucleic acid, buffer, and divalent cation activators (the volumes and concentrations to be indicated in the individual experiments) was placed in a Beckman semimicrocuvet (0.5-ml capacity) and following the addition of enzyme, optical density readings were taken at appropriate intervals, at the wavelength of maximal absorption of the nucleic acid in question. Since some of the reaction mixtures gradually became turbid upon prolonged standing (over 8 hr), simultaneous readings were taken at 420 mμ to detect the development of any such turbidity, which might obscure the results.

Results

The degradation of poly dA, poly dC, poly dT, and poly dG by enzymes A, B, C, and D was studied at two pH values depending upon the pH optimum of the nuclease. Nucleases A and B, with pH optimum between 8 and 9, were studied at pH 8.5, while nucleases C and D, with pH optima of 5–6 and 6–7 were studied at pH 7.1. In view of the conformational variations that occur in polynucleotides with changes in pH (Steiner and Beers, 1961; Brahms *et al.*, 1966), it was necessary to determine the total hyperchromicity of the four deoxyhomopolynucleotides, at these two pH values, in preliminary experiments. The results of such an experiment in which venom diesterase was used to hydrolyze the polymers to mononucleotides are shown in Table I. The values concur with those previously reported (Bollum, 1966; Inman, 1964).

Poly dA shows little difference in hyperchromic effect at the two pH values. No information is available concerning the conformation of this polymer. The hyperchromic effects of the oligonucleotides $d(pA)_2$, $d(pA)_3$, and $d(pA)_4$ were included in the table to indicate that they are unlike the corresponding fragments of poly A at neutral pH (Brahms *et al.*, 1966; Leng and Felsenfeld, 1966), *i.e.*, not those of a single-chain base-stacked helix in which small oligonucleotides contribute the major portion of the total hyperchromic effect of the polymer. For example, at the level of the tetramer, $d(pA)_4$ contributes only 32% of the hyperchromic effect of poly dA, whereas $(pA)_4$ contributes about 58% of that of poly A (Brahms *et al.*, 1966).

TABLE I: Total Hyperchromic Effect of Deoxyhomopolynucleotides at pH 7.1 and 8.5.[a]

Polymer, Oligomer	Wavelength (mμ)	% Hyperchromic Effect	
		pH 7.1	pH 8.5
Poly dA	259	65.0	61.0
Poly dC	271	31.7	20.1
Poly dT	267	19.0	15.9
Poly dG	252		30.4[b]
$d(pA)_2$	259	16.4	16.4
$d(pA)_3$	259	17.2	17.6
$d(pA)_4$	259	20.6	20.0
$d(pT)_4$	267	7.5	7.5

[a] The incubation mixture containing samples of the polymers (in 0.02 M Tris (pH 7.1 or 8.5) and 0.005 M $CaCl_2$ and $MgCl_2$) were hydrolyzed with venom diesterase at 30°, and the optical density followed in a Beckman DU spectrophotometer at the wavelengths indicated. [b] Poly dG is resistant to attack by venom diesterase. This value was calculated from the ε(p) values of dGMP at pH 8.0, and of poly dG in 0.006 M Tris buffer (pH 8.0) and 0.024 M $CaCl_2$ and $MgCl_2$.

Poly dC shows a 30% increase in hyperchromic effect when degraded at pH 7.1. This is consistent with the results of Inman (1964) which indicate that at pH values above 7.2, this polymer undergoes a transition from single-chain base-stacked helix to that of a random coil.

Poly dT exhibits a small hyperchromic effect similar to that of poly U, a polymer which is believed to be lacking in any ordered structure (Steiner and Beers, 1961). At pH 7.1, it shows a 26% increase in hyperchromic effect over that at pH 8.5. This increase is quite reproducible and may reflect a change in conformational structure.

Poly dG is resistant to degradation by venom diesterase. The hyperchromic effect value shown in the table was obtained by calculation from $\epsilon(p)$ values for poly dG and dGTP.

Degradation of Deoxyhomopolynucleotides by Nuclease A (Figure 1). At pH 8.5 nuclease A degrades

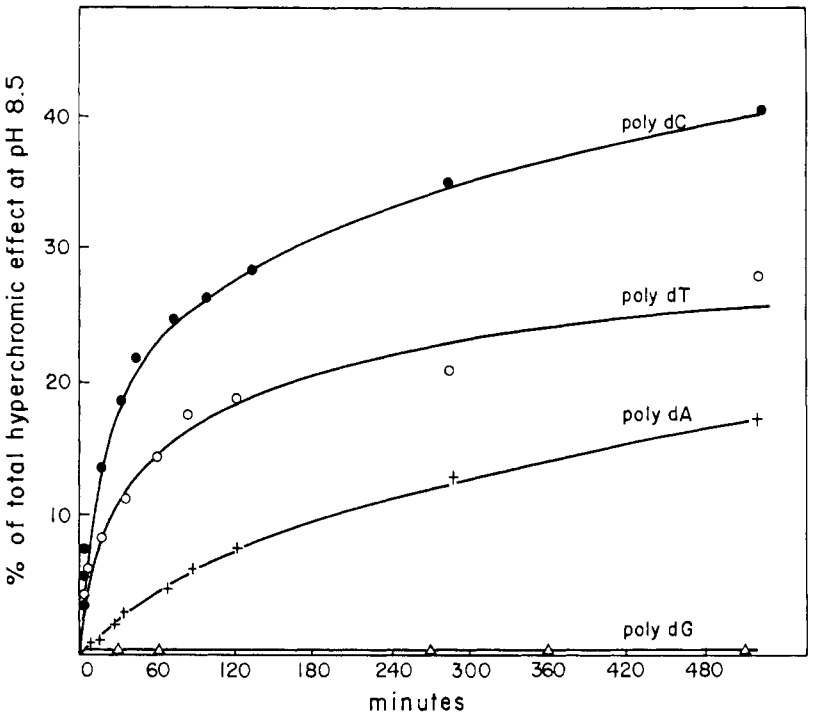

FIGURE 3: Degradation of deoxyhomopolynucleotides by nuclease B as measured by the hyperchromic effect assay. The reaction mixtures (total volume, 0.4 ml) at 30°, contained 0.1–0.3 ODU of deoxyhomopolynucleotide, 10 μmoles of Tris buffer (pH 8.5), 0.05 μmole of $CaCl_2$ and $MgCl_2$, and 14 units of enzyme.

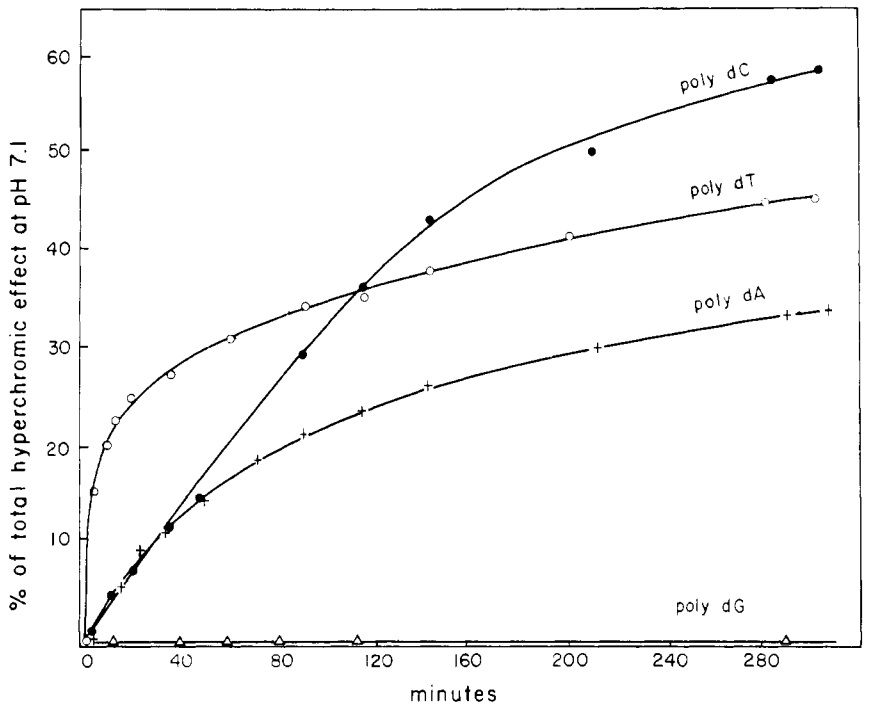

FIGURE 4: Degradation of deoxyhomopolynucleotides by nuclease C as measured by the hyperchromic effect assay The reaction mixtures (total volume, 0.4 ml) at 30°, contained 0.1–0.3 ODU of deoxyhomopolynucleotide, 10 μmoles of Tris buffer (pH 7.1), 0.10 μmole of $CaCl_2$ and $MgCl_2$, and 4 units of enzyme.

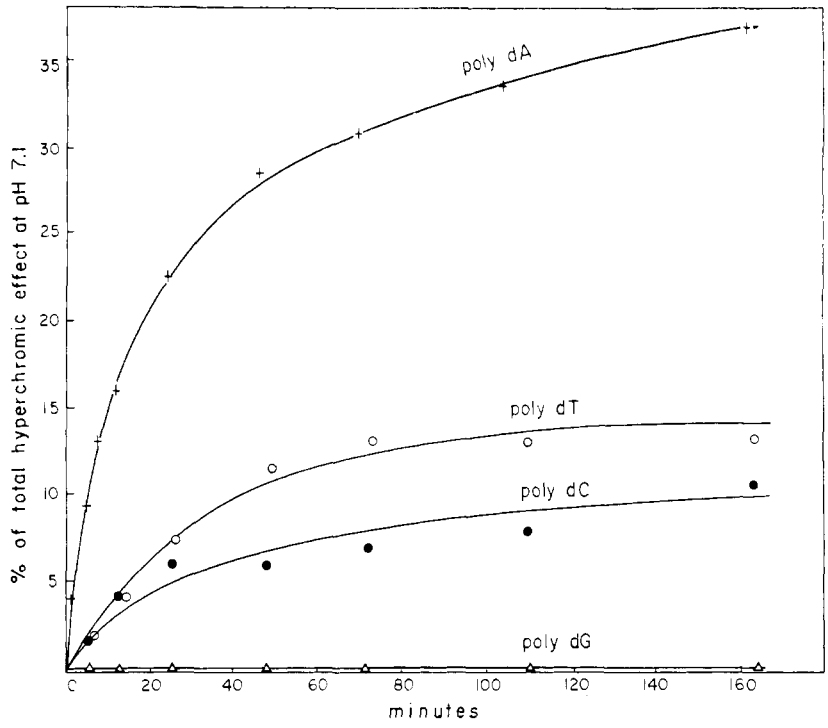

FIGURE 5: Degradation of deoxyhomopolynucleotides by nuclease D as measured by the hyperchromic effect assay. The reaction mixtures (total volume, 0.4 ml) at 30°, contained 0.1–0.3 ODU of deoxyhomopolynucleotide, 10 μmoles of Tris buffer (pH 7.1), 0.1 μmole of $CaCl_2$ and $MgCl_2$, and 6 units of enzyme.

poly dA and poly dT but not poly dC or poly dG. The initial rate on poly dA is greater than that on poly dT. After a 30-min incubation about 12% of the total hyperchromic effect of poly dA is achieved, as at which time only 2% is achieved by poly dT. The rate on poly dA may be slightly overestimated since, at pH 8.5, the secondary structure of poly dA may contribute appreciably to the total hyperchromic effect while that of poly dT may not.

Since at pH 8.5 poly dC is in the form of a random coil, the lack of any detectable degradation of this polymer by the A enzyme may have been caused by the fact that the enzyme requires an ordered structure for its action. To test this possibility the experiment in Figure 1 was repeated at pH 7.1. As shown in Figure 2 the poly dA and poly dT curves are comparable to those in Figure 1. Poly dC is also degraded but apparently at a slower rate. The rate on poly dC may be an underestimate in terms of bonds cleaved per unit time, since at pH 7.1 this polymer should be in the form of a single-chain base-stacked helix.

Degradation of Deoxyhomopolynucleotides by Nuclease B (Figure 3). Nuclease B degrades the deoxyhomopolynucleotides at rates different from those of nuclease A. It attacks poly dC with a high initial rate, followed by poly dT and poly dA. Poly dG is resistant to its action. At pH 8.5 poly dC and poly dT are both in the form of random coils and therefore the relative increases in absorbancy shown are good reflections of the rates in terms of bonds cleaved. The rate shown for poly dA, however, may actually be slower since small oligonucleotides do not contribute appreciably to the total hyperchromic effect of this polymer (Table I).

Degradation of Deoxyhomopolynucleotides by Nuclease C (Figure 4). Nuclease C degrades poly dT with a high initial rate which, however, sharply decreases so that after prolonged incubation, only 45% of the total hyperchromic effect is achieved as compared to 58 and 34% for poly dC and poly dA, respectively. At pH 7.1, poly dT exhibits 26% more hyperchromic effect than at pH 8.5, and it is therefore conceivable that the high initial rate shown is an overestimate caused by an appreciable contribution of the secondary structure of this polymer to the total hyperchromic effect. By the same token the rate shown on poly dA is probably also an overestimate because of the low contribution of small oligomers to the total hyperchromic effect (see Table I). The initial rate shown for poly dC is an underestimate since, at pH 7.1, this polymer is in the form of a single-chain base-stacked helix. Poly dG is resistant to degradation by nuclease C.

Degradation of Deoxyhomopolynucleotides by Nuclease D (Figure 5). Nuclease D attacks poly dA at a fast initial rate achieving 17% of the total hyperchromic effect in 10 min, at which time only 4 and 3% of the total hyperchromic effect of poly dT and poly dC are achieved, respectively. It is unable to degrade poly dG. Up to a 160-min incubation, the rate of degra-

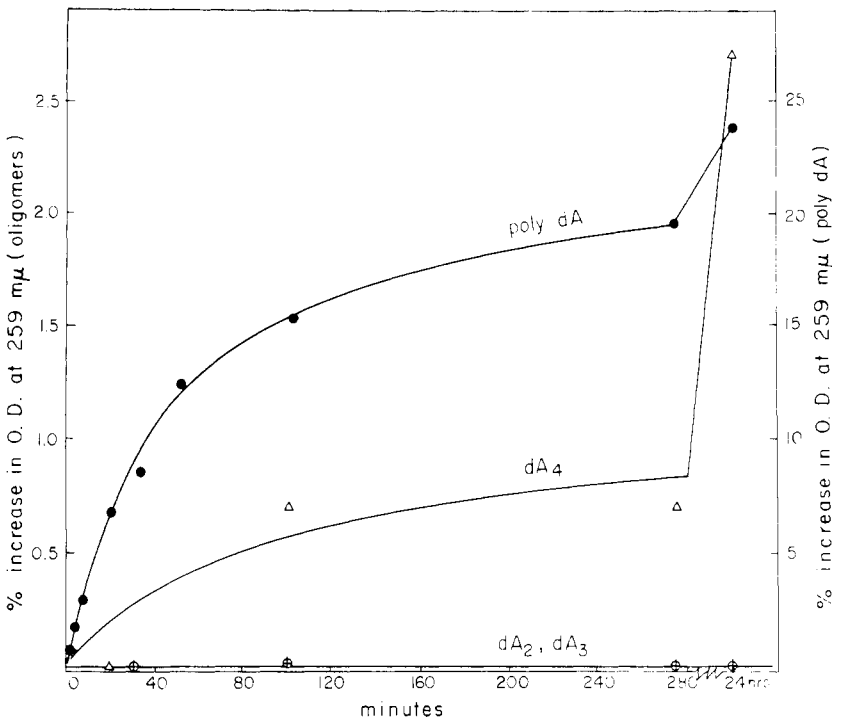

FIGURE 6: Degradation of adenylate deoxyoligonucleotides by nuclease A as measured by the hyperchromic effect assay. The reaction mixtures (total volume, 0.4 ml) at 30° contained 0.1–0.2 ODU of deoxyoligonucleotide, 8 μmoles of Tris buffer (pH 8.0), 2 μmoles of $CaCl_2$ and $MgCl_2$, and 3 units of enzyme.

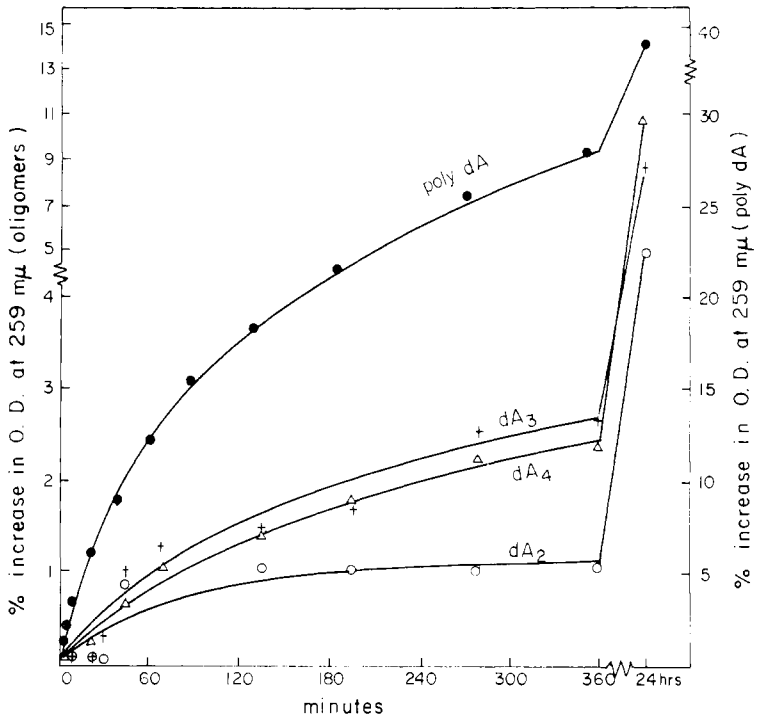

FIGURE 7: Degradation of adenylate deoxyoligonucleotides by nuclease B as measured by the hyperchromic effect assay. Reaction mixtures as in Figure 6 except that 70 units of enzyme was added.

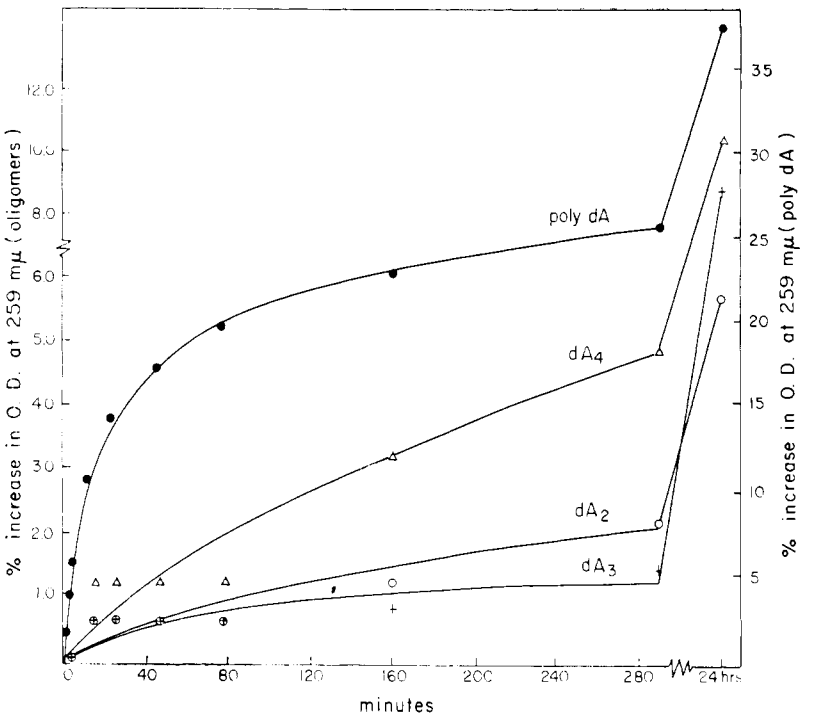

FIGURE 8: Degradation of adenylate deoxyoligonucleotides by nuclease C as measured by the hyperchromic effect assay. Reaction mixtures as in Figure 6 except that the pH was 7.1 and 4 units of enzyme was added.

dation of poly dA, with 37% of the total hyperchromic effect achieved, is still appreciable, whereas that of poly dT and poly dC, with only 13 and 10% of the total hyperchromic effect achieved, respectively, has almost ceased. These results strongly suggest the preference of the D enzyme for the degradation of poly dA.

Degradation of Deoxyadenylate Oligonucleotides by Nucleases A–D. The rate of degradation of the adenylate oligomers $d(pA)_2$, $d(pA)_3$, and $d(pA)_4$ was followed by the hyperchromic effect assay to determine the smallest fragment that can be degraded by each of the streptococcal nucleases. The results are shown in Figures 6–9, for enzymes A–D, respectively. Nuclease A degrades $d(pA)_4$ but not $d(pA)_2$ or $d(pA)_3$. After a 24-hr incubation only 2.7% hyperchromic effect is achieved by dA_4. This is equivalent to 13% of the total hyperchromic effect of this oligomer (see Table I). This resistance of small oligomers to attack by the enzyme is reflected in the action of the enzyme on poly dA since after 24 hr only 24% hyperchromic effect is achieved (or 38% of the total hyperchromic effect of the polymer), at which time the reaction has almost ceased. Enzymes B and C degrade all three oligomers at about the same rate. After a 24-hr incubation nuclease B achieved 5.1, 8.8, and 10.8% hyperchromic effect on $d(pA)_2$, $d(pA)_3$, and $d(pA)_4$, respectively, while nuclease C achieved 5.7, 8.3, and 10.3%, respectively. This greater extent of oligomer degradation by the two enzymes is again reflected in their action on poly dA. After a 24-hr incubation about 38% hyperchromic effect (or 60% of the total hyperchromic of poly dA) is achieved in both cases. Nuclease D degrades all three oligomers at a relatively fast rate with 5, 6, 7, and 30% hyperchromic effect achieved for $d(pA)_2$, $d(pA)_3$, $d(pA)_4$, and poly dA, respectively, after a 120-min incubation. This fast rate is consistent with the previous observation that the D enzyme has a preference for poly dA degradation.

The action of the four nucleases on adenylate dinucleotide $d(pA)_2$ was compared with that on the dinucleotide dephosphorylated by the action of the *Escherichia coli* phosphatase. The results indicated that the 5′-terminal phosphate was not essential for the action of any of the enzymes. This is in contrast to the exonucleolytic action of snake venom phosphodiesterase which is much less active on substrates without a 5′-phosphorylated terminus (Privat deGarilhe and Laskowski, 1956).

Discussion

The use of homopolynucleotides in the study of nuclease specificity is a relatively new approach. The ribohomopolynucleotides, first to be available commercially, have been used by a number of investigators for this purpose (Rushizky *et al.*, 1961; Bernardi, 1964). In spite of their simplicity these substrates have provided information on nuclease specificity which is consistent with that obtained by using the natural substrate (RNA) (Rushizky *et al.*, 1961; Yasmineh, 1966). The deoxyhomopolynucleotides, unavailable commercially, are relatively untested in studying nuclease specificity.

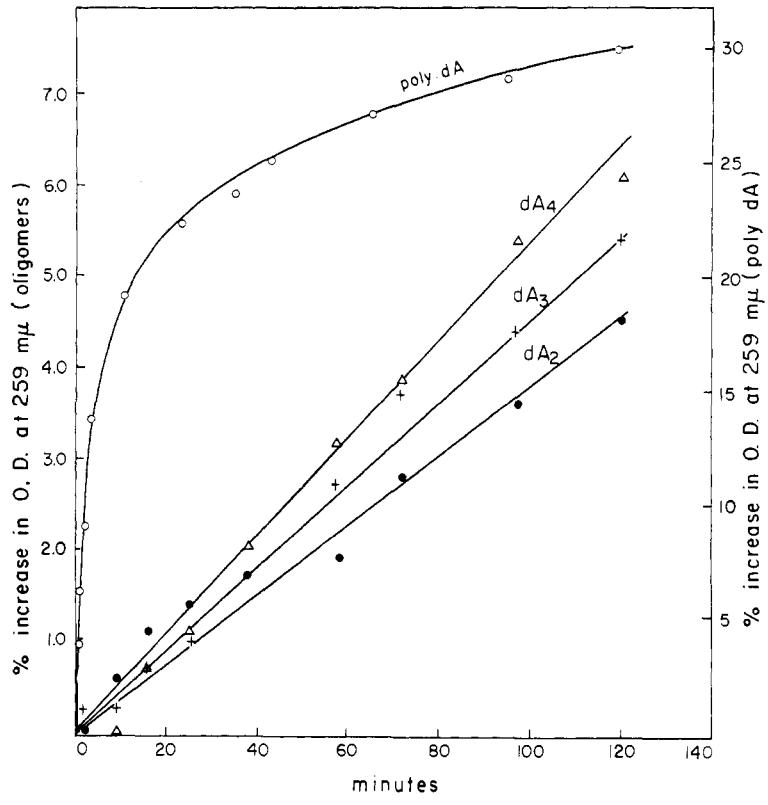

FIGURE 9: Degradation of adenylate deoxyoligonucleotides by nuclease D as measured by the hyperchromic effect assay. Same as in Figure 6 except that the pH was 7.1 and 28 units of enzyme was added.

In the present study only small amounts of the substrates were available. Consequently, the hyperchromic effect assay was used to measure nuclease activity to conserve substrate. Although this technique is probably unsuitable for precise kinetic measurements, it is adequate to detect enzyme preferences.

In the studies reported here, enzymes A and B showed a slight preference for the degradation of poly dA and poly dC, respectively, nuclease C showed little substrate preference, while nuclease D showed a preference for poly dA. The 6-amino base specificity of the RNase action of nuclease B and the preference of nuclease D for the degradation of poly C (see Gray and Yasmineh, 1967) have not been observed in the DNase action of enzymes B and D. This is of interest because it suggests a key role for the C-2′ hydrogen atom in the action of these enzymes; however, the physical state of the deoxypolymer may also be involved.

Specificity studies of the four streptococcal nucleases based on an examination of the products of exhaustive digests of thymus DNA (Winter and Bernheimer, 1964) have shown that nucleases A and B have a preference for the cleavage of the d-pXpG[2] bond, the C enzyme has little bond preference, while nuclease D has a preference for the d-pXpA bond. Comparison of these results with those obtained using homopolynucleotides reveals that the results of enzymes A and B differ while those of enzymes C and D are similar. The lack of agreement in the results obtained with the A and B enzymes may be due to the fact that Winter and Bernheimer used exhaustive digests. This technique may be inadequate when dealing with nucleases that change in specificity with changes in the chain length of the substrate. Such is the case with micrococcal nuclease (Sulkowski and Laskowski, 1962) which initially shows an endonucleolytic attack on DNA with a specificity for the d-XpTp and d-XpAp bonds until a chain length of seven to nine nucleotides is reached, at which time the 3′-terminal phosphate group starts to interfere with the endonucleolytic character of the enzyme and mono- and dinucleotides start to be formed. However, such a mechanism may not be operative in the case of nuclease B since dephosphorylation is without effect on the degradation of oligonucleotides. Another explanation for this lack of agreement is afforded by the resistance of poly dG to degradation by any of the enzymes, thus preventing the observation of d-pXpG preferences. It appears that poly dG has an unusual configuration rendering it resistant to attack by any of the streptococcal nucleases as well as pancreatic DNase and venom diesterase (Yasmineh, 1966; Ralph *et al.*, 1962). This conclusion is supported by the finding

[2] The notation indicates a deoxydinucleotide terminated in 5′-phosphate. X means any purine or pyrimidine nucleoside.

(Bollum, 1965) that both members of the homopolymer pair poly (dG:dC) are susceptible to attack by pancreatic DNase. Since poly (dG:dC) was prepared by using poly dC as template in the calf thymus DNA polymerase system, it would seem that the template prevented poly dG from assuming a configuration resistant to enzymic attack. This problem might be approached by using poly dI instead of poly dG as substrate. It appears that none of the streptococcal nucleases exhibit absolute specificity for internucleotide bonds. This lack of specificity is a property they share with all of the DNases that have been studied. Perhaps an enzyme which is capable of degrading DNA cannot be restricted in its specificity of action. The preferences observed might be reinforced or altered by the presence of divalent cations other than Ca^{2+} and Mg^{2+}. Such studies are presently underway.

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