Streptococcal Nucleases. IV. Some Properties and Specificities of the Ribonuclease Action of the B and D Enzymes*

E. D. Gray† and W. G. Yasmineh‡

ABSTRACT: Certain properties of the ribonuclease (RNase) activities of streptococcal nucleases B and D have been examined. The enzymes require divalent cations for activity. Nuclease B shows a broad pH optimum (5.5–7.5) while nuclease D shows a narrower range (6.5–7.0). These optima differ from those of the deoxyribonuclease (DNase) activities. The specificity of the two enzymes as well as that of pancreatic RNase was studied using ribohomopolynucleotides and, in the case of nuclease B, chemically deaminated yeast RNA. Nuclease B degrades polyadenylic acid (poly A) and polycytidylic acid (poly C) but not polyuridylic acid (poly U) or polyinosinic acid (poly I), indicating that the amino group on C-6 is essential for its activity. This specificity was confirmed by the demonstration that this enzyme degrades deaminated yeast RNA at a much slower rate than native RNA. Nuclease D shows a high affinity for poly C but can also degrade poly U and poly A at comparatively low rates. It does not degrade poly I. The kinetics of action of nuclease B and pancreatic RNase on natural and synthetic substrates have been compared. The K_m values of both enzymes are smaller for RNA than for any of the homopolymer substrates, despite the specificities exhibited above. The possibility that this is due to a conformational factor which enhances the affinity of the enzymes toward the natural substrate is considered in the light of available calculations for affinity constants and relative turnover numbers for the two enzymes.

The extracellular nucleases which are elaborated by *Streptococcus pyogenes* have been shown to consist of at least four distinct entities (Wannamaker, 1958, 1962; Winter and Bernheimer, 1964). Two of these enzymes originally designated as DNase B and DNase D have been demonstrated to possess RNase activity which strong evidence indicates is not due to contamination (Wannamaker, 1962; Yasmineh *et al.*, 1968). The ribonuclease activities are endonucleolytic in the initial stages of reaction but prolonged digestion results mainly in 5′-mononucleotide products (Gray *et al.*, 1964). The enzymes attack rRNA at a considerably faster rate than tRNA and this preference seems to be related to the secondary structure of tRNA (Yasmineh *et al.*, 1968). The purpose of the present study was to further examine the properties of these two ribonuclease activities and their specificities of action employing synthetic ribohomopolynucleotides.

Experimental Section

Materials

Yeast RNA and calf thymus DNA were prepared by Duponol extraction (Crestfield *et al.*, 1955; Zamenhof, 1957). Deaminated yeast RNA was prepared by the method of Takemura (1951).

Streptococcal nucleases B and D were prepared as described in the previous paper (Yasmineh *et al.*, 1968). Since the ratio of RNase to DNase activity, at least with respect to the B enzyme, is constant (Wannamaker and Yasmineh, 1968), the unit of RNase activity will be defined as was the unit of DNase activity in the previous paper (Yasmineh *et al.*, 1968). It is the amount of B or D 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.

The adenylate ribooligonucleotides $(pA)_2$, $(pA)_3$, and $(pA)_5$ were prepared by partial digestion of poly A with nuclease B, followed by chromatography on DEAE-cellulose in 0.02 M acetate buffer with a linear gradient of 0–0.4 M NaCl (Bollum, 1965). Desalting of the individual oligomers was accomplished by loading the samples on a DEAE-cellulose column (2 × 10 cm), washing with distilled water, and eluting first with 0.05 M triethylammonium bicarbonate (TEA–HCO_3)[1] to remove salt and then with 0.5 M TEA–HCO_3 to remove the oligomers. The oligomer fractions were pooled and the TEA–HCO_3 was removed by flash evaporation.

Uranium acetate–perchloric acid precipitating reagent was prepared as described by Hilmoe (1961). HCl–ethanol precipitating reagent was prepared ac-

* From the Departments of Pediatrics and Biochemistry, College of Medical Sciences, University of Minnesota, Minneapolis, Minnesota. *Received July 31, 1967.* This study was supported by grants from the U. S. Public Health Service (HE-01829-12), the American Heart Association, the Minnesota Heart Association, and the Graduate School of the University of Minnesota.

† Supported by U. S. Public Health Service, Cardiovascular Program Project Grant HE-06314.

‡ National Institutes of Health Predoctoral Fellow.

[1] Abbreviations used: TEA, triethylammonium; CMP and UMP, cytosine and uridine monophosphates.

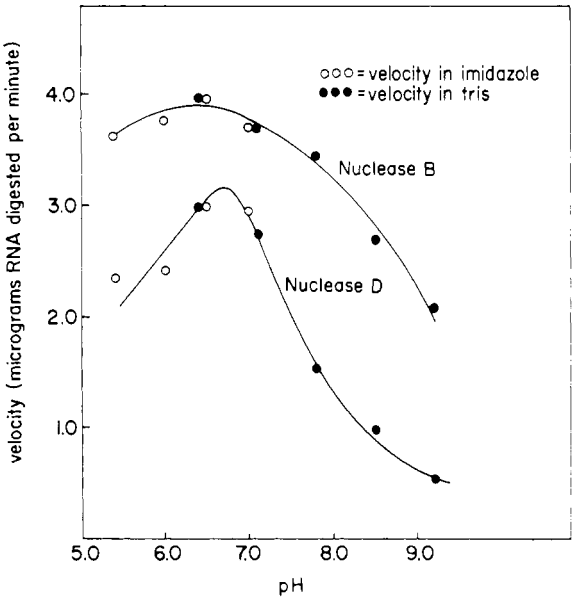

FIGURE 1: pH optima of RNase activity of nucleases B and D. The incubation mixtures (2.5-ml total volume) at 30° contained 0.8 mg of yeast RNA, 200 μmoles of Tris or imidazole buffer (at the pH values indicated), 10 μmoles of $CaCl_2$ and $MgCl_2$, and 200 units of nuclease B or 190 units of nuclease D. At various intervals after enzyme addition 0.5-ml aliquots were removed and added to 1 ml of cold 0.75 N HCl. Following centrifugation the optical density of the supernatant fluids were read at 260 mμ.

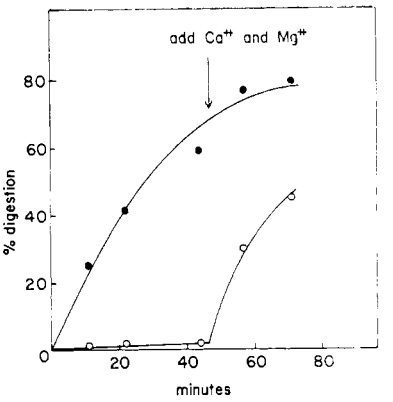

FIGURE 2: Inhibition of nuclease D by EDTA. The reaction mixture (5.0-ml total volume) at 37° contained 2 mg of yeast RNA, 10 μmoles of acetate buffer (pH 6.5), 5 μmoles of EDTA (pH 6.5), 0.5 μmole of $CaCl_2$ and $MgCl_2$, and 280 units of enzyme. Following enzyme addition 0.8-ml aliquots were removed at intervals and added to 0.8 ml of cold HCl–ethanol. The optical density of the supernatant fluids was measured at 260 mμ. $CaCl_2$ and $MgCl_2$ (10 μmoles each) were added (in 0.1-ml volume) to the incubation mixture at 47 min.

cording to Roth and Milstein (1952). The ribohomopolynucleotides poly A, poly U, poly C, and poly I were purchased from Miles Chemical Co., Elkhart, Ind. Pancreatic RNase and venom diesterase were purchased from Worthington Biochemical Corp., Freehold, N. J.

Methods

Assay for Ribonuclease Activity. Ribonuclease activity was measured by any one of three methods: pH-Stat titration, measurement of the nucleic acid rendered acid soluble, or measurement of the increase in ultraviolet absorption of the nucleic acid. The technique for pH-Stat titration has already been described (see Yasmineh *et al.*, 1968).

In the acid-solubility assay, the reaction mixture containing nucleic acid, buffer, and divalent cation activators (the concentrations to be indicated in the individual experiments) was incubated with enzyme at 30°. At various intervals aliquots were removed and added to cold HCl or alternately to uranium acetate–perchloric acid reagent. Following centrifugation the optical density of the supernatant fluid was measured at 260 mμ.

In the hyperchromic effect assay, the reaction mixture (as above) was placed in a Beckman cuvet and, following the addition of enzyme, optical density readings were taken at the wavelength of maximal absorption of the nucleic acid in question.

Results

pH Optima. The pH dependency of the RNase actions of nucleases B and D are shown in Figure 1. The points represent initial rates of reaction measured at the different pH values. Two buffer systems were employed in the study (imidazole and Tris). The actual rates determined in Tris buffer were somewhat lower than in imidazole at the same pH. This may be related to chelation by Tris and the requirement of both enzymes for Ca^{2+} and Mg^{2+} ions. A correction factor applied to the Tris values to give the same rates as imidazole buffer at overlapping pH values gave the smooth curves of Figure 1. The RNase action of nuclease B shows a broad optimum range of 5.5–7.5. Nuclease D is more pH dependent in its action with an optimum of 6.5–7.0.

Cation Requirements. It has been shown (Winter and Bernheimer, 1964) that the DNase activity of the B and D enzymes has an absolute requirement for divalent cations and a similar requirement has been reported (Wannamaker and Yasmineh, 1968) for the RNase activity of nuclease B. In Figure 2 are shown the results of a study examining the requirements of nuclease D. The enyzme was incubated with RNA and divalent cations in the presence and absence of EDTA sufficient to effectively bind all divalent cations present. EDTA almost completely inhibited the action of the D enzyme. When an excess of divalent cations was added to the same reaction mixture, RNA was degraded at a rate comparable to that of the control.

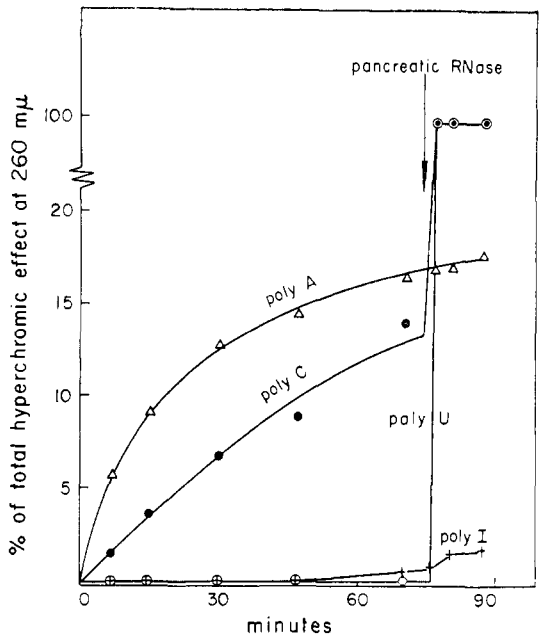

FIGURE 3: Degradation of ribohomopolynucleotides by nuclease B as measured by the hyperchromic effect assay. The reaction mixtures (3-ml total volume) at 30°, contained 0.3 μmole of ribohomopolynucleotide, 400 μmoles of NaCl, 1 μmole of $CaCl_2$ and $MgCl_2$, 8 μmoles of acetate buffer (pH 6.0), and 220 units of nuclease B. The hyperchromic effect was followed at 260 mμ. Pancreatic RNase (10 μg in 0.1-ml volume) was added after 75-min incubation.

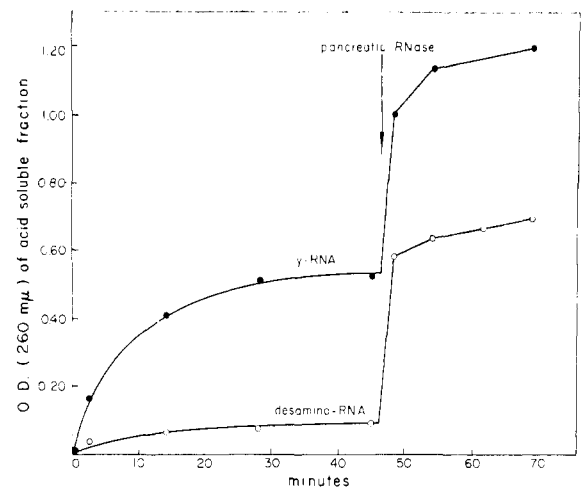

FIGURE 4: Degradation of desamino-RNA by nuclease B as measured by the acid-solubility assay. The reaction mixtures (total volume, 4.0 ml) at 30° contained 4 mg of substrate, 10 μmoles of $CaCl_2$ and $MgCl_2$, 100 μmoles of NaCl, 50 μmoles of acetate buffer (pH 6.0), and 70 units of nuclease B. Following enzyme addition undegraded RNA was precipitated with uranium acetate–perchloric acid and the optical density of the supernatant fluids was measured at 260 mμ. Pancreatic RNase (10 μg in 0.1-ml volume) was added at 46 min.

The RNase activities of both B and D enzymes evidently require divalent cations. This requirement is unusual for ribonuclease activity and lends support to the proposal that with respect to the D as well as the B enzyme both activities reside in a single protein.

Specificity of Action. The specificity of action of the RNase activities of enzymes B and D was studied using the ribohomopolymers poly A, poly U, poly I, and poly C as substrates. The results of such an experiment in which the activity of nuclease B on these polymers was measured by the hyperchromic effect assay are shown in Figure 3. This enzyme rapidly degrades poly A and to a lesser extent poly C but has no action on poly U and poly I. It was considered possible that these latter might somehow be unavailable for enzymic attack under the conditions of the experiment. However, when pancreatic RNase was added, there was a maximal increase in hyperchromic effect indicating a degradation of both poly U and poly C to monomers. Pancreatic RNase does not attack purine internucleotide bonds (Rushizky *et al.*, 1961); thus poly A and poly I were unaffected under the conditions of this experiment. It would appear that the specificity of action of nuclease B is directed toward the nucleotides with amino substituents at C-6, the 6-keto-substituted nucleotides being unaffected.

This is a type of specificity that has been previously unreported for ribonuclease. To obtain evidence in addition to that provided by the homopolymer studies, nuclease B was treated with RNA chemically deaminated with nitrous acid. Such treatment of RNA causes the deamination of cytidylic acid to uridylic acid and adenylic acid to inosinic acid and should provide a substrate less susceptible to attack than native RNA. The results of such an experiment are shown in Figure 4. The B enzyme attacks RNA at a much greater rate than desamino-RNA. The deaminated substrate is only slightly affected by the enzyme. After 45 min of incubation with nuclease B, addition of pancreatic RNase to both reaction mixtures led to an immediate breakdown of both substrates indicating that the desamino-RNA is not unavailable to enzymic attack. When the deaminated substrate was degraded to 2′,3′-mononucleotides by alkaline hydrolysis and the monomers separated by chromatography it was found that only 40–50% of the adenylic and cytidylic residues had been deaminated by the nitrous acid treatment. The resistance of this substrate to degradation by nuclease B suggests that the enzyme attacks only between adjacent susceptible residues.

Nuclease D exhibits a different specificity of action toward homopolymers. Figure 5 shows that the D enzyme attacks poly C very rapidly and is also active on poly U and poly A. After 7 min of incubation, 48% of the hyperchromic effect of poly C is attained while only 9 and 4% of the total hyperchromicity of poly U and poly A, respectively, are attained. Poly I is as resistant to enzyme D as it is to the B enzyme.

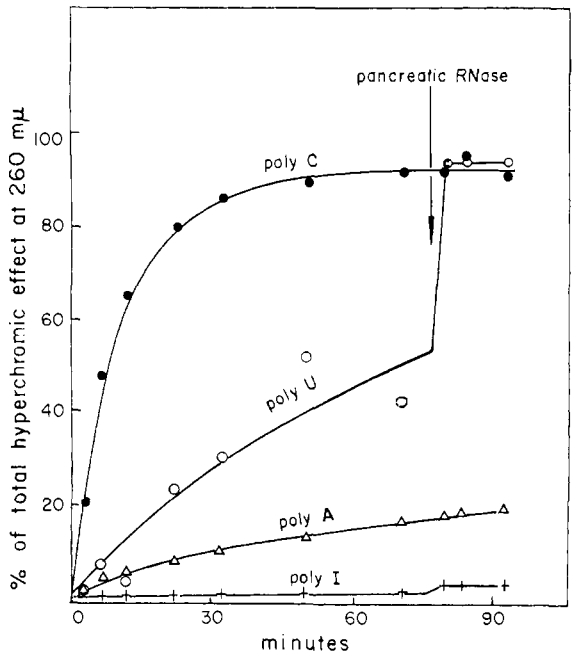

FIGURE 5: Degradation of ribohomopolynucleotides by nuclease D as measured by the hyperchromic effect assay. Experiment as in Figure 3 except that 280 units of nuclease D was added.

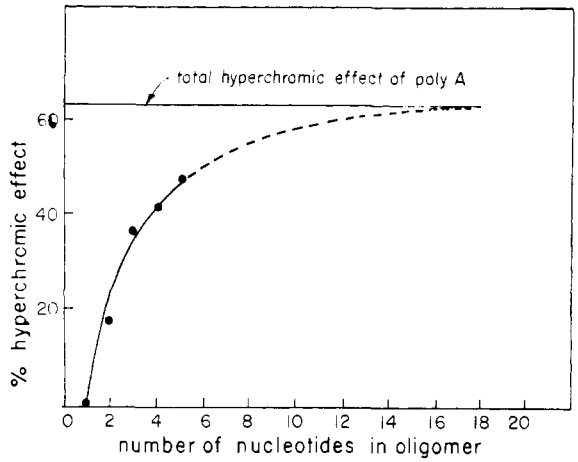

FIGURE 6: Hyperchromic effect as a function of the chain length of adenylate ribooligonucleotides. The reaction mixtures (2.2-ml total volume at 30° contained 0.9–1.1 ODU of oligomer, 100 μmoles of sodium acetate, and 40 μg of venom diesterase. The hyperchromic effect was followed at 259 mμ.

Following the addition of pancreatic RNase after 70-min incubation, poly U is rapidly and almost completely degraded while there is no further breakdown of poly C. This indicates that the action of nuclease D on poly C must have reduced the polymer mainly to mononucleotides.

To estimate in terms of bonds cleaved the extent and rate of degradation of both poly A and poly C as measured by the hyperchromic effect assay the secondary structure of the polymers must be considered. At the pH of the reaction mixtures (6.2) the polymers are in the form of single-chain base-stacked helices in which small oligonucleotides contribute substantially to the total hyperchromic effect (Inman, 1964; Brahms *et al.*, 1966; Yasmineh, 1966). An example of this is shown in Figure 6, which illustrates the hyperchromic effect on hydrolysis to mononucleotides of a series of adenylate oligomers. The trimer exhibits almost 40% hyperchromic effect while poly A itself shows 63%. Thus a considerable degradation of poly A (and poly C) would not be accompanied by a great increase in hyperchromic effect. It is only when the smaller oligomers are degraded that large increases in hyperchromic effect are obtained. In view of this, the action of nuclease D on poly C is even more drastic than would appear from Figure 5. The same conditions apply for the nuclease B results in Figure 3.

Kinetics of Action. The kinetics of action of nuclease B was compared with that of pancreatic RNase on various substrates. The pH-Stat was employed to measure rates of reaction as a more direct reflection of number of bonds cleaved than the hyperchromic effect assay allows. The pH-Stat titration results were consistent with those of the hyperchromic effect studies, *i.e.*, poly U and poly I are unaffected by nuclease B and poly A and poly I are not attacked by pancreatic RNase. The kinetics of these enzymes' action on susceptible substrates are shown in Figures 7 and 8.

In the case of nuclease B the kinetic studies indicate that with high substrate concentrations of RNA and poly C a decreased rate of action occurred (Figure 7). This might be ascribed to a "choking" of the substrate binding sites, but is more likely a reflection of the requirement of this enzyme for divalent cations. It has been reported that high concentrations of substrate require higher concentrations of cations for optimal activity of DNase I (Shack and Bynam, 1964). The present rate studies were carried out at concentrations of Ca^{2+} and Mg^{2+} which might have been limiting at higher concentrations of poly C and RNA. Pancreatic RNase does not require cation activators and does not show such an effect (Figure 8).

The K_m values for the action of the two enzymes on the various substrates are summarized in Table I. For both enzymes the Michaelis constants with RNA as substrate are smaller than with the ribohomopolynucleotides. The ratio K_m poly C/K_m RNA is 6.2 for RNase B and 2.6 for pancreatic RNase. In spite of the demonstrated preferences for bonds involving cytidylic acid, the natural substrate is apparently more strongly bound to both enzymes than the synthetic homopolymer. These results suggest that both enzymes require conformational factor(s) for optimal action, which are present in the natural substrate but lacking in the synthetic homopolymers.

The RNase actions of enzymes B and D appear to be endonucleolytic at least in the early stages of diges-

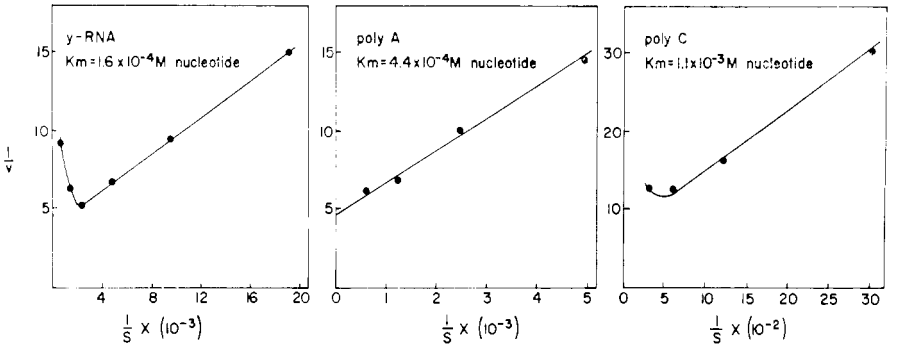

FIGURE 7: Double-reciprocal plots for nuclease B using various substrates; initial reaction rates determined by pH-Stat titration. Substrate concentrations are in moles per liter of nucleotide. The reaction mixtures (total volume, 1.1 ml; pH 6.5) contained substrate as indicated, 0.1 M NaCl, 0.01 M $CaCl_2$ and $MgCl_2$, and 98, 79, and 79 units of nuclease for RNA, poly A, and poly C as substrate, respectively. The concentrations of NaOH titrant was 10^{-4}, 10^{-5}, and 10^{-5} N, respectively. The velocity (v) is equal to milliliters of NaOH added per minute.

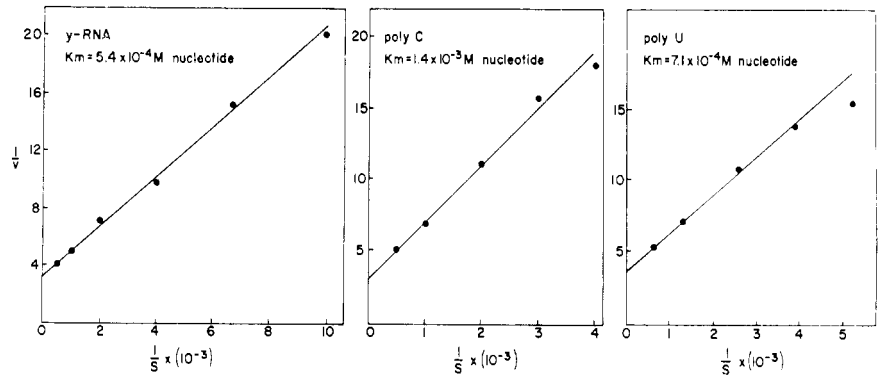

FIGURE 8: Double-reciprocal plots for pancreatic RNase using various substrates; initial reaction rates were determined by pH-Stat titration. Experiments performed as in Figure 7. The amounts of pancreatic RNase added were 4, 1, and 7 μg for RNA, poly C, and poly U as substrate, respectively. The concentration of NaOH titrant was 10^{-4} N.

tion (E. D. Gray, unpublished observations). In this respect they are similar to the DNase activities of these enzymes. It has been observed that upon prolonged action, the end products of both enzymes are 5'-mononucleotides (Gray *et al.*, 1964). To examine this exonucleolytic-like activity the B and D enzymes were treated with a series of small oligomers of adenylic acid. In Figure 9 are shown the rates of increase in hyperchromic effect catalyzed by nuclease B. Even though the enzyme concentration is an order of magnitude greater, the breakdown of the oligomers is very slow compared to the action of nuclease B on the polymer. Up to 24 hr of digestion the degradation is not complete and is apparently still proceeding at a constant rate. The rate of increase in hyperchromic effect of the dimer and trimer may be directly compared since the percentage increase resulting from the cleavage of these bonds is similar (see Figure 6). If one assumes that the substrate concentrations are saturating, the rate of breakdown of the trimer is almost twice that of the dimer.

The action of nuclease D on the oligonucleotides is much more rapid than that of the B enzyme (Figure 10). The dimer is almost completely degraded within 24 hr and the initial rates of attack on all of the substrates are higher than those of nuclease B, even though considerably less enzyme was added. As with nuclease B, the trimer is degraded at a faster rate than the dimer.

TABLE I: Michaelis Constants for Nuclease B and Pancreatic RNase with RNA and Ribohomopolynucleotides as Substrates.

	Nuclease B	Pancreatic RNase
RNA	1.6×10^{-4}	5.4×10^{-4}
Poly A	4.4×10^{-4}	Not active
Poly C	1.1×10^{-3}	1.4×10^{-3}
Poly U	Not active	7.1×10^{-4}
Poly I	Not active	Not active

The pentamer is attacked most readily by both enzymes but the rate cannot be directly compared since cleavage of the internucleotide bonds of oligomers of greater size than trimer produce considerably less hyperchromic increase (Figure 6). It is evident that both enzymes are active on fragments as small as dinucleotides and that the ultimate products of digestion are mononucleotides.

Discussion

The streptococcal enzymes B and D may be considered similar to micrococcal nuclease in that they all possess both DNase and RNase activities. The recent purification and crystallization of micrococcal nuclease has shown that both of these activities are resident in a single protein (Sulkowski and Laskowski, 1966; Taniuchi and Anfinsen, 1966). Various lines of evidence indicate that this is also the case with the streptococcal enzymes (Wannamaker and Yasmineh, 1968; Yasmineh *et al.*, 1968).

The requirement for divalent cation activators by nuclease B and D is unusual for ribonucleases. Most such enzymes do not require these for activity and indeed may be inhibited by them (Anfinsen and White, 1961). This is additional evidence linking the RNase and DNase activities of the B and D enzymes. The pH optima, however, indicate that the RNase and DNase (Wannamaker, 1958; Wannamaker and Yasmineh, 1968) activities of both enzymes differ in their pH dependency. This may be a reflection of the properties of the differing substrates. The end products of degradation of RNA by the B and D enzymes are mononucleotides (Gray *et al.*, 1964). The DNase activities produce a certain proportion of mononucleotides but even on exhaustive digestion the main products are di-, tri-, and tetranucleotides (Gray *et al.*, 1964; Winter and Bernheimer, 1964). This difference again might be considered a result of the properties of the different substrates.

The specificity of nuclease B is unusual in that it appears to attack those internucleotide bonds involving 6-amino-substituted nucleotides. The enzyme had no activity on the 6-keto polynucleotides. Since these studies were carried out on homopolynucleotide substrates, this specificity might not be exerted on the natural substrate. The experiment employing deaminated RNA, however, showed that the removal of 6-amino groups made RNA a poorer substrate for nuclease B action.

The specificity of the D enzyme is also of considerable interest. The initial rate of degradation of poly C is many times that on poly A and poly U although these are degraded at significant rates. The preferential action of this enzyme on cytidylic acid linkages might be made more specific by alteration of the reaction conditions. The substitution of other cation activators or incubation at lower temperatures might enhance the specificity and make this enzyme a useful tool in nucleotide sequence studies (Bollum, 1965; Holley *et al.*, 1965). Under the conditions used in the present

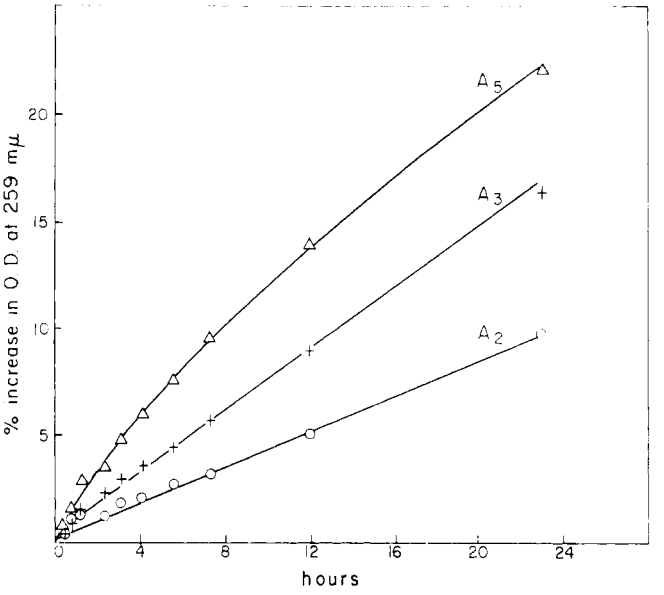

FIGURE 9: Degradation of adenylate ribooligonucleotides by nuclease B as measured by the hyperchromic effect assay. The reaction mixtures (total volume, 0.5 ml) at 30°, contained 0.2–0.3 ODU of ribooligonucleotide, 0.5 μmole of $CaCl_2$ and $MgCl_2$, 6 μmoles of acetate buffer (pH 6.2), and 280 units of enzyme. The hyperchromic effect was followed at 259 mμ.

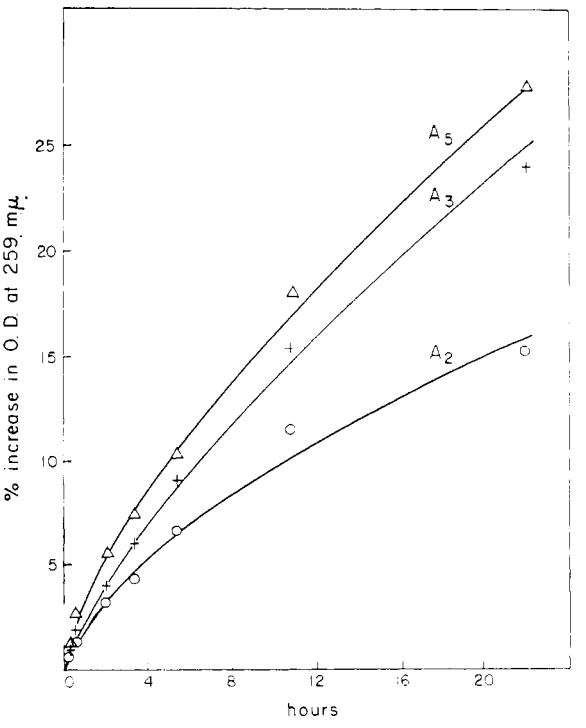

FIGURE 10: Degradation of adenylate ribooligonucleotides by nuclease D as measured by the hyperchromic effect assay. Experiment as in Figure 6 except that 56 units of nuclease D was added.

study both nucleases B and D exhibit only preferential specificities. Since the final products of action are mononucleotides, clearly both enzymes are capable of cleaving all types of internucleotide bonds.

The kinetic studies of nuclease B and pancreatic RNase have revealed some interesting points of similarity and difference between the two enzymes. The K_m values of both enzymes are smaller for RNA than for any of the homopolymer substrates. This greater affinity for RNA is amplified when it is considered that the specificity of action of the two enzymes makes a proportion of the internucleotide bonds in RNA less available for cleavage. It might be expected in view of the specificities of pancreatic RNase and nuclease B that they would exhibit a greater affinity toward homopolymers replete with susceptible bonds. Evidently there exists another factor, possibly conformational, which enhances the affinity of the enzymes toward the natural substrate.

These arguments are based on the assumption that the "affinity constant" is equal to the reciprocal of the Michaelis constant. Although this assumption is true in many instances, its validity ultimately depends upon whether the turnover number k_3 is negligible in comparison with the affinity constant k_1/k_2.[2] The pH-Stat data available from this study do not allow the calculation of k_1 and k_2; however, k_3 can be calculated if the molecular weight of the enzyme is known $V_{max} = k_3E_t$, where V_{max} is equal to the maximal velocity of the reaction, and E_t the total concentration of enzyme. The turnover numbers of nuclease B and pancreatic RNase obtained in this manner are listed in Table II.

Although it is difficult to decide whether the turnover numbers of pancreatic RNase are small enough to justify the equation of the Michaelis constant to the reciprocal of the "affinity constant" they are enlightening in other respects. The turnover number of pancreatic RNase with poly C as substrate is five times greater than that with poly U. This is consistent with the findings of Rushizky *et al.* (1961), who showed that upon digestion of RNA with pancreatic RNase, 3′-CMP is released at a faster rate than 3′-UMP, and furthermore that the cyclic 2′,3′-cytidylic residues at the end of purine oligonucleotides are hydrolyzed to 3′-phosphates at a faster rate than the corresponding terminal cyclic 2′,3′-uridylic residues. When the turnover numbers of pancreatic RNase are compared with the corresponding Michaelis constants it is found that whereas the lowest Michaelis constant is obtained with yeast RNA (and poly U) as substrate, the greatest turnover number is obtained with poly C. This implies that the low affinity suggested by the high K_m with poly C as substrate is only a reflection of the high turnover number. The situation with nuclease B is different. In this case the lowest Michaelis constant and greatest turnover number are obtained with yeast RNA. Such a situation can occur only if the k_3 values (or turnover numbers) are negligible in comparison with the k_2 values, since only under such a condition can yeast RNA have the highest affinity for the enzyme and yet be degraded by it at the highest rate.

One of the characteristics of nuclease action, the autoretardation effect (Laskowski, 1967), is also demonstrated in the present experiments. It has been proposed that this progressive decrease in the rate of nuclease action is due to the formation of small resistant oligomers. This interpretation would seem to be supported by the present experiments since such retardation in rate is not observed when small oligomers are the substrate.

TABLE II: Turnover Numbers of Nuclease B and Pancreatic RNase with RNA and Ribohomopolynucleotides as Substrates.

Substrate	Yeast RNA (min^{-1})	Poly A (min^{-1})	Poly C (min^{-1})	Poly U (min^{-1})
Pancreatic RNase	100		470	93
Nuclease B	5.2[a]	1.5[a]	1.0[a]	

[a] These are relative turnover numbers with the value for poly C arbitrarily set at unity. The absolute turnover numbers could not be calculated because the molecular weight of nuclease B is unknown.

[2] These constants were obtained from the simplified Michaelis–Menten equation for hydrolytic systems

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \longrightarrow E + P$$

k_1/k_2 being the affinity constant, k_3 the turnover number, and $(k_2 + k_3)/k_1$ the Michaelis constant.

References

Anfinsen, C. B., and White, F. H. (1961), *Enzymes 5*, 95.

Bollum, F. (1965), *J. Biol. Chem. 240*, 2599.

Brahms, J., Michelson, A. M., and Van Holde, K. E. (1966), *J. Mol. Biol. 15*, 467.

Crestfield, A. M., Smith, K. C., and Allen, F. W. (1955), *J. Biol. Chem. 216*, 185.

Gray, E. D., Yasmineh, W. G., and Wannamaker, L. W. (1964), *Federation Proc. 23*, 697.

Hilmoe, R. J. (1961), *Biochem. Prepn. 8*, 105.

Holley, R. W., Apgar, J., Everett, G. A., Madison, J. T., Marquisee, M., Merrill, S. H., Penswick, J. R., and Zamir, A. (1965), *Science 147*, 1962.

Inman, R. B. (1964), *J. Mol. Biol. 9*, 624.

Laskowski, M. (1967), *Advan. Enzymol. 29*, 165.

Roth, J. S., and Milstein, S. W. (1952), *J. Biol. Chem. 196*, 489.

Rushizky, G. W., Knight, C. A., and Sober, H. A. (1961), *J. Biol. Chem. 236*, 2732.

Shack, J., and Bynam, B. S. (1964), *J. Biol. Chem. 239*, 3843.
Sulkowski, E., and Laskowski, M. (1966), *J. Biol. Chem. 241*, 4386.
Takemura, S. (1951), *J. Chem. Soc. Japan 72*, 674.
Taniuchi, H., and Anfinsen, C. B. (1966), *J. Biol. Chem. 241*, 4366.
Wannamaker, L. W. (1958), *J. Exptl. Med. 107*, 797.
Wannamaker, L. W. (1962), *Federation Proc. 21*, 231.
Wannamaker, L. W., and Yasmineh, W. G. (1968), *J. Exptl. Med.* (in press).
Winter, J., and Bernheimer, A. W. (1964), *J. Biol. Chem. 239*, 215.
Yasmineh, W. G. (1966), Ph.D. Thesis, University of Minnesota, Minneapolis, Minn.
Yasmineh, W. G., Gray, E. D., and Wannamaker, L. W. (1968), *Biochemistry 7*, 91 (this issue; preceding paper).
Zamenhof, S. (1957), *Methods Enzymol. 3*, 696.

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

W. G. Yasmineh† and E. D. Gray‡

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-

* From the Department of Pediatrics and Biochemistry, College of Medical Sciences, University of Minnesota, Minneapolis, Minnesota. *Received July 31, 1967.* This study was supported by grants from the U. S. Public Health Service (HE-01829-12), the American Heart Association, the Minnesota Heart Association, and the Graduate School of the University of Minnesota.

† National Institutes of Health Predoctoral Fellow.

‡ Supported by U. S. Public Health Service, Cardiovascular Program Project Grant HE-06314.

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