Effects of Divalent Cations on Activity and Specificity of Streptococcal Nucleases B and D*

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ABSTRACT: The effect of divalent cations on the RNase activity and specificity of streptococcal nucleases B and D was studied using several divalent cation activators. The degradation of both yeast RNA and the ribohomopolynucleotides poly A, poly C, poly U, and poly I was investigated. The relative initial rates of attack on the polynucleotides were measured by both the hyperchromic effect assay and the acid-soluble assay.

The effects of divalent cations on nuclease B and D action are expressed in alteration of: (1) activity (differential rates of breakdown) toward ribopolynucleotides, both ribohomopolymers and RNA; (2) activity toward smaller low molecular weight polynucleotides as evidenced by the onset of autoretardation; and (3) specificity of the nucleases for internucleotide bonds adjacent to specific bases. Near absolute specificity of nuclease B for the internucleotide bond adjacent to the uridylate nucleotide was achieved when Hg^{2+} was the activating divalent cation.

Of the four extracellular nucleases elaborated by group A strains of streptococci, enzymes B and D possess RNase as well as DNase activity. These enzymes act endonucleolytically and form products terminated in 5′-phosphates. The RNase activities of both enzymes are dependent on the presence and concentration of divalent cations (Wannamaker, 1958; Winter and Bernheimer, 1964; Wannamaker and Yasmineh, 1967).

The specificity of nucleases A, B, C, and D against deoxyhomopolynucleotides has been studied using Ca^{2+} and Mg^{2+} as the divalent cation activator (Yasmineh and Gray, 1968). Nuclease D showed a preference for the degradation of poly dA as did nuclease A while nuclease C showed the highest initial rate of breakdown for poly dT. Nuclease B showed a preference for poly dC and poly dT. The degradation of ribohomopolynucleotides by nuclease D when activated by Ca^{2+} and Mg^{2+} showed a substrate preference for poly C (Gray and Yasmineh, 1968).

In studies done largely on nucleases obtained from other sources it has been shown that the activity of nucleases is strongly dependent on the concentration and character of divalent cations. The optimal concentration varies with pH, ionic strength and substrate concentrations (Shack and Bynum, 1964), and it has been demonstrated that divalent cations vary considerably in their ability to activate different nucleases (Wiberg, 1958; Desreux *et al.*, 1962).

Bollum (1965) indicated that different divalent cation activators may alter the relative specificity of DNase I. The purpose of the present study was to examine the effect of divalent metal cation activators on the activity and specificity of streptococcal nucleases B and D toward ribohomopolynucleotides.

Experimental Section

Materials. The ribohomopolynucleotides poly A, poly C, poly U, and poly I were purchased from Miles Chemical Co., Elkhart, Ind. The average molecular weight of the homopolymers was not less than 100,000 as determined by the supplier. Yeast RNA was prepared by the method of Crestfield *et al.* (1955).

Streptococcal nucleases were prepared by the method of Wannamaker (1958) with the exception that the final separation of the enzyme was achieved by DEAE-cellulose column chromatography instead of by starch zone electrophoresis (E. D. Gray, unpublished results). Nucleases B and D were further purified by additional DEAE-cellulose column chromatography. The enzyme was homogeneous with respect to streptococcal nuclease activity as determined by specific rabbit antisera neutralization.

Concentration of enzyme is presented as units of activity. One unit of activity is equivalent to the amount of enzyme capable of causing an initial rate of RNA breakdown of 1%/min at standard conditions employed in the hyperchromic effect assay when activated by Ca^{2+} plus Mg^{2+}.

Methods. ASSAY FOR RIBONUCLEASE ACTIVITY. The RNase activity of nucleases B and D was measured by either the increase in absorbance at 260 nm due to degradation of the polynucleotide (hyperchromic effect assay) or by measurement of the nucleic acid rendered acid soluble (acid-soluble assay).

The hyperchromic effect assay was the primary assay used in this work. The total volume of the reaction mixture was 3 ml and had a concentration of 0.11 mM substrate nucleotide (RNA or ribohomopolymer), 133.0 mM NaCl, 2.66 mM sodium acetate buffer at pH 6.2, and 0.33 mM divalent cation. The reaction mixture was allowed to equilibrate for 30 min at 30° before enzyme was added. Varying amounts of enzyme were

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TABLE I: The Relative Initial Rates of Breakdown of RNA by Nucleases B and D.[a]

Divalent Cation	Nuclease B	Nuclease D
$Ca^{2+} + Mg^{2+}$	1.00	1.00
Ca^{2+}	0.64	0.90
Mg^{2+}	0.31	0.34
Sr^{2+}	0.40	0.34
Cd^{2+}	0.01	0.01
Co^{2+}	0.64	0.56
Mn^{2+}	0.56	0.43
Ni^{2+}	0.11	0.16
Zn^{2+}	0.58	0.01
Hg^{2+}	0.01	0.01
Hg^{2+} (I:R = 30:1)	0.23	

[a] All reaction mixtures contained one unit of enzyme. The molar ratio of cation to substrate nucleotide (I:R) was 3:1 except as noted.

TABLE II: The Relative Initial Rates of Poly A Breakdown by Nucleases B and D.[a]

Divalent Cation	Nuclease B	Nuclease D
$Ca^{2+} + Mg^{2+}$	1.00	1.00
Ca^{2+}	0.34	0.83
Mg^{2+}	0.97	0.87
Sr^{2+}	0.50	0.51
Cd^{2+}	0.82	0.17
Co^{2+}	1.97	0.58
Mn^{2+}	1.62	0.36
Ni^{2+}	0.35	0.25
Zn^{2+}	0.22	0.33
Hg^{2+}	0.10	0.32

[a] The rates are relative to poly A breakdown when the enzymes are activated by Ca^{2+} plus Mg^{2+} and are corrected to correspond to one unit of enzyme in each assay. The ratio I:R was 3:1 in all reactions.

added as noted in each figure. The temperature was maintained at 30° and the hyperchromic effect followed at 260 nm.

The acid-soluble assay reaction mixture had a total volume of 5 ml and a concentration of 0.50 mM substrate nucleotide, 75.0 mM NaCl, 1.25 mM sodium acetate buffer at pH 6.2, and 1.50 mM divalent cation. The reaction mixture was equilibrated for 30 min before addition of enzyme. The amount of enzyme added is indicated in the figures. The temperature was maintained at 30° throughout the reaction. An equal volume of uranium acetate–perchloric acid reagent (250 g of uranium acetate and 4.17 ml of 60% perchloric acid are dissolved in 100 ml of distilled water) was added to 1-ml aliquots of the reaction mixture taken at various time intervals and chilled for 30 min at 0° before being centrifuged at 11,000*g* for 10 min. Absorbance at 260 nm of the supernatant was measured to determine the amount of substrate rendered acid soluble.

The divalent cation to ribonucleotide ratio (I:R) in the acid-soluble assay as well as in the hyperchromic effect assay was 3:1. The relatively low hyperchromic effect of poly U proved to be entirely adequate for use as an assay of nuclease action and the results obtained were always confirmable by acid-soluble assay.

The specificity of nuclease B toward heteropolynucleotides was studied by analysis of enzymic digests of yeast RNA. Yeast RNA was digested by nuclease B, alkali hydrolyzed, desalted, and the products separated by two-dimensional paper chromatography. Since this nuclease is a 5′-monoester former, the nucleotides on the 3′ side of the cleaved internucleotide bond are found in the nucleoside fraction after alkaline hydrolysis while the nucleotides on the 5′ side are in the nucleoside diphosphate fraction. The reaction mixture had a volume of 14 ml and concentrations of 0.36 mM RNA nucleotide, 17 mM KCl, 0.57 mM sodium acetate buffer at pH 6.2, and 11 mM divalent metal cation. Ten units of enzyme was added to the reaction mixture, and the digest incubated at 30° for 12 or 36 hr. Hg^{2+} or Ca^{2+} plus Mg^{2+} were the divalent cation activators studied. Equivalent amounts of Hg^{2+} were added to the Ca^{2+} and Mg^{2+} digests before base hydrolysis to obviate artifacts due to Hg^{2+} ions. An amount of KI was added equivalent to twice the concentration of Hg^{2+} to remove the latter by precipitation. The supernatant was made 0.3 N with respect to KOH and incubated at 37° for 13 hr. This digest was then neutralized and desalted according to the procedure of Frankel-Conrat and Singer (1962). The residue was solubilized in distilled water and chromatographed in two dimensions on Whatman No./3MM paper according to the method of Felix *et al.* (1960). The nucleosides were located by ultraviolet light and eluted with 0.01 N HCl. In the case of the Hg^{2+} 12-hr digest, only uridine was visible; the other nucleosides were eluted from areas corresponding to the positions of standards on another paper. Nucleosides were quantified from their absorbance at 260 nm as measured using a Beckman DU spectrophotometer.

Results

The effects of various divalent cations on the activity of nuclease B and D are shown in Table I. The values are expressed relative to the initial rate of breakdown of RNA when the enzymes are activated by Ca^{2+} and Mg^{2+} which are the most effective activators of both enzymes. Cd^{2+} and Ni^{2+} are significantly less effective and Zn^{2+} appears to activate only nuclease B. With Hg^{2+} an increased ion to nucleotide ratio favors an increased initial rate of breakdown with nuclease B which is somewhat unusual. The general effect of increasing or decreasing the divalent cation to nucleotide ratio is to retard activity.

When ribohomopolymers are employed as substrates the divalent cations have markedly differing effects on nuclease activity, as shown in Table II. These are exemplified by a comparison of relative rates of nuclease action on poly A. The differential rates of breakdown of poly A under the same conditions of temperature, pH, ionic strength, concentration, and with various divalent cation activators reflect the differing specificity of nucleases B and D. Thus enzyme B is induced to degrade poly A at much greater rates when activated by Co^{2+}

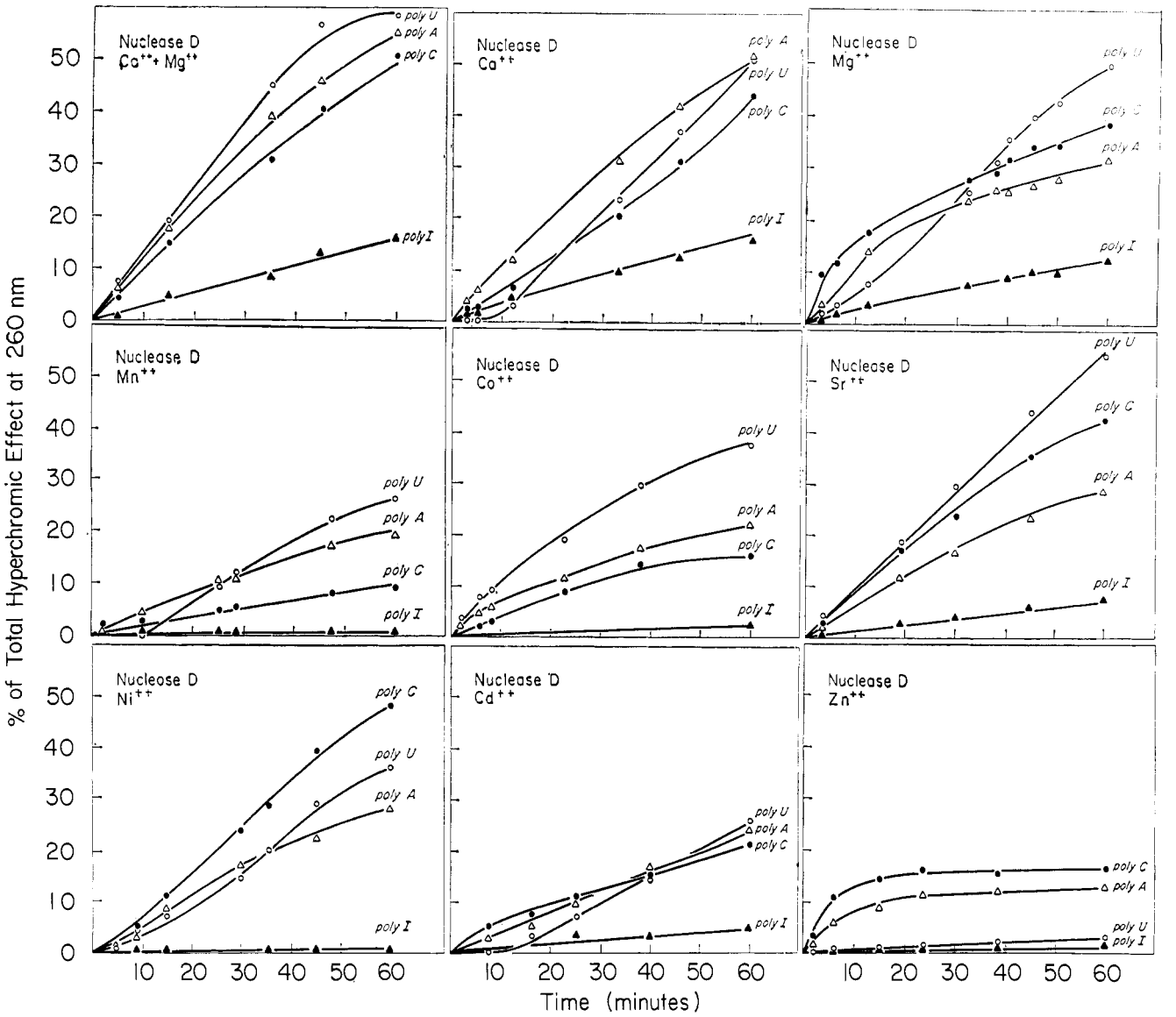

FIGURE 1: Degradation of ribohomopolymers by streptococcal nuclease D. Reaction mixtures as described in Methods contained 2 units of enzyme for Ca^{2+} and Mg^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Sr^{2+}; 4 units of enzyme for Ni^{2+}, Cd^{2+}, Zn^{2+}. Extent of degradation measured at various times during incubation is expressed in terms of percentage of total hyperchromicity of each ribohomopolymer. The ratio I/R was 3:1 in all reactions.

and Mn^{2+} while these cations are only moderately effective activators of enzyme D.

The relative rates (Table II) of nuclease action on poly A were derived from the data graphically represented in the figures. These figures illustrate particular characteristics of enzymic activity in the presence of many of the cation activators, which are not evident in a table of initial rates of action. Although mainly the results of hyperchromic effect assays are presented, these were in all cases confirmed by acid-soluble assays. Both assay methods demonstrated the same relative susceptibility of the homopolymers to nucleases B and D.

Nuclease D. Nuclease D when activated by Ca^{2+} and Mg^{2+} (Figure 1) shows little difference in the rate of attack on poly U, poly A, and poly C while the rate of poly I degradation is significantly lower. Ca^{2+} or Mg^{2+} alone, however, show quite different patterns. Ca^{2+} causes a definite lag in poly U breakdown before a rate of action similar to that on poly A and poly C is achieved. The rate of poly C degradation is somewhat lower than poly A or poly U breakdown but significantly higher than the rate of poly I degradation. Mg^{2+} alone enhances the rate of attack on poly C. There is a lag before poly U degradation commences and the initial rates of attack on poly U and poly A are 30% lower than that on poly C. The initial rate of attack on poly I is 80 to 90% lower than that on poly C. Mn^{2+}, Co^{2+}, and Sr^{2+} are effective activators of nuclease D and all show enhancement of poly U degradation although Mn^{2+} causes an initial lag in breakdown of this substrate. The order of preference with Sr^{2+} is poly U, poly C, poly A, and again at a significantly lower rate, poly I. Ni^{2+} causes a slight lag in poly U degradation while the rate of poly C breakdown is enhanced over the other homopolymers and there is essentially no breakdown of poly I.

TABLE III: Relative Molar Ratios of Nucleosides from 3′ Termini of Fragments from Nuclease B Digests of RNA.

	U	A	C	G
	36-hr digest			
$Ca^{2+} + Mg^{2+}$	1	1.62	1.02	0.47
Hg^{2+}	1	0.72	0.76	0.20
	12-hr digest			
$Ca^{2+} + Mg^{2+}$	1	1.33	1.01	0.37
Hg^{2+}	1	0.11	0.15	0.20

Cd^{2+} and Zn^{2+} are not as effective activators of nuclease D as the other divalent cations. The breakdown of poly U and poly I is only minimal when nuclease D is activated by Zn^{2+}. There is a lag before breakdown of poly U when Cd^{2+} is the activator. Poly C degradation is enhanced by both Zn^{2+} and Cd^{2+}.

The action of nuclease D in the presence of Hg^{2+} was followed using the acid-soluble assay since the hyperchromic effect assay proved to be unsuitable due to high absorbance of the Hg^{2+} containing reaction mixtures. Hg^{2+} (Figure 2) is a moderately effective activator of nuclease D and enhances the initial rate of poly C breakdown. The initial rate of attack on poly U is lower than that on poly C while the overall degradation of poly U is highest. Studies with the mercuric ion showed that increasing or decreasing the ion to nucleotide ratio (I:R) tended to lower overall activity and did not significantly alter the relative initial rates of attack on the four ribohomopolymers by nuclease D.

The autoretardation effect, characteristic of nuclease action, is observed with Mg^{2+} at about 15% and with Zn^{2+} at about 5% of polynucleotide degradation. The onset of autoretardation with other activators was not apparent prior to 50% degradation.

Nuclease B. The action of nuclease B on homopolymer substrates with these activating divalent cations is shown in Figure 3. With Ca^{2+} and Mg^{2+} together as the divalent cation activators, nuclease B shows the highest initial rate of attack and overall breakdown of poly A followed by poly C then poly U, and at a significantly lower initial rate and overall breakdown, poly I. Ca^{2+} alone enhances the initial rate of poly A degradation with poly C next. Although the overall rate of poly U is higher than poly C there is a lag before initiation of poly U degradation. The autoretardation effect is not observed with Ca^{2+} plus Mg^{2+} or Ca^{2+} alone under these conditions. With Mg^{2+}, however, the autoretardation effect is observed between 20 and 30% degradation. The autoretardation effect can also be observed with Ca^{2+}, Mn^{2+}, and Ni^{2+} at between 40 and 50% total degradation with Ca^{2+} or Mn^{2+} and at 10% with Ni^{2+}. It was difficult to discriminate between the initial preference of nuclease B for poly A, poly C, or poly U when Mg^{2+} was the activator while nuclease B had little effect on poly I. Co^{2+} and Mn^{2+} enhanced poly U degradation, Ni^{2+} enhanced poly C degradation, and Sr^{2+} showed no significant difference in poly A, poly U, or poly C breakdown. Cd^{2+} enhanced poly A degradation, showed no autoretardation up to 50% degradation, and caused a lag before initial breakdown of poly U. The activity of nuclease B was at a low level in the presence of Zn^{2+}.

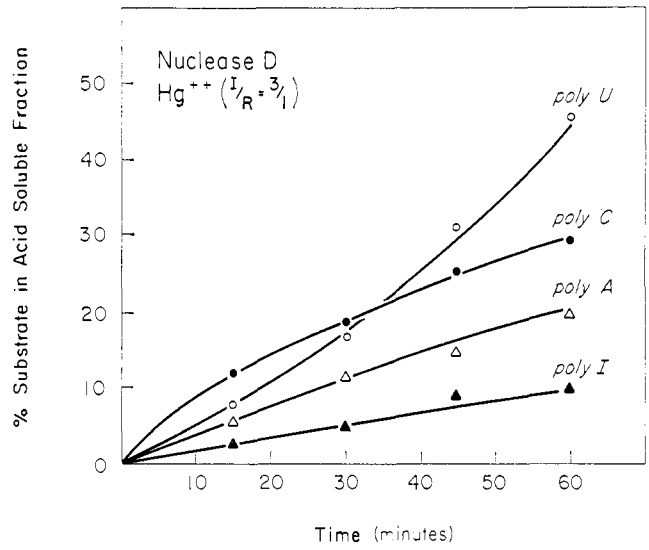

FIGURE 2: Degradation of ribohomopolymers by streptococcal nuclease D in the presence of Hg^{2+}. Reaction mixtures contained 8 units of enzyme and were assayed by the acid-soluble method. Results are expressed as percentage of total substrate converted into acid-soluble form. The ratio I:R was 3:1.

The mercuric ion effected the largest disproportion in relative rates, showing a sixfold enhancement of poly U degradation. The effect of increasing or decreasing concentrations of mercuric ion was studied next. Decreasing concentrations of Hg^{2+} significantly decreased the activity of nuclease B toward all of the ribohomopolymers. Increasing Hg^{2+} concentration appeared to further lower the rates of poly A, poly C, and poly I breakdown while not altering significantly the rate or extent of poly U degradation. With the ion to nucleotide ratio (I:R) equal to 10:1 there did not seem to be a significant change in the activity or specificity of nuclease B. With an I:R ratio of 20:1 there was virtually no lag before enzyme attack on poly U and relatively no change in the overall degradation of poly U while the rate of nuclease action, on poly A, poly C, and poly I was lower. The rate of poly U degradation was now 7-fold greater than that on any of the other homopolymers. When the ion to nucleotide ratio was 60:1 the rate of poly U degradation was 21-fold greater than that of the other homopolymers.

To determine whether the mercuric ion actually enhanced the rate of cleavage of internucleotide bonds with adjacent uridylic nucleotides in RNA, nuclease B digests of RNA were analyzed to determine the relative proportions of the various nucleotides at the 3′ side of the cleaved internucleotide bond. Table III shows the relative molar ratios of the nucleotides from the nuclease B digest after base hydrolysis. The 12-hr and 36-hr digests are shown for both the Ca^{2+} plus Mg^{2+}, and Hg^{2+} divalent cation activators. The 12-hr Hg^{2+}-activated digest showed that the uridylic nucleotide was present in sixfold greater proportion than either guanosine, adenosine, or cytidine, indicating that an internucleotide bond with a uridylic nucleotide on the 3′ side was cleaved at an overall rate six times greater than any other type of internucleotide bond.

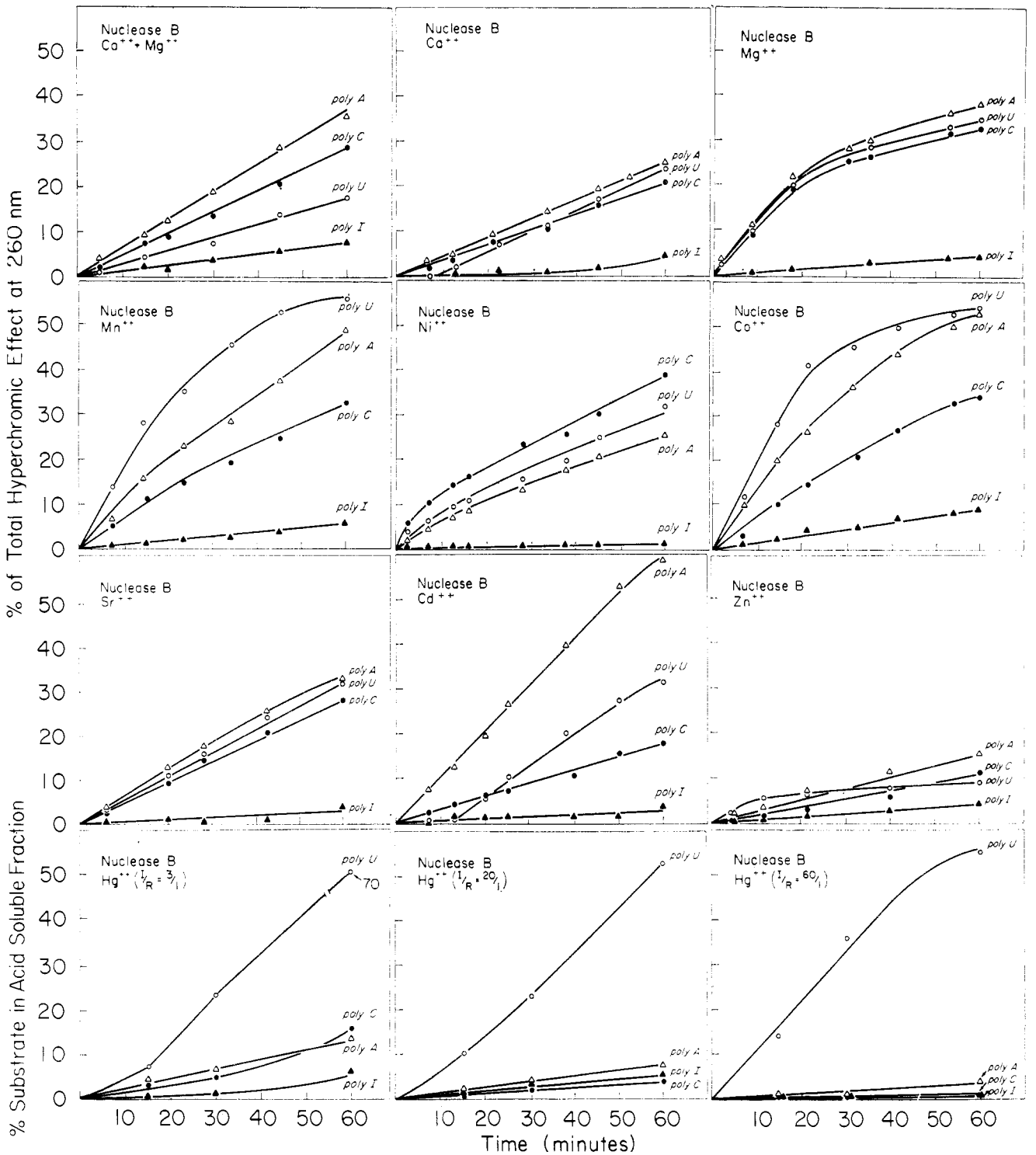

FIGURE 3: Degradation of ribohomopolymers by streptococcal nuclease B. Reaction mixtures contained 2 units of enzyme for Ca^{2+} and Mg^{2+}, Mn^{2+}, Co^{2+}; 4 units of enzyme for Ca^{2+}, Mg^{2+}, Ni^{2+}, Sr^{2+}, Cd^{2+}, Zn^{2+}; 8 units of enzyme for Hg^{2+}. All reactions except those with Hg^{2+} were followed by hyperchromic effect assay, Hg^{2+} by acid-soluble method. The ratio I:R was 3:1 except as noted.

Discussion

Each divalent cation appears to be unique in its effect on nuclease action. While some may elicit similar specificities (similar relative initial rates of homopolymer breakdown), they differ in either the rate of overall degradation or the onset of autoretardation or may cause a lag before attack on poly U. Nuclease D specificity was affected by the various ionic activators but there were few examples of marked preference for any of the homopolymer substrates. With nuclease B, however, there are greater variations in the relative initial rates of attack on the ribohomopolymers. Manga-

nese showed a twofold enhancement of poly U breakdown, and the initial rate of poly C breakdown was enhanced twofold by Ni^{2+}. The mercuric ion effected the greatest disproportionment and caused a sixfold enhancement of poly U degradation. Increasing the divalent mercuric cation to nucleotide ratio (I:R) from 3:1 to 20:1 to 60:1, showed increased specificity of nuclease B for the uridylic nucleotide. The rate of poly U degradation was 21-fold greater than that of any of the other homopolymers at an I:R ratio of 60:1. The increasing divalent cation concentration has only a marginal effect on the activity of nuclease B toward poly U. The only effect seems to be the elimination of the lag before poly U breakdown. The enhancement of the relative rate of poly U degradation by increasing divalent cation concentrations appears to be by virtue of lowering the rates of poly A, poly C, and poly I breakdown rather than increasing the absolute rate of poly U degradation.

It has been shown (Eichhorn and Butzow, 1965) that the nonenzymic breakdown of polynucleotides is facilitated in the presence of various divalent cations. A differential susceptibility to hydrolysis exists among the various internucleotide bonds dependent on the particular divalent cation present. The unusual enhancement of nuclease B specificity by Hg^{2+} is probably not ascribable to this phenomenon since similar concentrations of Hg^{2+} do not affect the specificity of nuclease D.

The rate of attack by both enzymes on poly I as compared to the other homopolymers is significantly lower with all of the divalent cation activators. The effect of secondary structure on the availability of substrate for nuclease attack may be responsible for the relative resistance of poly I. The well-ordered triple-helix structure of poly I under these conditions (Inman, 1964) may render it unsuitable as a substrate for nuclease action.The secondary structure of both poly C and poly A (Brahms *et al.*, 1966; Fasman *et al.*, 1964) may be described as a single-strand stacked-base helix, while poly U possesses very little secondary structure (Steiner and Beers, 1961). If the secondary structure of the substrate is important to the action of these nucleases, then similar activity toward poly A and poly C but not poly U would be expected. The activity of the nuclease toward poly U is actually very comparable to that toward poly A and poly C in most cases. Therefore, it appears that the effect of the divalent cation is on the specificity of the nuclease for the nucleotide base itself rather than on the substrate secondary structure in the case of poly A, poly C, and poly U.

The isolation and quantification of nucleosides after alkaline hydrolysis of the nuclease B digest of RNA (Table III) indicates the specificity of the enzyme toward the internucleotide bonds of the heteropolymer substrate. The 36-hr digests with Ca^{2+} plus Mg^{2+} and Hg^{2+} as activators showed that cytidine was the preferred nucleotide. This seems contrary to the results obtained using homopolymers. However, the extensive digestion during the prolonged incubation not only reflects nuclease B specificity toward the higher molecular weight polynucleotides, but also reflects specificity toward oligonucleotides, which may be considerably different. The 12-hr digests are also fairly extensive and with Ca^{2+} plus Mg^{2+} as activators show similar specificities to the 36-hr digest. The guanosine levels were lower in every experiment indicating a reduced preference for the guanylate nucleotide. This suggests that the lower rates of attack on poly I are not due solely to the secondary structure of the homopolymer, but represent the specificity of the enzyme.

The molar nucleoside ratios of the 12-hr Hg^{2+} digest clearly indicate an enhanced specificity for the uridylate nucleotide when nuclease B is activated by Hg^{2+} and the substrates are polynucleotides larger than small oligomers. The initial specificity may be and probably is even greater on high molecular weight polynucleotides. This enhanced specificity may be due to an increased affinity of the enzyme for the uridylate internucleotide bond, however, it appears more likely that it is a result of the lowered affinity for the guanylate, adenylate, and cytidylate nucleotides.

The autoretardation effect (Laskowski, 1967) evident in many of the figures is characteristic of nuclease action and occurs in the presence of several divalent cation activators before the extent of degradation exceeds 50%. It is thought that the decrease in rate of nuclease action is due to the formation of oligonucleotides which are attacked at a lower rate than the longer polynucleotides. With nuclease D and Mg^{2+} it appears that larger oligomers may be resistant because the retardation effect occurs at about 25% degradation of poly A or poly C. There is a lag before poly U degradation, and autoretardation does not occur until 40% degradation.

With nuclease B the autoretardation effects occur after 40% to 50% degradation with Mn^{2+} or Co^{2+}. This would seem to indicate that the K_m of nuclease B is higher for dinucleotide substrates than for substrates of higher molecular weight. With other divalent cations, such as Mg^{2+}, autoretardation begins after only 20% degradation which indicates a lowered affinity for the di- and trinucleotides. Nuclease B does not show autoretardation until after 30% degradation with Ca^{2+} plus Mg^{2+}, Sr^{2+}, or Co^{2+} as the divalent cation activator. This effect is somewhat dependent on the divalent cation and it is interesting to note that all four homopolymers are generally affected in the same way as seen with Mg^{2+} or Ni^{2+}. Autoretardation begins at 10% with Ni^{2+} indicating an effect on nuclease B activity toward even larger polynucleotides. The lag before poly U breakdown when Ca^{2+}, Cd^{2+}, or Hg^{2+} are the activators seems unrelated to this general phenomena and is difficult to interpret.

These effects are not due to a differential hyperchromicity of the various oligomers since similar effects have been noted when acid-soluble assays were used. Furthermore the varying point of onset of autoretardation is not compatible with such an explanation. The divalent cations apparently not only affect the activity in terms of initial rate of degradation but also in terms of the susceptibility of oligomers of differential length.

The specificity of nuclease D can be altered in the presence of various divalent cation activators to produce differing base preferences but in no case can absolute specificity be observed. The effect of Hg^{2+} on the action of nuclease B however suggests that this may prove a useful reagent in nucleotide sequence analysis.

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Aminoethylation of Thiopyrimidine Nucleosides

Brian R. Reid

ABSTRACT: Thiopyrimidine nucleosides can be readily alkylated by ethylenimine at pH 8. The reaction of 4-thiouridine, 2-thiouridine, 2-thiocytidine, and 2,4-dithiouridine has been studied. In contrast to cyanoethylation with acrylonitrile the 2-thionucleosides could be aminoethylated at readily detectable rates although proceeding at approximately one-fourth the rate of the 4-thionucleosides. 2,4-Dithiouridine showed both the rapid rate characteristic of 4-thionucleosides and the slower rate characteristic of 2-thionucleosides. Spectral analysis of the 4-thiouridine reaction product yields a typical cytidine derivative spectrum. Investigation of this reaction with ^{14}C-labeled ethylenimine, coupled with the pH dependence of the product spectrum, indicates that the final product of the reaction is N^4-β-thioethyl-*S*-β-aminoethylcytidine which is produced by a side-chain rearrangement reaction. The pH dependence of the reaction rate showed a discrete optimum at pH 8.1 and indicates the reactive species as being the thionucleoside anion and the ethylenimmonium cation. Pseudo-first-order rate constants at 25° with 0.19 M ethylenimine at pH 8 are reported as well as the second-order rate constant for the ethylenimmonium–thionucleoside anion reaction.

In the last few years thiopyrimidines have been shown to occur as minor constituents of bacterial tRNAs (Lipsett, 1965; Goehler and Doi, 1968; Carbon *et al.*, 1968). A variety of compounds containing suitably activated electrophilic double bonds have been found to alkylate certain nucleophilic thiopyrimidines—for instance, *N*-ethylmaleimide reacts with 4-thiouridine to form the *S*-NEM-4-thioU[1] adduct (Carbon and David, 1968). Similarly a detailed study of the reaction of acrylonitrile with 4-thiouridine demonstrated the formation of *S*-cyanoethyl-4-thioU (Ofengand, 1967). The pH dependence of the latter reaction showed a classical sigmoid rate curve with inflection at the pK_a of 4-thioU indicating that the thiopyrimidine anion was the reactive species. Both of the above reactions are Michael-type reactions and, as expected, the alkylated adduct was alkaline labile undergoing a reverse Michael reaction at high pH. The above two Michael reagents readily reacted with 4-thiouridine but did not react with 2-thiopyrimidine nucleosides (Ofengand, 1967; Carbon and David, 1968).

While investigating an unrelated problem I noted the facile aminoethylation of 4-thioU in *E. coli* tRNA by ethylenimine at pH 8 (Reid, 1968). The purpose of this communication is to report on the reaction of ethylenimine with a variety of thiopyrimidine nucleosides and to compare this aminoethylation reaction with the cyanoethylation of thiopyrimidine nucleosides reported by Ofengand.

Materials and Methods

Thionucleosides. 4-Thiouridine disulfide (lot R-5433) was purchased from Cyclo Chemical Corp., Los Angeles, and was reduced to 4-thiouridine with 1 mM dithiothreitol prior to use. 2-Thiocytidine and 2,4-dithiouridine were the kind gifts of Dr. Tohru Ueda, Faculty of Pharmaceutical Sciences, Hokkaido University, Japan. 2-Thiouridine was the kind gift of Dr. John Carbon, Department of Biological Sciences, University of California, Santa Barbara, and had been originally obtained by him from Dr. Mitsuji Sano, Analytical Division, Central Research Laboratory, Daiichi Seiyaku Co. Ltd., Edogawa-Ku, Tokyo, Japan. Ethylenimine was

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[1] Abbreviations used are: 4-thioU, 4-thiouridine, 2-thioU, 2-thiouridine, 2-thioC, 2-thiocytidine.