Streptococcal Nucleases. III. Kinetics of Action and Inhibition by Transfer Ribonucleic Acid*

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ABSTRACT: The Michaelis constants of the deoxyribonuclease (DNase) activity of streptococcal nucleases A–D with thymus deoxyribonucleic acid (DNA) as substrate [Wannamaker, L. W. (1958a), *J. Exptl. Med. 107*, 783; (1962), *Federation Proc. 21*, 213] have been compared using two assay systems, viscosimetry and pH-Stat titration. Although the K_m values for the A, B, and D enzymes as obtained by viscosimetry are severalfold greater than those obtained by pH-Stat titration (values are 6.5×10^{-4}, 1.8×10^{-3}, 6.8×10^{-4}, and 4.3×10^{-5}, respectively, by pH-Stat titration), their ratios within both assay systems are comparable. These findings suggest that both assay systems reflect similar kinetics of action of these enzymes. For nuclease C, the difference between the K_m values obtained by viscosimetry and by pH-Stat titration is greater (4.7×10^{-3} and 2.4×10^{-4}, respectively). With the viscosimetric technique, it can be shown that the inhibition of nuclease B by yeast and bacterial RNA [Yasmineh, W. G., Gray, E. D., and Wannamaker, L. W. (1965), *Federation Proc. 24*, 227] is due to transfer ribonucleic acid (tRNA). This inhibition appears to be competitive in nature. This and other findings [Wannamaker, L. W., and Yasmineh, W. G. (1967), *J. Exptl. Med. 126*, 475] suggest that nuclease B is a single protein possessing both DNase and ribonuclease (RNase) activity. The inhibition of nuclease D by tRNA is also competitive. The inhibitory effects of tRNA seem to be mediated through a specific association with the active site of the nucleases. Alteration of tRNA structure through heat denaturation reduces its effectiveness as an inhibitor.

The elaboration of nuclease activity by strains of group A streptococci was first demonstrated by McCarty (1948) and by Tillet *et al.* (1948). Wannamaker (1958a,b) grew a number of strains of group A streptococci on a dialysate medium which allowed the preparation of these enzymes in a relatively pure form, free from medium contaminants. By starch zone electrophoresis four immunologically distinct nucleases were isolated, which were designated nucleases A–D (Wannamaker, 1958b, 1962; Winter and Bernheimer, 1964). All the nucleases have DNase activity. Nucleases B and D also possess RNase activity and are inhibited by yeast and bacterial RNA (Wannamaker and Yasmineh, 1967; Wannamaker *et al.*, 1967). There is evidence suggesting that the DNase and RNase activities of these nucleases reside in single proteins (Wannamaker and Yasmineh, 1967).

The purpose of the present study is to examine the kinetics of the four streptococcal nucleases and the nature of the inhibition of nucleases B and D by RNA, by an adaptation of the viscosimetric technique. Titration by pH-Stat is also used to support the results obtained by viscosimetry, since it measures the velocity directly in terms of bonds cleaved per unit time.

Experimental Section

Materials

Thymus DNA was prepared by the Duponol method of Zamenhof (1957) and stored in a solution of 0.1 M NaCl at −10°. Yeast RNA was prepared by the method of Crestfield *et al.* (1955).

RNA from *Streptococcus pyogenes* (strain C203S, a type 3 strain of group A streptococci) was prepared by the phenol method of Georgiev (1959). RNA from *Rhodopseudomonas spheroides* was prepared by phenol extraction (Scherrer and Darnell, 1962).

tRNA from group A streptococci, *R. spheroides*, and yeast was prepared by the fractionation of total RNA with 2 M LiCl according to the method of Barlow *et al.* (1963). tRNA from yeast was also prepared by the method of Holley *et al.* (1961). Crystalline pancreatic DNase was purchased from Worthington Biochemical Corp., Freehold, N. J.

Nucleases A–D were prepared by the method of Wannamaker (1958b), using starch zone electrophoresis for the final separation of the enzymes. The nuclease B and C preparations used in the pH-Stat experiments were prepared by the same method except that the final

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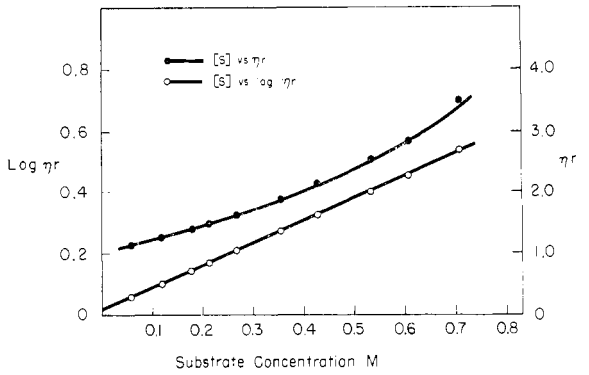

FIGURE 1: Relation of viscosity to DNA concentration. DNA concentrations are in moles per liter of nucleotide. Dilutions were made in a solution containing 0.05 M imidazole buffer (pH 7.8), 0.05 M NaCl, and 0.01 M Ca^{2+} and Mg^{2+}.

separation of the enzymes was achieved on DEAE-cellulose by a modification (E. D. Gray, unpublished data) of the method described by Dillon and Wannamaker (1965). Commercial Varidase (Lederle Laboratories), a mixture of streptococcal nuclease A and streptokinase, was used for nuclease A in the viscosimetric experiments.

The concentration of streptococcal nucleases as well as pancreatic DNase are designated in terms of units of activity. The unit is defined as the amount of enzyme that degrades thymus DNA releasing acid (in the pH-Stat) at an initial rate of 10^{-5} mequiv/min, at 30°, pH 6.5, and under the condition of substrate excess.

Methods

Viscosimetric Measurement of the Initial Rate of Reaction. Viscosity was measured in viscosimeters of the Cannon–Fenske type with water flow times of 47–67 sec. The method used was a modification of the method used by Wannamaker (1958b). The viscosimeter was placed in a water bath at 30°. Thymus DNA (5 ml) (0.3–2.4 mM nucleotide in 0.05 M imidazole buffer (pH 7.8), 0.05 M NaCl, 0.1 M $CaCl_2$, and $MgCl_2$) was delivered into the viscosimeter. After temperature equilibrium was attained the enzyme was added in 0.5 ml of 0.1% bovine albumin and the solution immediately was mixed by bubbling air through it. Viscosity measurements were then made every 2–5 min depending upon the concentration of substrate, the less concentrated solutions flowing more rapidly and therefore allowing more frequent measurements. The initial rate of reaction was obtained by plotting the common logarithm of the relative viscosity (log ηr) against time. Under the experimental conditions described, the function relating thymus DNA concentration to relative viscosity was exponential (see Figure 1). It follows therefore that initial velocity measurements made at various substrate concentrations (as in the determination of the Michaelis constant of a nuclease) should have the starting points of the velocity *vs.* time curves

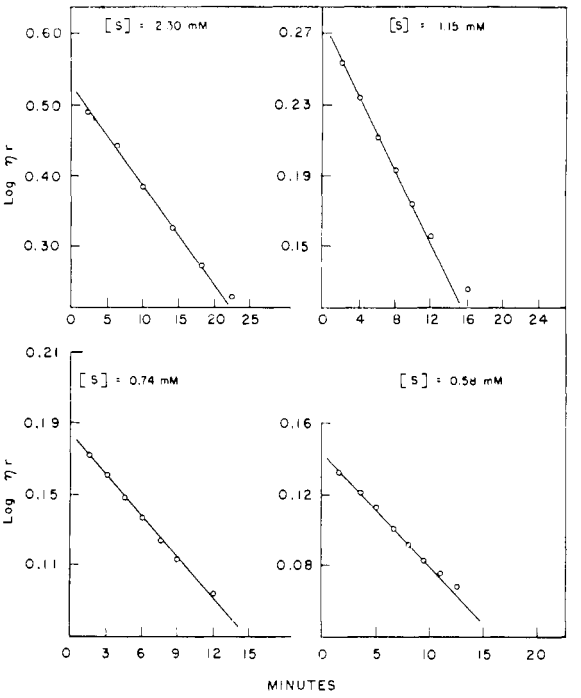

FIGURE 2: Viscosimetric measurement of the rate of degradation of thymus DNA by nuclease B at various substrate levels. DNA concentrations are in moles per liter of nucleotide. The incubation mixtures (total volume, 5.5 ml) contained thymus DNA as indicated, 0.05 M imidazole buffer (pH 7.8), 0.05 M NaCl, 0.01 M Ca^{2+} and Mg^{2+}, and 0.03 unit of enzyme.

related to log ηr rather than ηr. In addition, Laskowski and Seidel (1945) showed that the kinetics of the degradation of a nucleic acid by an endonuclease is first order. This implies that the function relating relative viscosity with time is also exponential and therefore that the measurement of the initial rate of reaction is greatly facilitated when log ηr is plotted against time, since such a plot would be expected to be linear over the initial phase studied. That this was indeed the case is illustrated in Figure 2, in which the degradation of thymus DNA by nuclease B is shown at four substrate levels.

pH-Stat Measurement of the Initial Rate of Reaction. The micro assembly of the pH-Stat (Radiometer, agents: London Co., Westlake, Ohio) was used. Thymus DNA solution (1.0 ml) (containing 1–100 × 10^{-5} M nucleotide, 0.1 M NaCl, 0.01 M $CaCl_2$, and $MgCl_2$) was delivered into the reaction vessel. The solution was then layered with a 0.25-in. heavy mineral oil to minimize CO_2 diffusion into the reaction mixture. After temperature equilibrium was attained at 30°, the pH was adjusted to 6.5 by the addition of 10^{-3} N alkali or acid. At zero time the nuclease (previously dialyzed against 0.1 M NaCl, 0.01 M $CaCl_2$, and $MgCl_2$ and adjusted to pH 6.5) was delivered in 0.03–0.10-ml volume from a 2-ml micrometer syringe (Kontes Glass Co., Vineland, N. J.) *via* an 8-in. piece of polyethylene

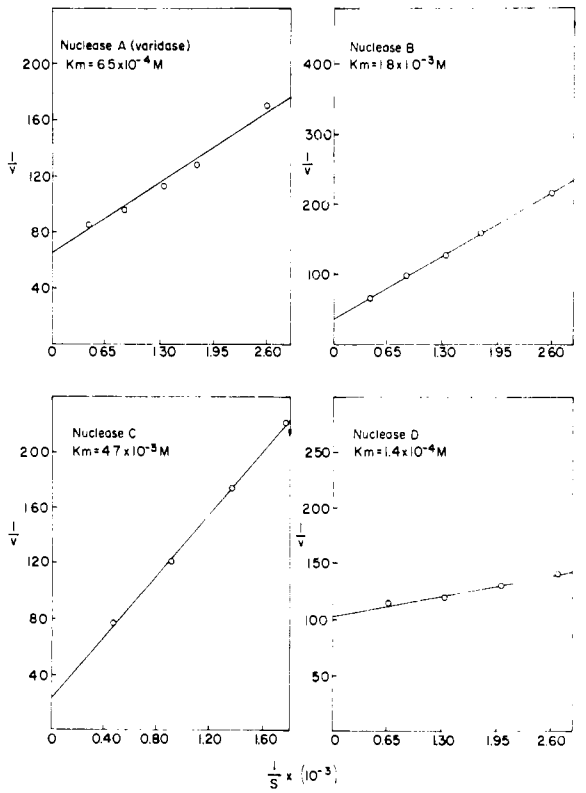

FIGURE 3: Double-reciprocal plots for nucleases A–D as determined viscosimetrically. DNA concentrations are in moles per liter of nucleotide. The incubation mixtures (total volume, 5.5 ml) contained thymus DNA as indicated, 0.05 M imidazole buffer (pH 7.8), 0.05 M NaCl, 0.01 M Ca^{2+} and Mg^{2+}, and approximately 0.03, 0.03, 0.03, and 0.01 unit of nuclease A, B, C, and D, respectively.

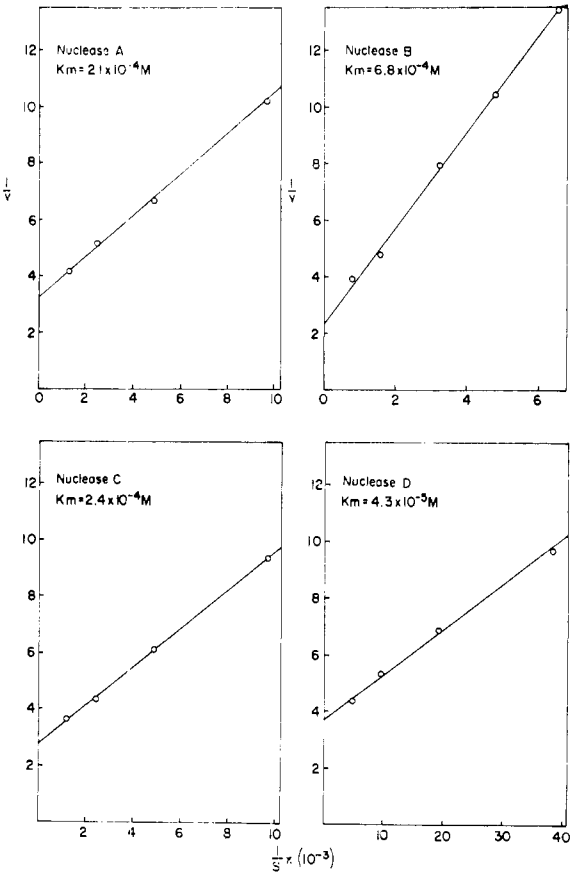

FIGURE 4: Double-reciprocal plots for nucleases A–D, as determined by pH-Stat titration. DNA concentrations are in moles per liter of nucleotide. The incubation mixtures (total volume, 1.1 ml; pH 6.5) contained thymus DNA as indicated, 0.1 M NaCl, 0.01 M Ca^{2+} and Mg^{2+}, and 3.2, 4.2, 3.6, and 1.3 units of enzymes A, B, C, and D, respectively. The concentrations of NaOH titrant were 1×10^{-4}, 1×10^{-4}, 1×10^{-4}, and 5×10^{-5} N, respectively, for enzymes A, B, C, and D.

tubing, to avoid disturbance of the electrodes. Titration of the acid released was performed with 10^{-4}–10^{-5} N NaOH. Since the concentration of such dilute solutions of alkali was found to be appreciably affected by atmospheric CO_2, the solutions were always freshly prepared from concentrated alkali and CO_2-free water, and placed in syringes that could be directly connected to the buret syringe assembly of the pH-Stat with rubber tubing. When treated in such a manner the concentration of NaOH was found to be invariable for at least 12 hr.

TABLE I: Michaelis Constants of Streptococcal Nucleases and DNase I.

Nuclease	K_m by Viscosimetry (M nucleotide)	K_m by pH-Stat (M nucleotide)
A	6.5×10^{-4}	2.1×10^{-4}
B	1.8×10^{-3}	6.8×10^{-4}
C	4.7×10^{-3}	2.4×10^{-4}
D	1.4×10^{-4}	4.3×10^{-5}
Pancreatic DNase	3.0×10^{-4}	7.0×10^{-5}

Results

Kinetic analysis of the action of streptococcal nucleases on DNA resulted in the data shown in Figure 3. The double-reciprocal plots of the viscosimetric data for all of the enzymes are linear over the substrate range (four- to sixfold) achievable by this technique. Similar studies using pH-Stat titration to follow reaction rates are presented in Figure 4. The substrate range in this case was wider (8–16-fold). The K_m values obtained by these methods are summarized in Table I. The values for nucleases A, B, and D determined by pH-Stat titration are two to three times smaller than

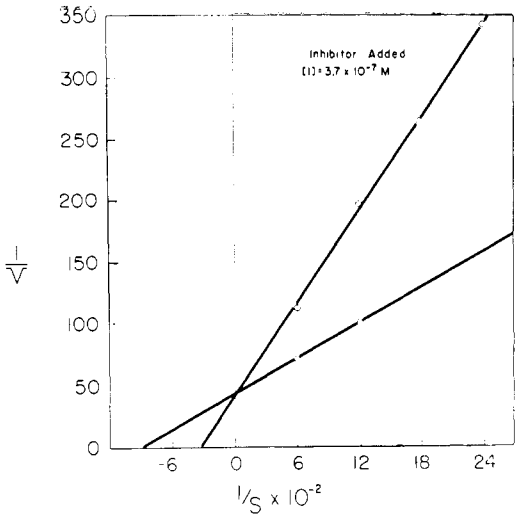

FIGURE 5: Double-reciprocal plots for the inhibition of nuclease B by streptococcal tRNA, as measured viscosimetrically. The incubation mixtures (total volume, 5.5 ml) contained thymus DNA as indicated, 0.05 M imidazole buffer (pH 7.8), 0.05 M NaCl, 0.01 M Ca^{2+} and Mg^{2+}, and 0.03 unit of enzyme. The tRNA was added in a 0.1-ml volume prior to the addition of enzyme.

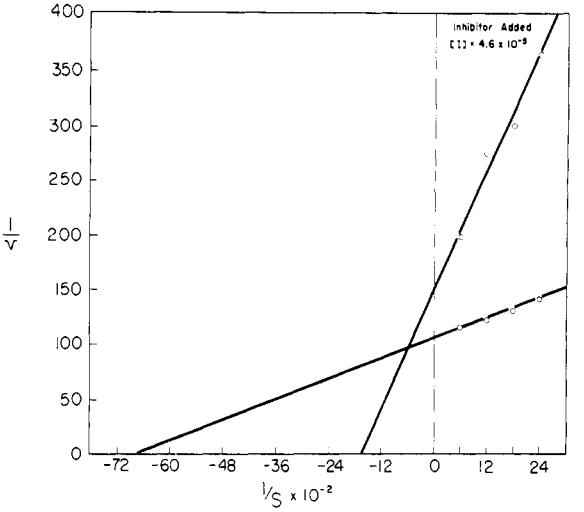

FIGURE 6: Double-reciprocal plots for the inhibition of nuclease D by streptococcal tRNA, as measured viscosimetrically. Proceeded as in Figure 5 except that 0.01 unit of enzyme was added.

those obtained by viscosimetry. The results for nuclease C show a greater disparity between the values obtained by the two methods. The K_m for pancreatic DNase I is also shown for comparison with the streptococcal enzymes. The K_m obtained by viscosimetry is about fourfold greater than by pH-Stat titration. Both values however, are in the range of values obtained for the streptococcal enzymes.

It has been observed that the DNase activity of those streptococcal nucleases that attack RNA (the B and D enzymes) is inhibited by RNA (Wannamaker, 1962; Yasmineh *et al.*, 1965). To examine this observation RNA was fractionated in order to study the relative DNase inhibitory effect of various classes of RNA. The results of such a study (Table II) demonstrate that the inhibitory activity resides almost entirely in the tRNA species. rRNA is lacking in inhibitory activity and the small effect observed might be ascribed to contaminant tRNA. The inhibition obtained with tRNA is 50–90-fold greater than for the same amount of rRNA. This observation was confirmed with RNA from species as variant as yeast, *S. pyogenes*, and *R. spheroides*. The homopolymer pair poly (A:U) was also tested and found to be devoid of inhibitory activity.

A study of the kinetics of tRNA inhibition of nucleases B and D is shown in Figures 5 and 6. The double-reciprocal plot for DNase B indicates the competitive nature of the inhibition. The results for DNase D are not as conclusive but also suggest that tRNA competitively inhibits the action of the enzyme. The K_i values for the inhibition of DNase B and D by streptococcal tRNA are 2.0×10^{-7} and 1.4×10^{-5} M, respectively. On comparing these with the corresponding K_m values it is evident that the inhibitory RNA is strongly bound to the substrate binding site.

It has been shown that the rate of degradation of RNA by both B and D enzymes is much slower than the DNase action of these enzymes (Wannamaker and Yasmineh, 1967). An analogous differential degradation of tRNA and rRNA might account for the difference in inhibitory activity of these species. In Figures 7 and 8 are presented the results of a study in which it is demonstrated, using the acid-soluble assay, that tRNA is broken down at a slower rate than rRNA by both nucleases B and D. The B enzyme attacks rRNA at a rate four times faster than tRNA. The difference in the rates of attack by nuclease D on these substrates is 66-fold. These values are probably under-

TABLE II: Relative Inhibitory Activity of Various Species of RNA to Nuclease B.

Source of RNA	% Inhibn/μg[a]		Ratio of Inhibn (tRNA/rRNA)
	rRNA	tRNA	
Yeast	0.1	5	50
S. pyogenes	1.0	92	92
R. spheroides	1.0	59	59

[a] The per cent inhibition was determined by the viscosimetric method. Concentration of thymus DNA was 2.4 mM and of nuclease B, 0.03 unit/ml. The inhibitor RNA was added to 0.1-ml volume prior to the addition of enzyme.

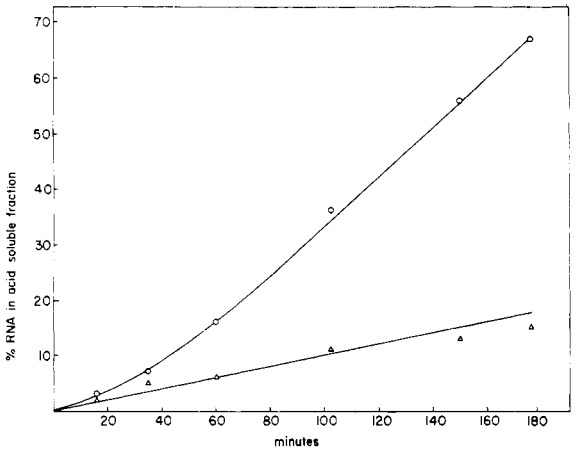

FIGURE 7: Degradation of yeast r- and tRNA by nuclease B. The incubation mixtures (3.5-ml total volume at 30°) contained 3 mg of RNA, 0.05 M imidazole buffer (pH 7.8), 0.005 M Ca^{2+} and Mg^{2+}, and 2 units of nuclease B. At various intervals after the addition of enzyme 0.5-ml aliquots were removed and added to 0.5 ml of cold 1 N HCl. The RNA in the acid-soluble fraction was estimated by the orcinol method (Mejbaum, 1939). (○—○) rRNA and (△—△) tRNA.

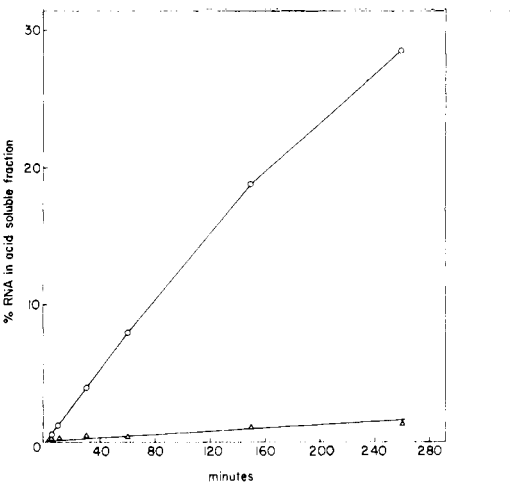

FIGURE 8: Degradation of yeast r- and tRNA by nuclease D. The incubation mixtures (5.5-ml total volume, at 30°) contained 5 mg of RNA, 0.05 M imidazole buffer (pH 7.8), 0.005 M Ca^{2+} and Mg^{2+}, and 400 units of enzyme. At various intervals after the addition of enzyme, 0.5-ml aliquots were removed and treated as in Figure 7. (○—○) rRNA and (△—△) tRNA.

estimates because of the greater extent of degradation required to reduce rRNA to the acid-soluble form. The resistance of tRNA to enzymic attack is probably involved in the mechanism of inhibition. However, at enzyme levels which degrade DNA, even ribosomal RNA is not markedly affected. It is only with the higher amounts of enzyme employed in the experiments of Figures 7 and 8 that rRNA is degraded at a rapid rate. rRNA fragmented by prolonged sonication[1] was found to be unaltered in inhibitory activity. Evidently the size difference between the two classes of RNA is not the sole factor in the differential inhibitory action. The relative ineffectiveness of rRNA as an inhibitor of DNase activity is probably related to its lower affinity for the substrate site.

Winter and Bernheimer (1964) have shown that nuclease B attacks denatured DNA at a much slower rate than native DNA. It was thought that the secondary structure of tRNA may play a role in the inhibitory mechanism. Accordingly the degree of inhibitory activity was measured in tRNA which was subjected to heat treatment for varying periods followed by quick cooling. The results are shown in Table III. Increasing time of heat treatment of tRNA results in a progressive increase in absorbance at 260 mμ indicating that there was a loss of secondary structure. The insignificant change in amount of acid soluble material demonstrates that the heating probably did not cleave covalent bonds. The inhibitory activity continuously decreases as the heating is prolonged. After 50 min of heat denaturation 20% of the inhibitory activity of tRNA is lost. At various time intervals during the heating process samples of tRNA were removed and their susceptibility to degradation by nuclease B was measured. After 1 hr of enzymic treatment 12% of the unheated tRNA is converted to the acid-soluble form. This proportion increases with heat denaturation until after 50 min of heating 20% of the tRNA is degraded. The heat denaturation of tRNA with resultant loss or alteration in secondary structure thus has demonstrable effects on the capacity of tRNA to inhibit nuclease B and on its resistance to enzymic attack.

[1] Treatment for 15 min in MSE ultrasonic oscillator.

Discussion

The viscosimetric method has been widely used to assay for nuclease activity. Unfortunately, with the exception of DNA, most of the other species of nucleic acid possess little measurable viscosity. Furthermore, despite the fact that various modifications of this technique have been proposed for the estimation of the number of bonds cleaved (Laskowski, 1965), these methods have been cumbersome, and any kinetic results obtained therefrom rather uncertain. By doing simultaneous kinetic studies with the pH-Stat, one can compare the results obtained by viscosimetry in terms of direct measurement of the number of bonds cleaved. Using a simple adaptation of the viscosimetric technique, relative K_m values obtained on five nucleases indicate that the kinetics of the reaction involved in the initial cleavages as measured by the viscosimetric assay are analogous to those involved in later cleavages measurable by the pH-Stat. An estimate of the extent of degradation using the two assay methods can be obtained by comparing the amounts of enzyme used (Figures 3

TABLE III: Inhibition of Nuclease B by Yeast tRNA Heat Denatured for Various Time Intervals.

Denaturation Time (min)[a]	% Increase in Hyperchromic Effect	% tRNA in Acid-Soluble Fraction	% Loss in Inhibn[b]	% tRNA in Acid-Soluble Fraction Following Treatment with Nuclease B[c]
0	0	0	0	12.0
10	0	1	3	11.2
30	2	1	14	14.5
50	6	1	20	19.8

[a] tRNA (1 mg/ml in 0.1 M phosphate buffer, pH 6.9) was heat denatured in a boiling-water bath and fast cooled in an alcohol–Dry Ice mixture. [b] The reaction mixtures (total volume, 4.3 ml) at 37° contained 1.2 mg of thymus DNA, 1.2 mg of tRNA, 0.03 M phosphate buffer (pH 6.9), 6×10^{-4} M Ca^{2+} and Mg^{2+}, and 700 units of nuclease B. At various intervals after the addition of enzyme aliquots were removed and the undigested DNA precipitated with an equal volume of HCl–ethanol (Roth and Milstein, 1952). [c] Proceeded as in *b*, with the exclusion of DNA from the incubation mixtures. After 60-min incubation the undigested tRNA was precipitated as above.

and 4). Thus the amount of nuclease used per ml of incubation mixture is approximately 500 times greater in the pH-Stat technique for all the streptococcal enzymes and for pancreatic DNase.

It is of interest that such correspondence should be obtained with the two assay methods. The viscosimetric assay measures the cleavage of very few bonds per substrate molecule, while titration with the pH-Stat is a much less sensitive assay and requires considerably more degradation. The initial rate of attack might be expected to be reflected by the viscosimetric technique; this seems to correspond reasonably well with the rates of more extensive cleavage. The greater disparity in the nuclease C results could indicate that the kinetics of early cleavages for this enzyme are different from those of later cleavages measurable by the pH-Stat.

The inhibition of streptococcal deoxyribonuclease activity by RNA was first shown by Bernheimer and Ruffier (1951). The inhibition of nucleases B and D by tRNA is not unique to streptococcal nucleases. Using the acid solubility technique Lehman *et al.* (1962) showed that *Escherichia coli* endonuclease I is competitively inhibited by *E. coli* tRNA with a K_i of 1×10^{-8} M RNA nucleotide. This value is comparable to that obtained for the inhibition of the B enzyme by streptococcal tRNA (2×10^{-7} M RNA nucleotide); however, unlike enzymes B and D, endonuclease I is not believed to possess RNase activity. Spleen DNase has also been reported to be competitively inhibited by tRNA and to a lesser extent by rRNA, poly U, and poly I (Bernardi, 1964). The homopolymer pair poly (A:U) is also a very efficient inhibitor of DNase II, although it has no such effect on nuclease B.

The study of the inhibition of nuclease B by heat denatured tRNA suggests that the inhibitory effects are related to the "native" structure of tRNA. After 50 min of heat denaturation only a 6% increase in the absorbance at 260 mμ was achieved. This value is much lower than that obtained by Takanami *et al.* (1961) (21% for rat liver tRNA), and by Brown and Zubay (1960) (16% for *E. coli* tRNA). When, in the present study, the increase in absorbance at 260 mμ was determined in 6 M urea (0.004 M phosphate buffer, pH 6.8), it was found to be 21%, suggesting that upon heat denaturation and subsequent fast cooling there may have been a disruption and subsequent reformation of hydrogen bonds, but not necessarily into the native configuration. It is difficult to determine from Table III whether such reformation resulted in the original structure of tRNA. Upon heat denaturation, there was only a 20% decrease in inhibitory activity and the rate of action of nuclease B on the heat-denatured tRNA was 60% greater than the rate on native tRNA. The findings would suggest that the former is basically different in secondary structure. It appears that the inhibitory activity of tRNA as well as its resistance to enzymic attack is a consequence of the conformation of the native structure. The competitive inhibition that the tRNA effects suggests that it is strongly bound to the substrate sites on both nucleases B and D. The resistance of the tRNA to degradation thus effectively prevents the binding and breakdown of substrate DNA. The failure of the duplex poly (A:U) to inhibit the DNase activity of nuclease B is suggestive that the particular conformation of native tRNA is involved and not simply a base-paired structure.

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