STREPTOCOCCAL NUCLEASES*

I. Further Studies on the A, B, and C Enzymes

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Earlier studies have indicated that Group A streptococci can produce several different deoxyribonucleases, previously designated streptococcal DNAses A, B, and C, which migrate separately on analysis by starch zone electrophoresis, are immunologically distinct, and show certain differences in inhibition by citrate and in optimal pH of activity (1).

The present report extends the observations on the properties of these first three enzymes, particularly the B enzyme, which is shown to manifest ribonuclease as well as deoxyribonuclease activity and to be specifically inhibited by bacterial ribonucleic acid.

Materials and Methods

Strains of Group A Streptococci.—The following strains were those most commonly used for the preparation of enzymes:

Strain H105 OP, an opaque variant of Tillet's "CO". This strain is an undesignated new type and produces high yields of streptokinase.

Strain D58, Colebrook's strain "Richards", type 3.

Strain SH42 and SH47A, type 49 strains isolated from a family epidemic of acute glomerulonephritis (2).

Strain Dematteo, a type 12 strain isolated from a patient with acute glomerulonephritis, supplied by Dr. Charles H. Rammelkamp.

C203S is a variant of strain C203 obtained from Dr. Alan Bernheimer. This strain possesses a type 3 M antigen and a type 1 T antigen.

Enzyme Preparations.—Concentrates of the extracellular products of Group A streptococci were prepared and subjected to separation by zone electrophoresis and by column chromatography as previously described (1, 3, 4). The preparations of deoxyribonuclease were characterized immunologically as well as by physicochemical means and were stored in the dried state under vacuum at 4°C (1, 3). It was found that enzymes were more stable if lyophilized

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in the presence of glycine buffer. Dry weights of enzyme preparations, unless otherwise noted, include buffer salts. Protein determinations done on several preparations indicated that they contained approximately 0.3% protein.

Enzyme Determinations.—Screening for deoxyribonuclease and ribonuclease activities was done by modifications (3) of the alcohol precipitation (5) and turbidimetric methods (6), respectively.

More precise measurement of deoxyribonuclease and ribonuclease activities were made by the viscosimetric (5, 1) and turbidimetric techniques, respectively.

For the turbidimetric assay of ribonuclease activity, a solution of 1.5 ml of yeast ribonucleic acid at a concentration of 2 mg/ml was mixed with 1.5 ml of enzyme dilution in neopeptone broth, pH 7.6 (7), and incubated for 30 min at 37°C. 3 ml of 1 N HCl were added and the resulting turbidity was read at 420 mμ on an Evelyn colorimeter (Rubicon Co., Philadelphia, Pa.). Blanks containing neopeptone instead of enzyme and distilled water instead of substrate solution were used. A normal curve with varying amounts of substrate was constructed. In the experiments where cations were added, the required divalent cation concentration was included in the RNA solution. In the experiments where inhibitor was added, it was added in 0.3 ml volumes prior to addition of enzyme.

In the viscosimetric assay for deoxyribonuclease activity, one unit was defined as the amount of enzyme which causes a fall of 1.0 in relative viscosity in a 20 min period (8). In the turbidimetric assay for ribonuclease activity, one unit was defined as the amount of enzyme which causes a fall in optical density of 0.001 in 15 min.

In other assays, HCl-ethanol supernatant fluids or precipitates (9) from digests were examined for ultraviolet absorption, phosphorus, and specific nucleic acids or their degradation products. More reproducible results for phosphorus values were obtained in precipitates than in supernatant fluids. By this method one unit of nuclease action was defined as the amount of enzyme which reduced the amount of precipitable substrate by 1 μg in 1 min at 37°C. Specific nucleic acids could be determined in mixed substrate systems by modifications of the methods of Webb and Levy (10) and of Webb (11). Evidence was obtained that the presence of one substrate did not interfere with the assay of the other.

For determination of DNA in mixed substrate systems, the colorimetric method of Webb and Levy (10), originally designed for the determination of DNA in tissues and microorganisms, was adapted as follows. A 1.5 ml volume of the sample to be analyzed was pipetted into a 16 × 150 mm tube calibrated to contain 10 ml. 1.5 ml of 10% trichloroacetic acid (TCA) was added and the contents mixed and placed in a boiling water bath for 30 min. Evaporation was minimized by covering the tubes with pyrex marbles (Arthur H. Thomas Co., Philadelphia, Pa.). A blank and standards containing from 20 to 200 μg of DNA were treated in a similar manner. The solutions were then made up to 10 ml with 5% TCA. After mixing, 6 ml aliquots were pipetted into 5 ml tubes and 0.3 ml of 0.5% *p*-nitrophenyldrazine (Eastman Kodak Co., Rochester, N. Y.) in ethanol was added. The tubes were mixed, covered with marbles, and placed in a boiling water bath for 20 min. After cooling in cold water, 20 ml of *n*-butyl acetate (purified, J. T. Baker Chemical Co., North Phillipsburg, N. J.) were added, and the tubes were stoppered with rubber stoppers and shaken for 10 min. Upon separation of the two phases, the upper *n*-butyl acetate phase was removed by suction and discarded. 4 ml aliquots of the colorless aqueous phase were pipetted into Evelyn tubes. The color reaction was obtained by adding 3 ml of 1 N NaOH. The per cent transmission was read on an Evelyn colorimeter using a 550 mμ filter. The color reached maximal intensity within 15 sec of the addition of NaOH, after which it started to fade at the rate of approximately 2%/min.

For RNA determinations in mixed substrate systems, the colorimetric method of Webb (11) was modified in the following manner. The RNA sample or standard (containing from 20 to 200 μg of RNA) was hydrolyzed in 3 ml of 5% TCA as described in the DNA method. After hydrolysis, 2 ml aliquots were pipetted into 16 × 150 mm tubes and 2 ml 8 N HCl, 2 ml xylene

(analytical reagent, Mallinckrodt Chemical Works, St. Louis, Mo.), plus enough NaCl crystals to saturate the solution (about 2 g) were added. The solutions were then mixed, covered with pyrex marbles, and placed in a boiling water bath for 3 hr. After cooling, 4 ml of xylene were added to each tube and the solutions mixed. 4 ml aliquots of the upper xylene layer were then placed into Evelyn tubes and 4 ml of a 2.5% solution of *p*-bromophenylhydrazine hydrochloride (Eastman Kodak Co.) in alcohol-HCl (2 ml of 37% HCl plus 100 ml 95% ethanol) were added. The solutions were mixed, incubated at 37°C for 1 hr and read at 440 mμ on an Evelyn colorimeter.

Preparation of Substrates and Inhibitors.—Deoxyribonucleic acid was extracted from calf thymus and purified by the method described by McCarty (5).

Yeast ribonucleic acid was a purified preparation of commercial yeast RNA (Nutritional Biochemical Co., Cleveland, Ohio). 10 g of commercial RNA were dissolved in 1 liter of 0.05 M phosphate buffer, pH 7.1. The solution was dialyzed against 0.05 M phosphate buffer for several days in the cold followed by dialysis for several days against distilled water. The RNA was precipitated by a mixture of ethyl alcohol and hydrochloric acid according to the method of Roth and Milstein (9). An 80% solution of ethanol, which was 1 N with respect to HCl, was added to the RNA solution in equal volume. The precipitated RNA was washed twice with the HCl-ethanol reagent followed by three washes with ether. After drying in the desiccator, the RNA was lyophilized and stored under vacuum at 4°C. The phoshporus content of the purified preparation was 7.8%.

Bacterial ribonucleic acid inhibitor was extracted from Group A streptococci by a modification of the method described by Bernheimer and Ruffier (12). Cells from Group A streptococci were washed twice with physiological saline, resuspended in 1 M saline, and disrupted in a Mickle disintegrator. Carbon dioxide was bubbled through the suspension for 15 min to acidify the suspension and thereby facilitate centrifugation. Cell walls and other debris were removed by centrifugation at 18,000 rpm for 30 min. The supernatant fluid containing the inhibitor was subjected to further purification. 0.1 volume of calcium chloride and 2 volumes of ethanol were added to the supernatant fluid. The precipitate was redissolved in distilled water and the resulting solution was deproteinized by vigorous shaking in the presence of chloroform and amyl alcohol, according to the method of Sevag et al. (13). The process was repeated until no further protein cake appeared. The aqueous phase was dialyzed against three changes of M/40 Veronal buffer containing M/100 magnesium sulfate. 50 micrograms of pancreatic deoxyribonuclease (Worthington Biochemical Corp., Freehold, N. J., 1 × crystallized) was added and the mixture was incubated at 37°C for 18 hr. The solution was subjected to the deproteinization procedure again. 2 volumes of ethanol were added and the precipitate was redissolved in distilled water and dialyzed against three changes of distilled water. The preparation was lyophilized and stored in a desiccator at 4°C. It appeared to be stable over a period of greater than 1 yr.

Chemical Determinations.—Phosphorus was determined by the method of Fiske and Subbarow (14). RNA and DNA determinations on the substrate and inhibitor preparations were done by the orcinol method (15) and a modification of the Dische method (16), respectively. Protein determinations were done by a modification of the Folin method (17) and by a modification of the micro-Kjeldahl method (18).

RESULTS

Heat Stability.—The stability of the three streptococcal deoxyribonucleases was examined by comparing enzyme activities before and after heating. Enzyme preparations were diluted in neopeptone dialysate broth to a concentration of approximately 40 units/ml, and subjected to heating at three different tem-

peratures for 10 and 30 min. Original and residual enzyme activities were measured by the viscosimetric technique after dilution to appropriate concentrations for testing. Results were expressed as per cent residual activity.

When examined in this way, all three enzymes appeared to be stable at temperatures up to 37°C. DNAse A seemed to be somewhat less stable at this temperature than DNAses B and C. At temperatures of 56°C or greater DNAse C appeared to be more stable than DNAses A or B (Table I).

Cation Requirements.—Studies of divalent cation activation of DNAse B indicated that either cobalt or manganese ions activated on a molar basis equally as well as magnesium ions. It was also observed that DNAse B preparations diluted in neopeptone dialysate as a stabilizer showed consistently higher enzyme activities than preparations diluted in 1 or 0.1% bovine serum albumin as a stabilizer. The higher activity in the former stabilizing diluent was traced to the calcium content of neopeptone diluent, which was found to be

TABLE I

Heat Stability of Streptococcal Deoxyribonucleases

Enzyme	Per cent residual enzyme activity					
	37°C		56°C		65°C	
	10 min	30 min	10 min	30 min	10 min	30 min
DNAse A	100	75	41	15	—	—
DNAse B	100	100	36	14	—	—
DNAse C	100	100	75	79	63	10

about 12 mg/100 ml. This suggested that calcium ions might potentiate magnesium ion activation of DNAse B in a manner similar to that previously reported for DNAse A (19, 20).

More definitive studies of the divalent cation requirements of DNAse B were therefore made with thymus deoxyribonucleic acid (DNA) which had been treated with sodium ethylenediaminetetraacetate (EDTA) and then exhaustively dialyzed to remove as much of trace contaminants of divalent cations as possible. With substrate treated in this manner, the enzyme activity obtained in the absence of added cations was quite low (Table II). Calcium ions alone resulted in only slight increase in activity, approximately twofold, when added in amounts up to those resulting in formation of precipitates which interferred with the determination. The addition of magnesium ions in a concentration as low as 0.0005 M resulted in a 10-fold increase in activity. The highest activity obtained with magnesium ions alone was 158 units/ml at a 0.001 M concentration. A mixed system with magnesium ions at this concentration and calcium ions at a concentration of only 0.0005 M showed an activity

of 216 units/ml, a potentiation of approximately 30% by the addition of the second cation.

The optimal concentration of magnesium ions appeared to vary with the concentration of calcium ions available and vice versa. This ranged from 0.0005 to 0.02 M for magnesium ions and from 0.0005 to 0.005 M for calcium ions. There is an indication that greater concentrations of calcium ions are required for maximal potentiation when the concentration of magnesium ions is below or above that which gives maximal activation alone. The marked potentiation of calcium ions when excessive amounts of magnesium ions are present suggests a possible protective effect.

These observations confirm and extend to multiple combinations of concentrations the findings of Winter and Bernheimer (21). Subsequent observations in this laboratory have indicated that calcium ions serve as a stabilizer as well as a potentiator of DNAse B activity.[1]

TABLE II

Activation of DNAse B by Various Combinations of Concentrations of Magnesium and Calcium Ions

Mg^{++} (M conc.)	Ca^{++} (M conc.)					
	0	5×10^{-4}	1×10^{-3}	5×10^{-3}	1×10^{-2}	2×10^{-2}
0	14*	34	26	33	ppt‡	ppt
5×10^{-4}	146	192	204	216	162	ppt
1×10^{-3}	158	216	206	190	190	158
5×10^{-3}	124	146	215	202	194	166
1×10^{-2}	96	174	190	218	174	170
2×10^{-2}	20	70	112	150	168	156

* Viscosimetric units/mg enzyme B preparation.

‡ Indicates that precipitation was obtained at this concentration of cations.

Specificity of the Bacterial Ribonucleic Acid Inhibitor.—A bacterial ribonucleic acid inhibitor of streptococcal deoxyribonuclease activity has previously been described by Bernheimer and Ruffier (12). In order to examine the behavior of the individual streptococcal deoxyribonucleases in the presence of this inhibitor, inhibitor was prepared from Group A streptococcal cells by a modification of the method employed by these authors. This crude preparation was subjected to further purification. The ultraviolet absorption pattern and orcinol reaction of the resulting preparation of inhibitor resembled rather closely that of a purified preparation of yeast ribonucleic acid (Figs. 1 and 2). Further comparative analyses of these two preparations are given in Table III.

[1] Gray, E. D., W. G. Yasmineh, and L. W. Wannamaker. Unpublished observations.

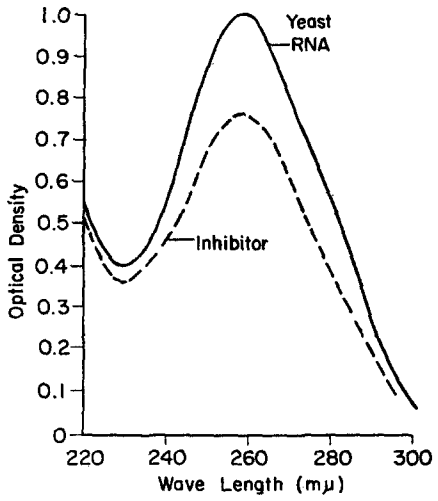

FIG. 1. Comparison of ultraviolet absorption pattern of a partially purified preparation of bacterial ribonucleic acid inhibitor (0.04 mg/ml) with that of a purified preparation of yeast ribonucleic acid (0.04 mg/ml), in glycine buffer, $\mu = 0.1$, pH 9.0.

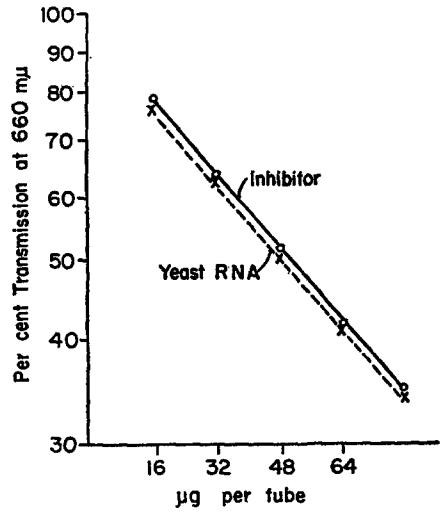

FIG. 2. Comparison of the orcinol reaction of a partially purified preparation of bacterial ribonucleic acid inhibitor with that of a purified preparation of yeast ribonucleic acid.

When the three streptococcal enzymes were examined for activity in the presence of this inhibitor, a striking difference was found. Even with inhibitor concentrations as high as 200 μg per ml, the activities of DNAses A and C were unaffected whereas the activity of DNAse B was markedly inhibited (Fig. 3).

Considerable inhibition of the B enzyme was also obtained with much lower concentrations of the inhibitor. Concentrations of 5 and 20 μg/ml showed approximately 50 and 80% inhibition, respectively, and inhibition was detectable with concentrations as low as 0.4 μg/ml (Fig. 4). In accordance with the

TABLE III

Comparative Analyses of Preparations of Bacterial and Yeast Ribonucleic Acids

Determination	Bacterial RNA inhibitor	Yeast RNA
ϵ(P)	8300	9150
Phosphorus, per cent	9.5	8.7
Atomic N/P ratio	3.9	3.7
DNA, per cent	8.0	2.5
Protein, per cent	5.5	1.4

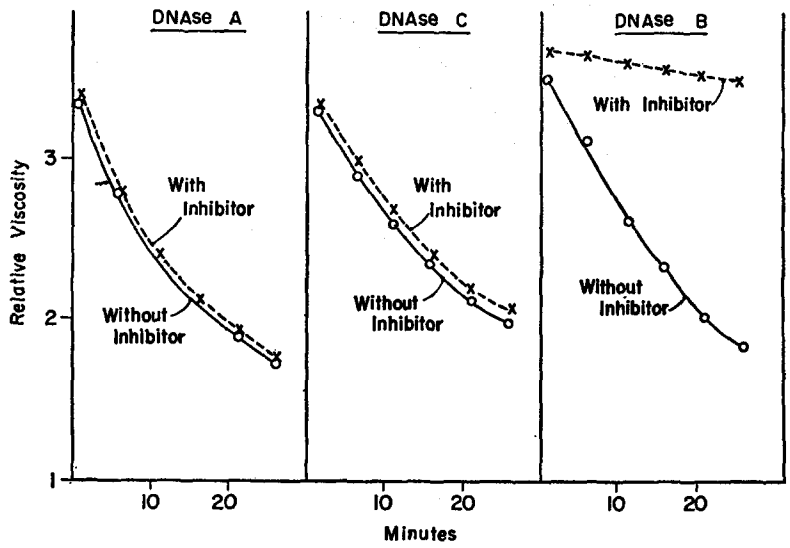

FIG. 3. Effect of bacterial ribonucleic acid inhibitor (200 μg/ml) on the activities of the three streptococcal deoxyribonucleases as measured viscosimetrically.

findings of Bernheimer and Ruffier (12), the prior incubation of the inhibitor with pancreatic ribonuclease, in a concentration of 10 μg/ml, resulted in inactivation of the inhibitor (Fig. 5). The specificity of inhibition of the B enzyme could be confirmed using the alcohol precipitation test of McCarty (5).

The possibility that the A and C enzymes might fail to be inhibited because of rapid inactivation of the inhibitor in their presence was examined. Preincuba-

tion of the inhibitor with either DNAse A or DNAse C had no effect on its ability to inhibit the activity of DNAse B (Fig. 5).

In order to examine the effect of DNAse B on the inhibitor, an amount of B enzyme equivalent in deoxyribonuclease activity to that used for the A and C enzymes above (approximately 10 times the amount normally used in viscosimetric assays) was incubated with the inhibitor, and residual B enzyme was removed by vigorous shaking with chloroform according to the method of Sevag et al. (13). Inhibitor preparations treated in this manner retained their ability to inhibit the deoxyribonuclease activity of the B enzyme (Fig. 6). Thus, under the conditions of this experiment, the biological activity of the inhibitor was apparently unimpaired by prior exposure to the enzyme, although in

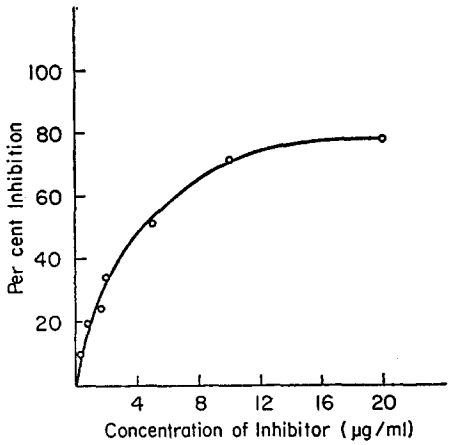

FIG. 4. Effect of various concentrations of bacterial ribonucleic acid inhibitor on the activity of DNAse B as measured viscosimetrically.

subsequent studies with larger amounts of enzyme and incubation over a long period of time, evidence of both chemical and biological destruction of the bacterial ribonucleic acid inhibitor was obtained (see below).

Ribonuclease Activity.—Since this inhibitor, which had the characteristics of a bacterial ribonucleic acid, appeared to be relatively resistant to all of the three streptococcal enzymes, it was of interest to determine whether these enzymes would destroy ribonucleic acid from other sources. Preparations of DNAses A and C showed no activity against yeast ribonucleic acid, whereas preparations of DNAse B showed definite destruction of this substrate. Divalent cations are required for activation of RNA digestion by preparations of the B enzyme, just as when DNA is used as substrate. Ribonuclease activity was completely inhibited by the addition of ethylenediaminetetraacetate (EDTA) (Fig. 7).

With relatively large amounts of the B enzyme, bacterial as well as yeast

ribonucleic acid was digested (Fig. 8). A concentration of DNAse B about 5-fold greater was required to achieve digestion of bacterial ribonucleic acid equivalent to that obtained with yeast ribonucleic acid. Subsequent studies (22), to be published in detail elsewhere, have suggested that the nature of the

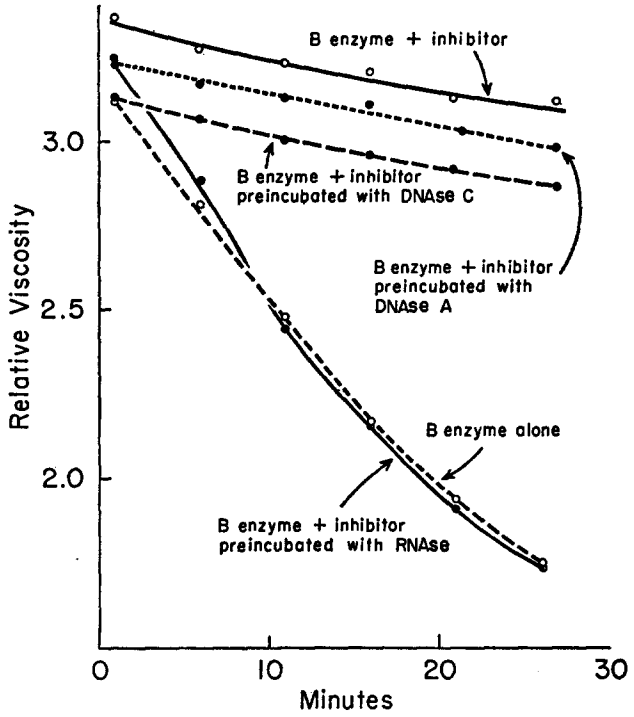

FIG. 5. Effect of preincubation with pancreatic ribonuclease, with DNAse A and with DNAse C, on the ability of bacterial ribonucleic acid to inhibit DNAse B as measured viscosimetrically. Concentration of enzymes in the preincubation mixture was 10 μg/ml for pancreatic ribonuclease, 16 units/ml for DNAse A and 7 units/ml for DNAse C. The concentration of inhibitor in the preincubation mixture was 80 μg/ml. A concentration of inhibitor or its digest equivalent to 16 μg/ml of bacterial ribonucleic acid was present in the final mixture tested for inhibition. The concentration of DNAse B in the final mixture was 0.3 units/ml.

ribonucleic acid (i.e., ribosomal or soluble RNA) is more important than the source of ribonucleic acid in determining susceptibility to digestion by DNAse B.

Prolonged digestion of preparations of bacterial ribonucleic acid with relatively large amounts of the B enzyme also resulted in appreciable destruction of its biological capacity to inhibit (Fig. 9). In this experiment, 10 ml of a solution of bacterial RNA (2 mg/ml) in 0.05 M imidazole buffer, pH 7.8, with

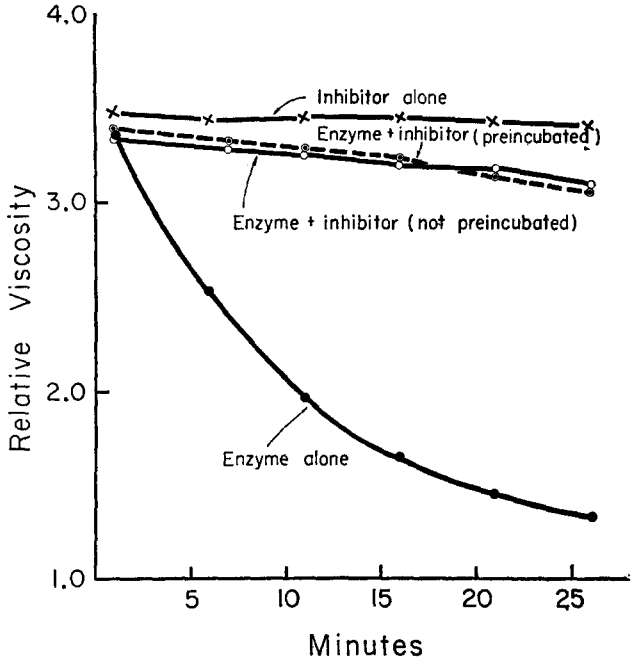

FIG. 6. Effect of preincubation with DNAse B (3 viscosimetric units/ml incubated with 80 μg/ml inhibitor for 30 min at 37°C) on the ability of bacterial ribonucleic acid (final concentration 16 μg/ml) to inhibit this enzyme as measured viscosimetrically. Final concentration of DNAse B was 0.3 units/ml.

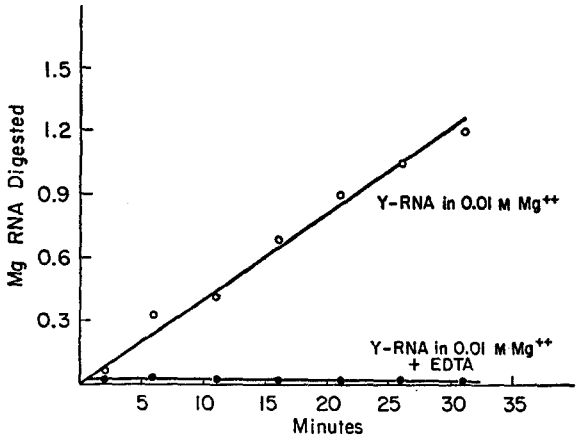

FIG. 7. Digestion of yeast ribonucleic acid by DNAse B in the presence and absence of activation by magnesium ions. Final concentration of B enzyme was 68 viscosimetric units/ml, of yeast ribonucleic acid 1 mg/ml, of EDTA 0.015 M.

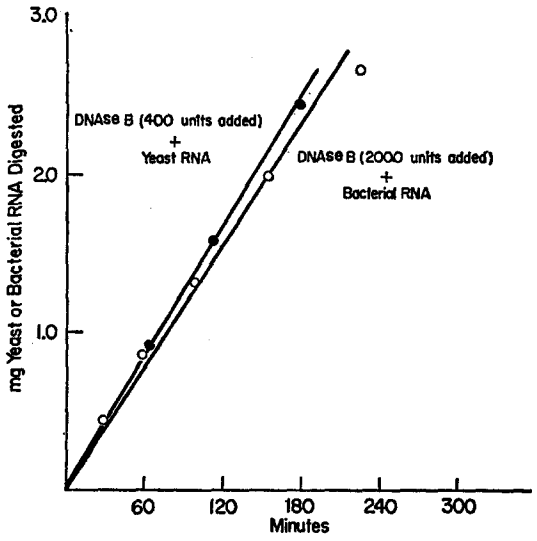

FIG. 8. Comparison of the digestion of yeast ribonucleic acid and bacterial ribonucleic acid. Final concentration of ribonucleic acids was 1 mg/ml. Total amounts of enzyme added are indicated in viscosimetric units.

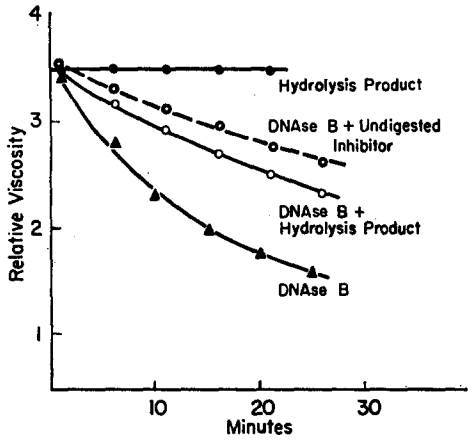

FIG. 9. Residual inhibitory effect on DNAse B activity after prolonged hydrolysis of bacterial ribonucleic acid by large amounts of the same preparation of enzyme. The hydrolysis product was tested at a concentration of approximately 6.5-fold greater than the undigested inhibitor (see text).

0.01 M Ca^{++} and 0.01 M Mg^{++} was incubated with 2 ml of a solution of B enzyme (1500 viscosimetric units/ml or 30 μg protein/ml) for 18 hr at 37°C. After this exhaustive digestion, approximately 90% of the RNA was rendered acid-alcohol soluble. The hydrolysis product was deproteinized by shaking with chloroform

according to the method of Sevag et al. (13) until there was no residual DNAse B activity. Residual inhibitor activity of the deproteinized hydrolysis product was tested viscosimetrically. Untreated inhibitor showed 63% inhibition at a final concentration in the viscosimeter of 5 μg/ml, while digested inhibitor showed 48% inhibition at a final concentration equivalent to 33 μg/ml. The calculated biological destruction was approximately 90% and therefore equal to the demonstrated chemical destruction. This experiment is similar to that depicted in Fig. 6 except that the ratio of enzyme to inhibitor used in obtaining the hydrolysis product is 20-fold greater and the incubation time used in obtaining the hydrolysis product is 18 hr rather than 30 min.

Repeated attempts to separate the deoxyribonuclease and the ribonuclease activities of the B enzyme by zone electrophoresis and column chromatography under varying conditions have been unsuccessful, but they did reveal the presence of a fourth nuclease which is discussed in the paper that follows (23). Examples of two such attempts are given in Figs. 10 and 11.

The ratios of ribonuclease to deoxyribonuclease activity were determined in several different preparations and were found to be similar (Table IV). Both deoxyribonuclease and ribonuclease activities of the B enzyme were relatively stable when tested at 4°, 25°, and 37°C, whereas loss of both activities occurred at 56° and 65°C. Following heat inactivation at 56°C, the ratios of ribonuclease to deoxyribonuclease activity did not change (Table V).

The ability of preparations of the B enzyme to destroy yeast ribonucleic acid was inhibited by bacterial ribonucleic acid in a manner similar to that previously shown for its deoxyribonuclease activity. Concentrations of inhibitor about 20 μg/ml showed rather marked inhibition (Fig. 12).

The foregoing results suggested the possibility that the B enzyme might be a single nuclease which had the ability to destroy both deoxyribonucleic and ribonucleic acids. It was therefore of interest to explore whether inhibition by bacterial ribonucleic acid was of a competitive or noncompetitive nature and whether the two substrates would show mutual inhibition.

When varying amounts of deoxyribonucleate substrate with a constant amount of bacterial inhibitor were used in a viscosimetric assay, the results suggested a competitive effect (Fig. 13). In a final inhibitor concentration of 5 μg/ml, the per cent inhibition was less when the substrate concentration was 1.8 mg/ml (59% inhibition) than when the substrate concentration was 0.6 mg/ml (80% inhibition). These results have been subsequently confirmed by more precise kinetic techniques (24, 25).

In contrast to the report of Bernheimer (26), studies in our laboratory suggested that yeast ribonucleic acid as well as bacterial ribonucleic acid showed significant inhibition of DNAse B activity although the latter appeared to be a much more potent inhibitor (Fig. 14). Subsequent findings (22) have indicated that the method of preparation and consequently the kind of ribonucleic acid

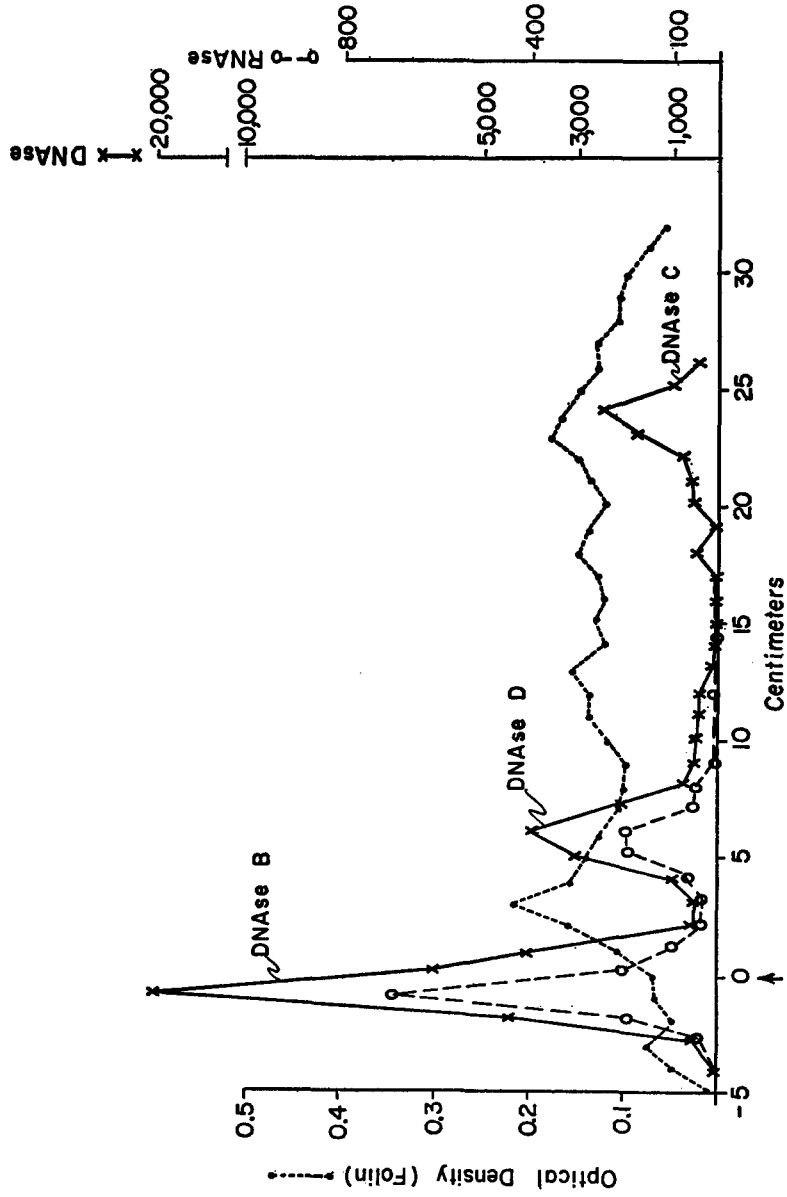

FIG. 10. Separation of the extracellular products of a strain of Group A streptococcus by starch zone electrophoresis (glycine buffer, $\mu = 0.1$, pH 9.0, 400 v for 16 hr) (3).

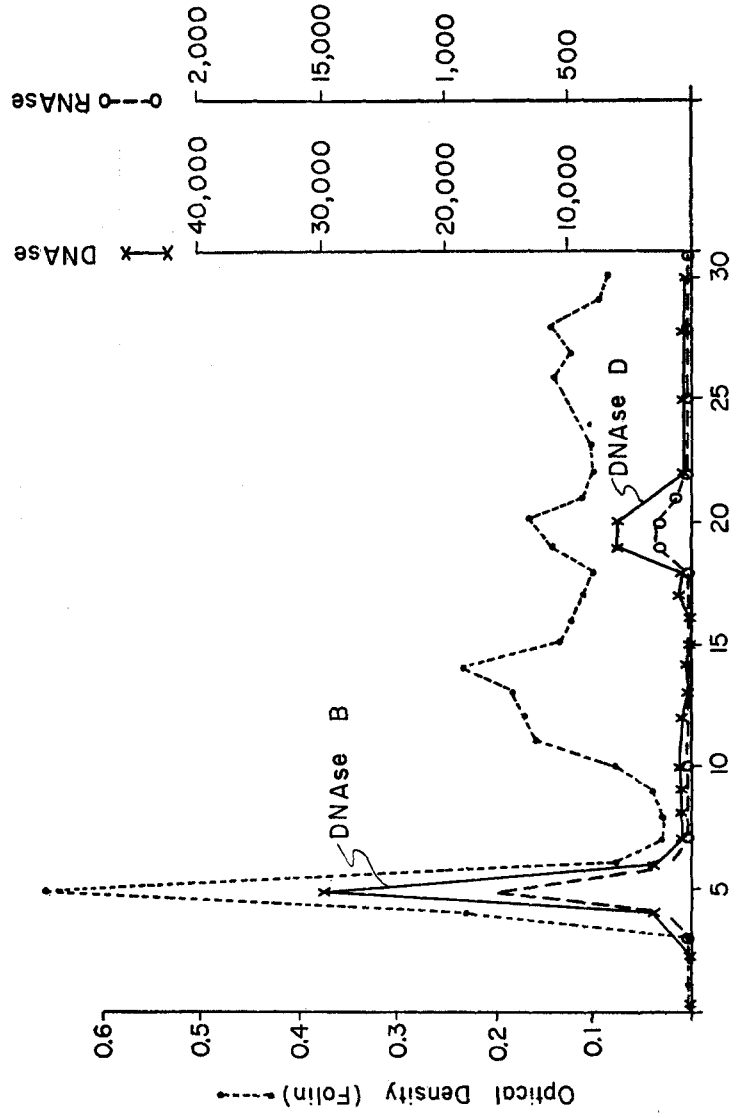

FIG. 11. Separation of the extracellular products of a strain of Group A streptococcus by column chromatography on DEAE-cellulose (4).

obtained (ribosomal or soluble RNA) are more important in determining inhibition than the source of the ribonucleic acid.

The observation that yeast RNA could inhibit the digestion of thymus DNA by the B enzyme in the viscosimetric assay suggested that the two substrates might show mutual inhibition. Studies were therefore carried out with the B enzyme in mixed substrate systems by techniques which permitted assay of both nucleic acids. The classical assay procedure of susceptibility to alkaline hydroly-

TABLE IV

Relative RNAse and DNAse Activities in Various Preparations of the B Enzyme

Preparation (lot No.)	RNAse activity (units)*	DNAse activity (units)‡	Ratio (RNAse/DNAse)
48	78	93	0.84
48	71	62	1.14
51	190	275	0.69
51	137	189	0.72
56	261	360	0.72
57	188	270	0.72

* Turbidimetric units/mg enzyme B preparation.

‡ Viscosimetric units/mg enzyme B preparation.

TABLE V

Relative RNAse and DNAse Activities in a Preparation of B Enzyme before and after Heating

Preparation (Lot No.) and treatment	RNAse activity	DNAse activity	Ratio (RNAse/DNAse)
	*units**	*units**	
48, unheated	500	3500	0.14
48, heated at 56°C for 30 min	150	950	0.16

* Micrograms of substrate digested per min/mg of enzyme B preparation, based on total phosphorus in the acid-alcohol precipitate.

sis (Schmidt-Thannhauser procedure) proved unsatisfactory when applied to HCl-ethanol precipitates of digests since the enzyme appeared to render precipitable deoxyribonucleic acid more susceptible to alkaline hydrolysis. The procedures of Webb (11) and of Webb and Levy (10) for the determination of ribonucleic acid and deoxyribonucleic acid, respectively, were modified so that determinations of the two nucleic acids could be made in the presence of each other.

With these methods, it was possible to show that each substrate inhibits the digestion of the other. In the experiment depicted in Figure 15, the deoxyribo-

nucleic acid concentration was kept constant and the concentration of yeast ribonucleic acid was varied. Considerable inhibition was obtained with a concentration of yeast ribonucleic acid (0.5 mg/ml) which was only half that used for the deoxyribonucleic acid (1 mg/ml). In a reverse experiment, the concentration of ribonucleic acid was kept constant and the concentration of deoxyribonucleic varied (Fig. 16). Definite inhibition occurred but it was less striking than that observed in the previous experiment. These studies indicate that mutual inhibition occurs in mixed substrate systems.

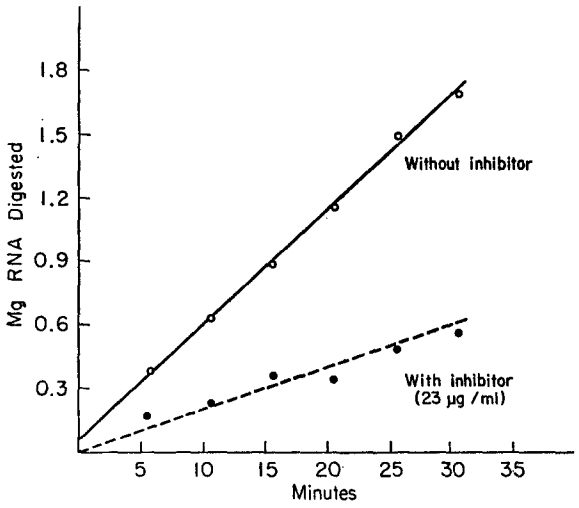

FIG. 12. Effect of bacterial ribonucleic acid inhibitor on the ribonucleic activity of a preparation of the B enzyme.

DISCUSSION

In addition to the distinguishing properties previously reported (1), the observations detailed here indicate differences in heat stability of the three streptococcal deoxyribonucleases, in their behavior in the presence of a bacterial ribonucleic acid inhibitor and in their association with ribonuclease activity.

The original observation of Bernheimer and Ruffier (12) that the bacterial ribonucleic acid inhibitor is effective against deoxyribonuclease formed by Group A streptococci but not against that of Group C streptococci is clarified by the findings reported here. The B enzyme, which is shown here to be inhibited by relatively small amounts of bacterial ribonucleic acid, is the deoxyribonuclease produced in largest quantity by most strains of Group A streptococci (1). On the other hand, the A enzyme, which along with the C enzyme is not inhibited even by large amounts of bacterial ribonucleic acid, is the deoxyribonuclease produced by Group C strains. The observation by Bernheimer and

Ruffier (12) that the deoxyribonuclease activity of crude supernatant fluids of Group A strains is only partially inhibited is consistent with our previously reported studies which indicate that these strains usually produce several other deoxyribonucleases in addition to the B enzyme (1).

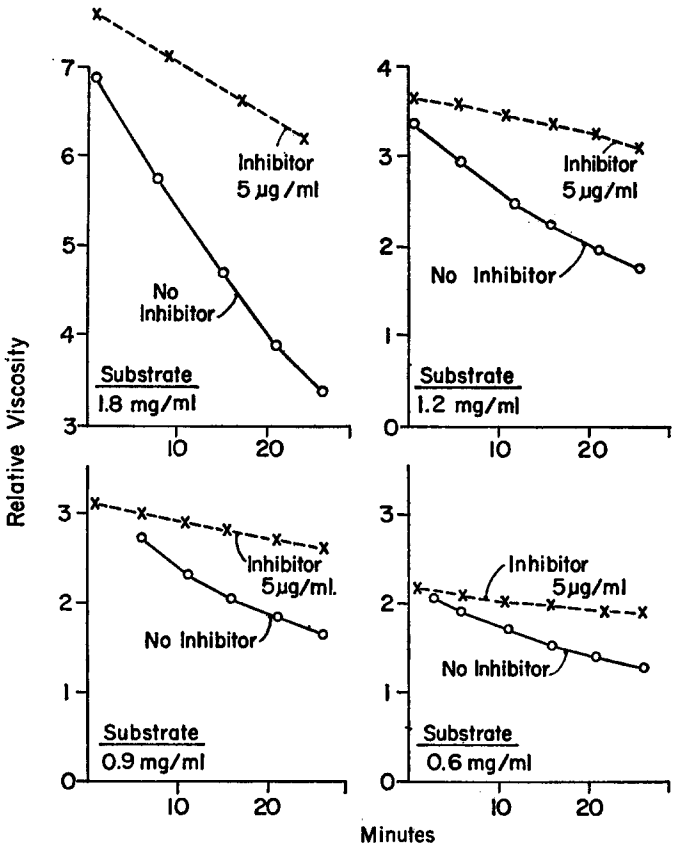

FIG. 13. Effect of varying amounts of deoxyribonucleic acid substrate with a constant amount of bacterial ribonucleic acid on the activity of the B enzyme as measured viscosimetrically.

In the studies reported here, DNAses A and C appear to be indifferent to the bacterial ribonucleic acid inhibitor. Their failure to be inhibited could not be explained by any demonstrable effect on the biological activity of the inhibitor. Equivalent concentrations of DNAse B, though markedly inhibited, also had no demonstrable effect on biological activity. However, prolonged digestion with relatively large amounts of B enzyme preparations resulted in significant chemical and biological destruction of the ribonucleic acid inhibitor. This

suggested that the inhibitory capacity of the preparation was associated with some fraction relatively resistant to degradation by streptococcal nucleases. The subsequent demonstration (22) that the inhibitor is specifically associated with the S-RNA, or soluble ribonucleic acid fraction, is consistent with these findings. The difference between the preparations of yeast and bacterial ribo-

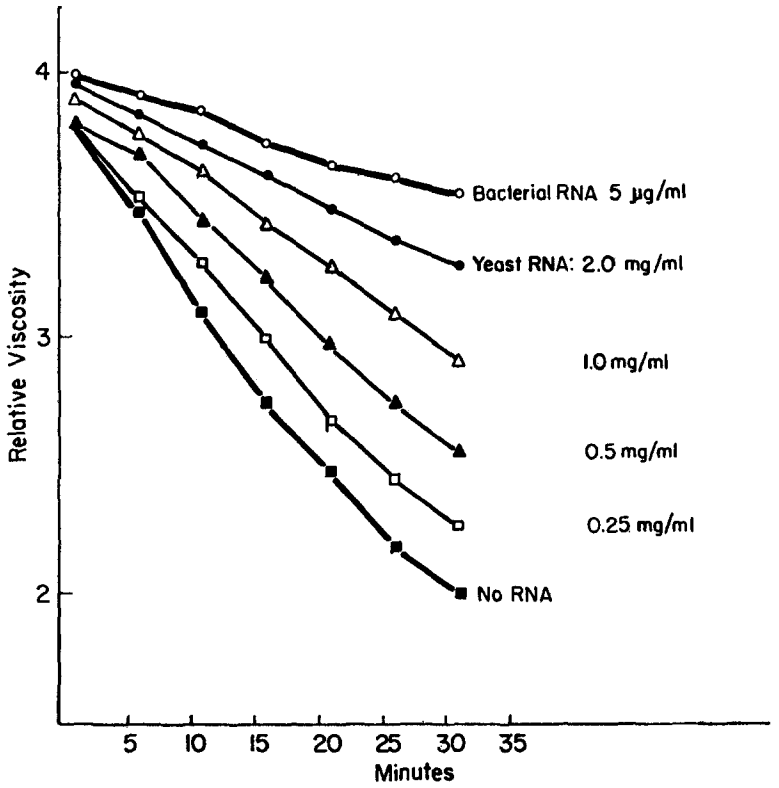

FIG. 14. Comparison of the inhibition of DNAse B activity by yeast ribonucleic acid in various amounts with the inhibition by bacterial ribonucleic acid. Deoxyribonuclease activity was measured viscosimetrically.

nucleic acids used here in their susceptibility to digestion by DNAse B might also be explained on this basis.

The relative resistance of the biological activity of the inhibitor to degradation by DNAse B is perhaps surprising in view of the constant association of ribonuclease activity with preparations of the B enzyme. This suggests that the ribonuclease activity associated with the B enzyme is different from that of pancreatic ribonuclease, which rapidly destroys the biological activity of the

inhibitor. The ribonuclease activity associated with the streptococcal B enzyme also differs from the pancreatic enzyme in its requirement for divalent cations for activation.

The constant association of ribonuclease with preparations of the B enzyme suggested that this might be more than just a chance contaminant. A number of other findings were consistent with the view that the B enzyme may be a

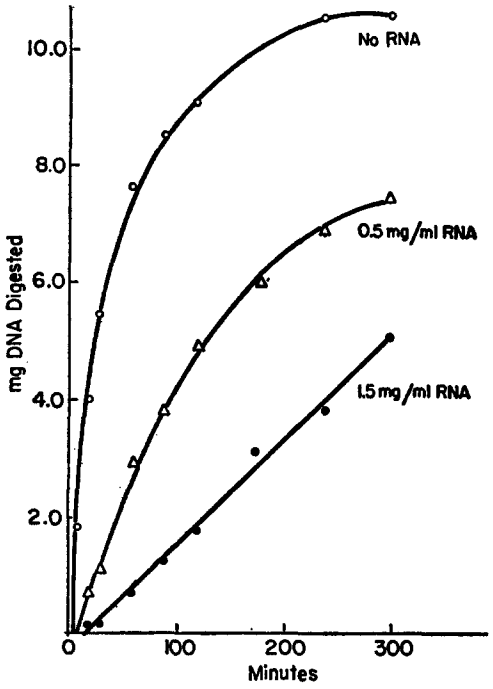

FIG. 15. Digestion of deoxyribonucleic acid in a mixed substrate system containing a constant amount of deoxyribonucleic acid (1 mg/ml) and varying amounts of yeast ribonucleic acid. The final concentration of B enzyme was 15 viscosimetric units/ml. The amount of deoxyribonucleic acid rendered soluble in acid-alcohol was measured by the procedure of Webb and Levy (10).

single nuclease which can attack both deoxyribonucleic and ribonucleic acids: (*a*) Numerous attempts to separate these two activities by zone electrophoresis and column chromatography were unsuccessful. (*b*) The ratio of ribonuclease to deoxyribonuclease activity was constant in many different enzyme preparations, and the ratio of the two enzyme activities was unaltered after partial heat inactivation. (*c*) Divalent cations were required for the ribonuclease as well as the deoxyribonuclease activity associated with the B enzyme. (*d*) Ribonuclease activity associated with preparations of the B enzyme, as demonstrated by its ability to depolymerize yeast ribonucleic acid, was inhibited by the bacterial ribonucleic acid inhibitor in a manner similar to the inhibition of the deoxyri-

bonuclease activity of these preparations. (*e*) Yeast ribonucleic acid, though apparently less efficient than bacterial ribonucleic acid, could also be shown to inhibit the deoxyribonuclease activity of the B enzyme. (*f*) Conversely, deoxyribonucleic acid could be shown to inhibit the ribonuclease activity of preparations of the B enzyme.

The inability to separate or otherwise distinguish between the properties of

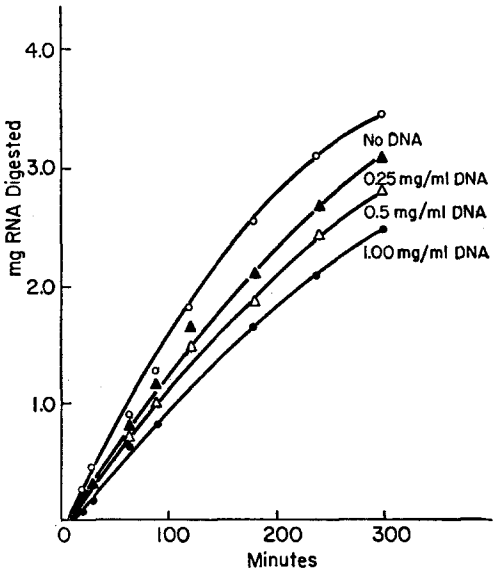

FIG. 16. Digestion of yeast ribonucleic acid in a mixed substrate system containing a constant amount of yeast ribonucleic acid (0.5 mg/ml) and varying amounts of deoxyribonucleic acid. The final concentration of B enzyme was 15 viscosimetric units/ml. The amount of ribonucleic acid rendered soluble in acid-alcohol was measured by the method of Webb (11).

the two enzyme activities and their mutual inhibition in mixed substrate systems are compatible with the single enzyme hypothesis but do not necessarily indicate that they are associated with an identical active site. If the single enzyme hypothesis is correct, then the term streptococcal nuclease B appears to be preferable to that previously suggested, streptococcal deoxyribonuclease B.

Nucleases with broad specificity for both major kinds of nucleic acid have been postulated for enzymes associated with staphylococci and with *Escherichia coli*, and evidence has been obtained consistent with this point of view (27, 28, 29). Bacterial ribonucleic acid has been shown to inhibit one of the deoxyribonucleases of *E. coli* (30, 31) and deoxyribonucleases of other organisms (see review, reference 32). These properties therefore do not appear to be unique for the streptococcal enzyme.

SUMMARY

Streptococcal DNAse C is more resistant to heat inactivation than the A or B enzyme. DNAses A and C are indifferent to the bacterial ribonucleic acid inhibitor whereas the B enzyme is markedly inhibited. Prolonged digestion with relatively large amounts of DNAse B results in chemical and biological destruction of the inhibitor.

Ribonuclease as well as deoxyribonuclease activity is associated with the B enzyme. Both activities require divalent cations and both are inhibited by bacterial ribonucleic acid. The ratios of the two activities are constant in various preparations and after partial heat inactivation. Mutual inhibition of the two activities can be demonstrated in mixed substrate systems. The evidence presented is consistent with the view that the B enzyme is a single nuclease which can attack both deoxyribonucleic and ribonucleic acids.

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