STREPTOCOCCAL NUCLEASES*

II. Characterization of DNAse D

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The existence of a fourth extracellular deoxyribonuclease produced by Group A streptococci was recognized independently by Winter and Bernheimer (1) and in this laboratory (2, 3). The present report details evidence for immunological specificity of this nuclease, thereby strengthening its designation as a distinct enzyme, DNAse D, and describes other characteristics which indicate its relationship to the other streptococcal nucleases, particularly the B enzyme.

Materials and Methods

The materials and methods were the same as those used in the companion paper (4). Strain C203S of Group A streptococcus was used for most preparations of DNAse D employed in these studies.

For the production of antisera, rabbits were immunized with fractions obtained by zone electrophoresis or column chromatography, according to methods previously described (5, 6). Absorption was not necessary. Antibody neutralization of streptococcal deoxyribonucleases was measured by a viscosimetric technique originally described by McCarty (7) as modified in an earlier publication (5). Double diffusion plates used in immunological studies contained 0.8% agar (special Noble) with 0.85% NaCl and Merthiolate at a final concentration of 1:10,000.

RESULTS

In the course of attempts to partition the deoxyribonuclease and ribonuclease activities of streptococcal DNAse B, a fourth extracellular nuclease was encountered which could be separated from the three nucleases previously described (5) by either zone electrophoresis or column chromatography (2, 3,

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4). When examined by zone electrophoresis, this new nuclease migrated toward the cathode more rapidly than DNAse B but less rapidly than the other two nucleases. (See Fig. 10, accompanying paper. DNAse A was not present in the pattern shown.) On column chromatography, on DEAE-cellulose, the new enzyme eluted after DNAse B (See Fig. 11, accompanying paper).

Antisera prepared against streptococcal DNAses A, B, and C failed to neutralize the deoxyribonuclease activity of this enzyme when examined by the

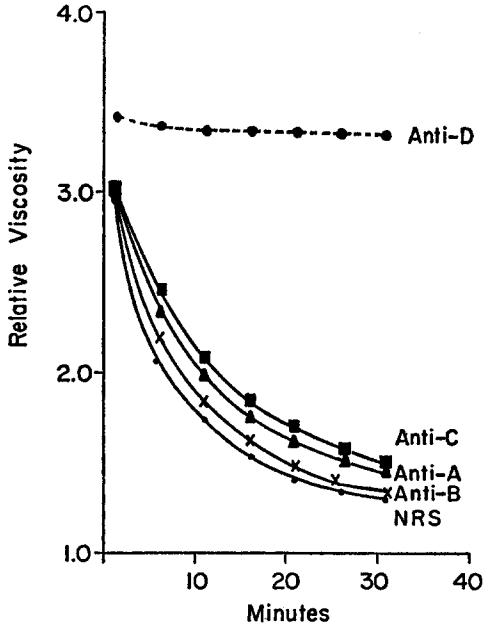

FIG. 1. DNAse D activity in the presence of homologous rabbit antiserum, antisera to heterologous streptococcal nucleases and normal rabbit serum (NRS), as demonstrated viscosimetrically. The DNA concentration was 1 mg/ml and the final enzyme concentration approximately 1 viscosimetric unit/ml.

viscosimetric technique (Fig. 1). Rabbits injected with electrophoretically purified preparations of this enzyme developed antibody which inhibited the activity of the homologous enzyme (Fig. 1) but failed to neutralize the activity of the other three streptococcal deoxyribonucleases (Fig. 2). This immunologically specific fourth deoxyribonuclease was therefore designated DNase D.

Analysis of the immune rabbit sera by zone electrophoresis revealed that the specific inhibitor of the D enzyme, as well as the other three enzymes, was found in the gamma globulin fraction and could be completely or partially separated from serum nuclease activity which migrated somewhat more rapidly toward the anode.

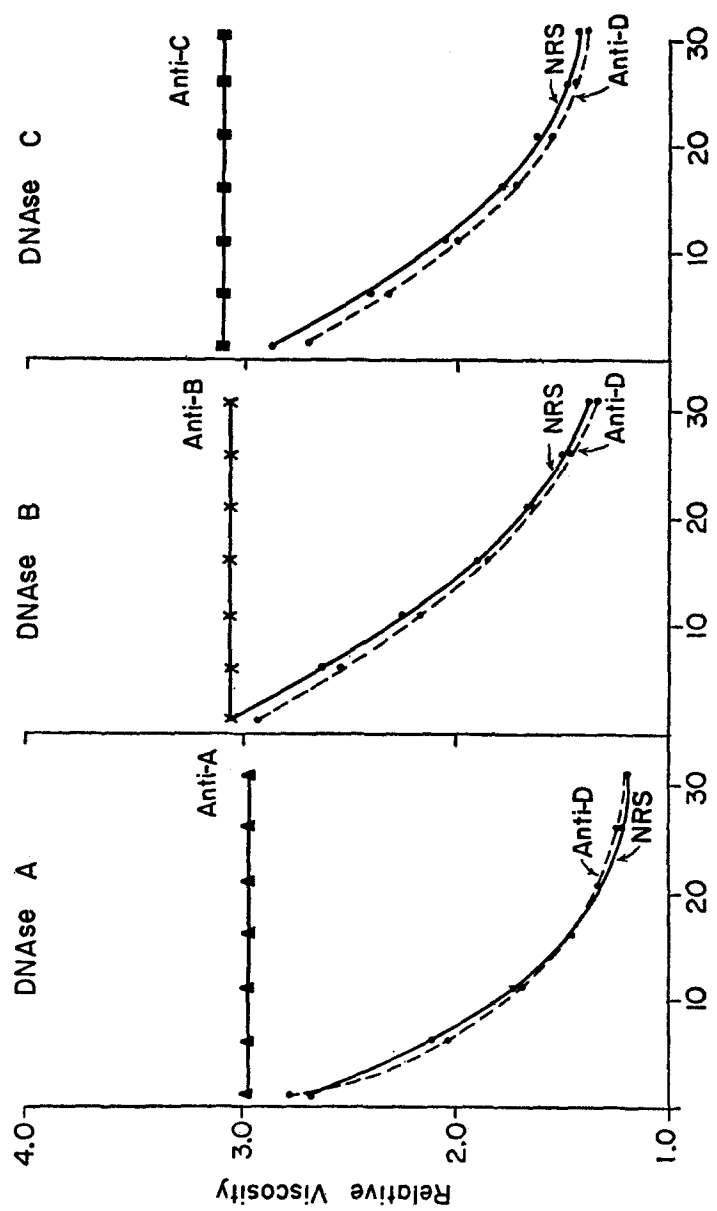

FIG. 2. Effect of DNAse D antiserum, homologous antisera, and normal rabbit serum (NRS) on streptococcal DNAses A, B, and C. The experimental conditions and concentrations of enzyme and substrate were the same as in Fig. 1.

DNAse D was found to be active over a pH range of 6.5–9.5. Magnesium ions are required for activation. Activity is completely inhibited by sodium ethylenediaminetetraacetic acid (EDTA) at a 0.03 M concentration and partially inhibited by sodium citrate at a 0.03 M concentration (Fig. 3).

The specific activity of the D enzyme was measured viscosimetrically at pH 7.8 and 37°C in a system containing sodium deoxyribonucleate (1 mg/ml), magnesium ions (0.01 M) and calcium ions (0.01 M). When examined in this manner, a preparation of the D enzyme gave a specific activity of 62 units/μg of protein as compared with 16 and 21 units/μg of protein in preparations of

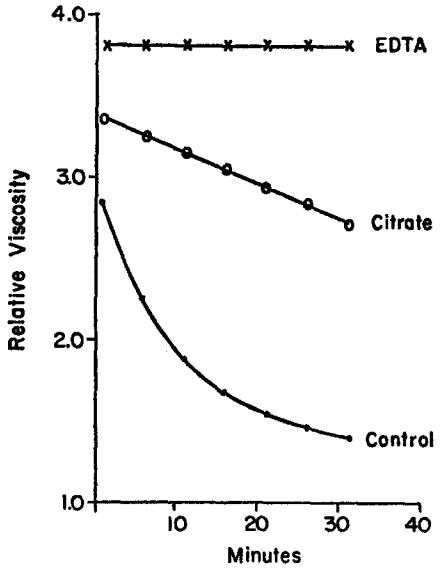

FIG. 3. Effect of sodium ethylenediaminetetraacetic acid (EDTA) and sodium citrate on DNAse D activity, as measured viscosimetrically.

streptococcal DNAse B and pancreatic deoxyribonuclease (Worthington Biochemical Corp., Freehold, N. J., 1 × crystallized), respectively.

Some but not all preparations of the D enzyme gave a single line of precipitation when examined in a double diffusion system (Fig. 4). In the experiment depicted, 12 mg of a preparation of DNAse D was dissolved in 0.7 ml buffered saline (1 part Veronal buffer, 4 parts 0.85% saline). Twofold dilutions were made in buffered saline and 0.1 ml aliquots were placed in the peripheral wells. 0.2 ml of antiserum from rabbits immunized with DNAse D was placed in the center well. Plates were placed under a glass jar, with a beaker containing water to maintain humidity, and allowed to stand at room temperature.

Native (double-stranded) and heat-denatured (single-stranded) deoxyribo-

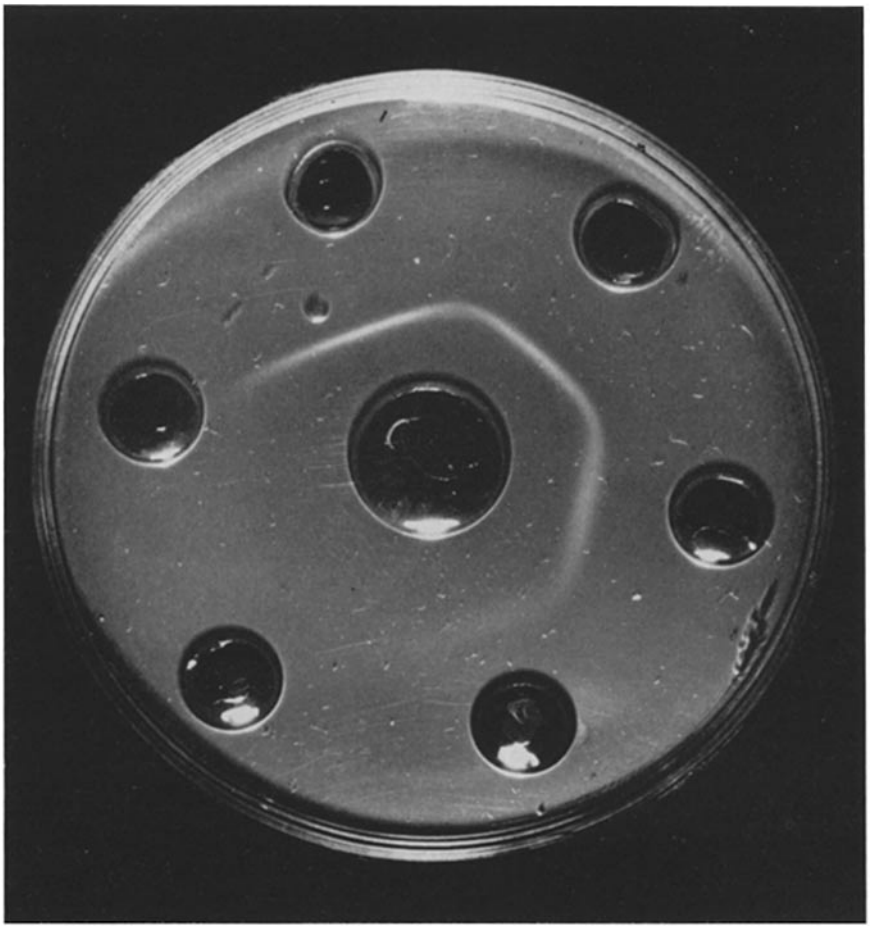

FIG. 4. Preparation of DNAse D and homologous antibody as examined in a double diffusion system in agar. Homologous antiserum was placed in the center well. DNAse D was placed in the peripheral wells, in twofold decreasing dilutions beginning with the well in the left upper corner and proceeding in a clockwise fashion. The photograph was taken after 72 hr at room temperature.

nucleic acids were compared as substrates for streptococcal DNAses A, B, C, and D. Release of acid-alcohol soluble products was used to monitor the rates of digestion. In confirmation of the findings of Winter and Bernheimer (1), our studies indicated that all four enzymes attacked both kinds of substrates. In the initial stages of digestion, the native substrate was attacked more readily than the heat-denatured substrate. The differences were of the order of two- to threefold. In the later stages of digestion, no difference could be demonstrated.

Preparations of DNAse D purified by zone electrophoresis or by column chromatography showed ribonuclease as well as deoxyribonuclease activity (Fig. 5). The activity of a preparation of this nuclease was examined on several substrates with respect to release of acid-alcohol soluble products. Relatively

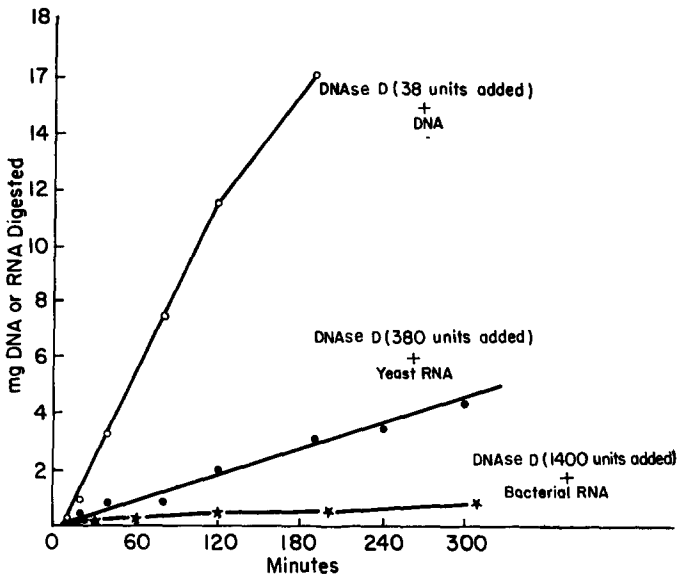

FIG. 5. Digestion of DNA, yeast RNA, and bacterial RNA by a preparation of DNAse D, as determined by release of acid-alcohol soluble products. The final concentration of nucleic acid was 1 mg/ml. Total amounts of enzyme added are indicated in viscosimetric units.

TABLE I

Comparison of Specific Activities of the B and D Enzymes on Three Different Substrates*

Enzyme	Substrate		
	Thymus DNA	Yeast RNA	Bacterial RNA
B	6.6×10^4	4.4×10^3	9.6×10^2
D	2.1×10^5	4.4×10^3	4.8×10^1

* Micrograms of nucleic acid digested per min/mg protein.

large amounts of enzyme were required for digestion of ribonucleic acid, particularly preparations of bacterial ribonucleic acid (Fig. 5).

A comparison was made of the specific activities of preparations of the B and D enzymes on three different substrates(Table I). In accord with the above findings by the viscosimetric technique, the specific activity of the preparation

of D enzyme on native thymus DNA was several times higher than the preparation of B enzyme as measured by release of substrate into the acid-alcohol soluble fraction. The specific activities of the two enzyme preparations were equal with respect to yeast ribonucleic acid, but both showed considerably less activity on this substrate than on thymus deoxyribonucleic acid. Both enzymes exhibited even less specific activity on a preparation of bacterial ribonucleic acid, but the specific activity of the B enzyme on this substrate was approximately 20 times higher than that of the D enzyme.

Bacterial ribonucleic acid inhibits the digestion of deoxyribonucleic acid by the D enzyme. Relatively large amounts of the inhibitor are required to demon-

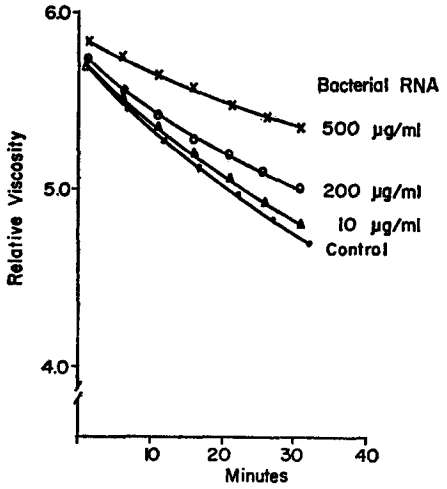

FIG. 6. Inhibition of deoxyribonuclease activity of the D enzyme by various concentrations of bacterial RNA, as demonstrated viscosimetrically.

strate appreciable inhibition. In a viscosimetric system (Fig. 6) a purified preparation of streptococcal ribonucleic acid at a final concentration of 200 µg/ml inhibited the depolymerization of calf thymus deoxyribonucleic acid by DNAse D approximately 27%. A final concentration of 500 µg/ml of inhibitor resulted in approximately 62% inhibition. Detectable inhibition was obtained with inhibitor concentrations of 10 µg/ml.

Inhibition of digestion of yeast RNA by the D enzyme also occurred in the presence of bacterial RNA. When monitored by release of substrate into the acid-alcohol soluble fraction, the digestion of yeast RNA by the D enzyme was inhibited approximately 57% at an inhibitor concentration of 66 µg/ml (Fig. 7).

As with the B enzyme, inhibition of the deoxyribonuclease activity of the D enzyme could be demonstrated in the presence of yeast as well as bacterial

ribonucleic acid, when large amounts were added. In a viscosimetric assay of DNAse D activity, the addition of yeast RNA at a final concentration of 2 mg/ml resulted in 35% inhibition (Fig. 8).

DISCUSSION

A variety of methods can be used to separate DNAse D from the other nucleases of Group A streptococci. Winter and Bernheimer (1) used continuous

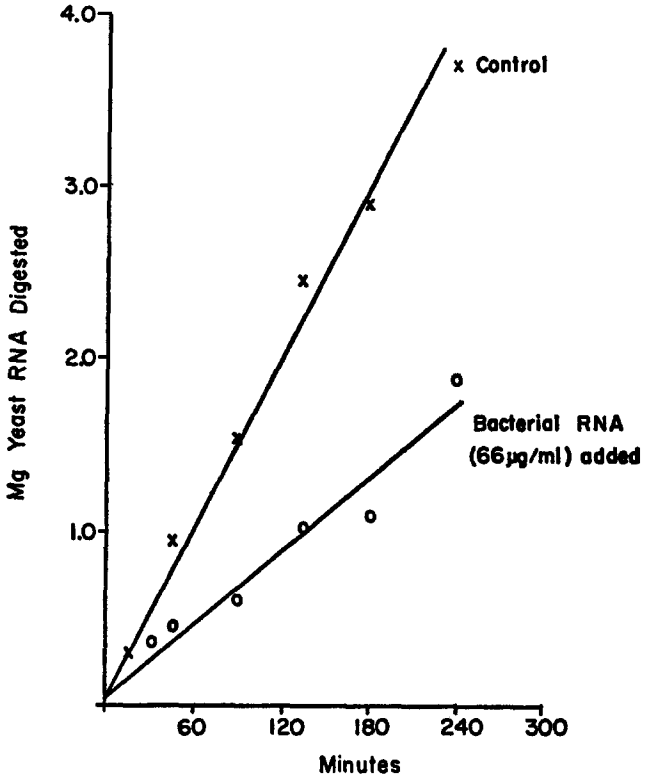

FIG. 7. Inhibition of digestion of yeast RNA by preparations of the D enzyme in the presence of bacterial RNA. Final concentration of yeast RNA was 1 mg/ml, of enzyme 1 µg protein/ml (or 40 viscosimetric units/ml).

flow paper electrophoresis followed by electrophoresis in a sucrose gradient. Partial separation of DNAses B and D was achieved by these techniques. As illustrated in the companion paper (4), virtually complete separation of DNAse D from other streptococcal nuclease activity can be achieved by zone electrophoresis and an even more striking separation can be accomplished by column chromatography.

Not only is it possible to obtain preparations of DNAse D free of other streptococcal nucleases but preparations of this enzyme are also apparently less likely than certain other nucleases to be contaminated by other streptococcal products. Preparations of DNAse B are commonly contaminated with streptococcal NADase and streptolysin O, and preparations of DNAse A may contain streptokinase (5, 6). Usually no evidence of other streptococcal products can be found in preparations of DNAse D, and some preparations show a single line of precipitation in double diffusion against sera from rabbits immunized with these preparations. This higher degree of purity is perhaps reflected in the higher specific activity when compared with preparations of streptococcal DNAse B and even with preparations of crystalline pancreatic deoxyribonuclease.

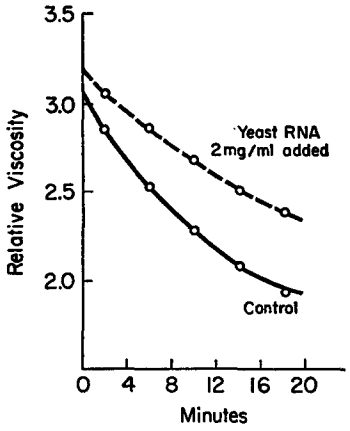

FIG. 8. Inhibition of deoxyribonuclease activity of DNAse D by yeast RNA, as determined viscosimetrically.

Like DNAse B, preparations of DNAse D regularly show ribonuclease activity. Whether this is a contaminant or a single nuclease capable of attacking both major kinds of nucleic acid must await further studies of the kind presented for DNAse B in the accompany paper (4). However, all of the evidence obtained so far, including the observation that both bacterial and yeast RNA can inhibit the deoxyribonuclease activity of this enzyme, is consistent with the single enzyme hypothesis.

When preparations of DNAses B and D were compared as to their ability to depolymerize various substrates, approximately equal specific activities were found with respect to yeast RNA. DNAse D showed a greater specific activity than DNAse B for thymus DNA when measured by either of two methods.

Conversely, DNAse B showed a greater specific activity than DNAse D for bacterial RNA. The meaning of these differences is not clear but it may relate to the amount of specific inhibitor present in various preparations of ribonucleic acid (8).

Bacterial ribonucleic acid, which itself is poorly hydrolyzed by preparations of DNAse D, has the capacity to inhibit the digestion of other substrates by this enzyme. When added in very large amounts, yeast ribonucleic acid could also be shown to inhibit the deoxyribonuclease activity of DNAse D. In these respects, also, the D enzyme resembles the B enzyme.

Relatively large amounts of bacterial RNA are required to inhibit significantly the digestion of yeast RNA by preparations of either DNAse B or DNAse D. Even larger amounts of the inhibitor are required to bring about appreciable inhibition of the deoxyribonuclease activity of DNAse D. This is in contrast to the relatively small amounts of inhibitor required to affect the deoxyribonuclease activity of DNAse B. Thus, whereas bacterial ribonucleic acid at a concentration of 2 μg/ml achieved a 25% inhibition of the deoxyribonuclease activity of DNAse B (4), a concentration of 200 μg/ml was required to achieve a comparable inhibition of the deoxyribonuclease activity of DNAse D. Since DNAse B depolymerizes bacterial RNA more rapidly than DNAse D, the ability of relatively small amounts of inhibitor to influence markedly the behavior of the B enzyme cannot be explained on this basis. Although unexplained, this quantitative difference in the ability of bacterial ribonucleic acid to inhibit the deoxyribonuclease activity of DNAse B and DNAse D reflects one of the most striking and distinguishing features in the behavior of these two nucleases.

The requirement of divalent cations for activity and the degradation of the native DNA at faster rates than heat-denatured DNA have been demonstrated for DNAse D as well as the other streptococcal nucleases. These observations are in agreement with the previous publication of Winter and Bernheimer (1) and preliminary reports from our laboratory (2). The former workers reported potentiation of activity of all four enzymes in the presence of both magnesium and calcium ions.

The pH optimum for DNAse D extending from 6.5 to 9.5 as reported here and previously (3) from our laboratory is somewhat broader than that observed by Winter and Bernheimer (1) who found an optimal pH of 6 to 7 for this enzyme. Both laboratories have agreed in previous reports (9) that the end products of deoxyribonuclease digestion by all four enzymes are terminated in 5′-phosphate. Winter and Bernheimer (1964) have further recorded that DNAse D acts preferentially on pXpA linkages. Observations in our laboratory[1] on

[1] Gray, E. D. Unpublished observations.

dinucleotides of a D limit digest indicate a preference for pTpG bond cleavage.

That DNAse D is immunologically distinct from the other three streptococcal nucleases seems clear from the evidence obtained here in neutralization studies with antisera produced in experimental animals. This suggests that specific neutralizing antibodies for DNAse D, like those for the other streptococcal nucleases, may be found in human sera. Although extensive studies have not yet been done, the relative infrequency with which this enzyme has been identified in association with Group A strains would suggest that neutralizing antibodies may develop uncommonly after natural streptococcal infection in man.

SUMMARY

Preparations of streptococcal DNAse D with high specific activity and free of other streptococcal nucleases have been obtained by zone electrophoresis and column chromatography. Antisera prepared by injecting rabbits with such preparations specifically neutralize the activity of this enzyme.

As with DNAse B, preparations of DNAse D regularly exhibit ribonuclease activity. For both B and D enzymes, the order of substrate preference is thymus DNA, yeast RNA, bacterial RNA; but the specific activity of the D enzyme is higher than that of the B enzyme with respect to thymus DNA and lower with respect to bacterial RNA.

Both the deoxyribonuclease and the ribonuclease activities exhibited by preparations of both enzymes are inhibited by bacterial RNA, but approximately 100-fold greater concentrations of bacterial RNA are required to achieve inhibition of the deoxyribonuclease activity of the D enzyme equivalent to the inhibition of the B enzyme. The deoxyribonuclease activity of the D enzyme is also inhibited by yeast RNA, but even larger amounts are required.

These observations indicate that the D enzyme is immunologically distinct from the other streptococcal nucleases and that it differs quantitatively from the B enzyme with respect to relative specific activities on different substrates and behavior in the presence of the bacterial ribonucleic acid inhibitor.

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