THE OCCURRENCE OF NUCLEASES IN CULTURE FILTRATES OF GROUP A HEMOLYTIC STREPTOCOCCI

By MACLYN McCARTY, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

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The enzymes which are released into the environment by pathogenic microorganisms are of importance in a consideration of the mechanism of disease processes, since they may play a rôle in the virulence and invasiveness of the bacteria as well as in specific tissue injuries. In this connection, it is of interest that certain of the bacterial exotoxins have recently been demonstrated to be enzymatic in character. In the case of group A hemolytic streptococci, a number of extracellular products possessing a wide variety of biological activities have been recognized. Some of these substances, for example, streptolysin O, streptokinase, and streptococcal proteinase, have been subjected to intensive study, and a considerable body of information is available concerning their chemical and biological properties and their possible relationship to the pathogenesis of streptococcal disease. Because of the value of this type of study and the probability that other hitherto unrecognized substances accumulate in the environment of growing streptococci, the enzymatic activities of culture supernates of these organisms were investigated further. In the course of these studies, it was found that both ribonuclease and desoxyribonuclease occur with great regularity in culture media in which group A hemolytic streptococci are grown. The present paper deals with the extracellular occurrence and enzymatic activity of the two nucleases released from streptococcal cells during growth in fluid media.

Materials and Methods

Strains of Group A Hemolytic Streptococci.—Thirty-six different strains of group A hemolytic streptococci were studied for their ability to form nucleases. Sixteen serological types were represented and strains were included with special biological characteristics such as formation of large amounts of streptokinase or streptococcal proteinase, formation of hyaluronidase, failure to form erythrogenic toxin or streptolysin S, and requirement of the purine growth factor described by Wilson (1). A summary of the strains employed is given in Table I. The strains are designated by the numbers used in this laboratory, and the serological type is indicated in parentheses.

Bacteriological Media.—Three different media were employed: (*a*) Todd-Hewitt buffered broth (2), (*b*) neopeptone dialysate broth prepared according to the method of Dole (3), and (*c*) the partially defined medium developed by Adams and Roe for growth of pneumococci (4).

Preparation of Cultures.—16 to 18 hour cultures were used for the most part. The bacterial cells were removed by centrifugation, and in many experiments the supernates were tested without sterilization. While this procedure required that certain precautions be observed in

the handling and disposal of the material, it proved simpler than attempting to sterilize by filtration as a routine. However, it was found that the culture supernates could be passed through Coors P3 filters without loss of enzymatic activity, and this method was used to obtain bacteria-free filtrates.

Measurement of Ribonuclease.—A turbidimetric method was employed as the routine procedure for measuring ribonuclease activity, since the other methods available are not readily adaptable to use with nutrient broth. The method is based on the fact that under the

TABLE I

Strains of Group A Streptococci Tested for Production of Ribonuclease and Desoxyribonuclease

Special biological characteristics	Strain designation and serological type
Produces high yield of streptokinase	H105 (Tillett's "Co"; undesignated new type)
Produces high yield of streptococcal proteinase	B220 (Elliott's strain 5797; type 8 T antigen, no M antigen identified)
Produce extracellular hyaluronidase	C748 (type 4); B247 (type 22)
Stock strain for production of streptolysin O	D58 (Colebrook's strain "Richards," type 3)
Produce no streptolysin S	C439, C440, C441 (Colebrook's strains, all type 12)
Widely used for production of erythrogenic toxin	NY5 (type 12*)
Produces no erythrogenic toxin	C998 (Todd's "Cooper 3122," type 3)
Require Wilson's purine growth factor	C811, C812, C813, C272 (all type 19); C660 (type 19, no T antigen)
Do not require Wilson's purine growth factor	C817, C820, S24 (all type 19); C655 (type 19, no T antigen)
Recently isolated from scarlet fever patients	1GL19 (type 3); 1GL21 (type 17); 1GL4 (type 19); 1GL49 (type 30); 1GL22 (type 30)
Stock strains of various serological types	Tl (type 1); C203 (type 3, types 1 and 3 T antigens); S43 (type 6); S23 (type 14); J17E (type 17); J17F (type 26); T28 (type 28); D23 (type 29); D24 (type 30); London (undesignated new type)

* Originally classified as type 10 on the basis of typing by agglutination.

conditions employed the end-products of the reaction are soluble in acid, in contrast to the insolubility of the undigested nucleic acid. Ribonucleic acid from yeast was purified by the method of Kunitz (5). A stock solution containing 5 mg. per cc. was prepared by adding to an aqueous suspension of the nucleic acid just enough 1 N NaOH to cause complete solution. A solution so prepared has a pH of approximately 5.0 and is quite stable at refrigerator temperatures. A fivefold dilution of the stock solution in 0.025 M veronal buffer pH 7.5 is used as substrate for the test. The substrate solution is mixed with an equal volume of culture supernate and incubated in a water bath at 37°C. At intervals, 1.0 cc. of the reaction mixture is removed, mixed with 1.0 cc. of 1 N HCl, and the optical density of the resulting precipitate determined in a Coleman junior spectrophotometer at wave length 425 mμ. Turbidity controls for each of the reagents are employed. At appropriate enzyme concentrations the decrease in turbidity is linear with time over a period of 20 minutes. Contrary to expectation

on the basis of published data concerning the instability of yeast nucleic acid at alkaline pH, the substrate shows no spontaneous loss of acid precipitability in the absence of enzyme. Even after several hours at 37°C. and pH 7.5, the optical density after addition of HCl is identical with that of freshly prepared solutions.

Measurement of Desoxyribonuclease.—For routine testing of desoxyribonuclease a turbidimetric method identical with the ribonuclease method was used, except that the substrate solution was a 0.1 per cent solution of sodium desoxyribonucleate from calf thymus in 0.025 M veronal buffer pH 7.5 containing 0.01 M $MgSO_4$. In general, because of the higher activity of this enzyme, it was necessary to use dilutions of the culture filtrate to obtain linear rates of decrease in turbidity. Control tests of undigested substrate frequently give fibrous precipitates on the addition of acid which interfere with turbidimetric readings, but the results of enzymatic tests are unequivocal.

The turbidimetric method was supplemented by the more quantitative viscosimetric method previously described for estimating desoxyribonuclease activity (6). By this means, it was possible to make quite accurate comparisons of the potency of streptococcal nuclease with nuclease from other sources, *e.g.*, beef pancreas.

EXPERIMENTAL

Nuclease Activity of Culture Supernates.—All thirty-six strains studied produced both ribonuclease and desoxyribonuclease, and no relation was detected to the other biological characteristics of the strains. Although there were quantitative differences in the amount of the two enzymes elaborated by different strains, the variations were not great and were not studied in detail. Supernates of cultures in neopeptone dialysate broth uniformly showed higher activity than those in Todd-Hewitt broth. This difference is not referable to greater growth of the organisms in dialysate broth, but is due, in part at least, to the presence of inhibitory substances in the Todd-Hewitt broth.

Ribonuclease activity was relatively low and dilution of the culture supernates was not necessary. The results of a test of a typical culture supernate are presented graphically in Fig. 1. The effect is comparable to that obtained with 0.01 μg. of crystalline ribonuclease from beef pancreas as tested by the same method. In comparison with ribonuclease, desoxyribonuclease activity was surprisingly high. The data plotted in Fig. 1 show that a fiftyfold dilution of the culture supernate was required to give a decrease in acid precipitability comparable to that obtained with ribonucleic acid and undiluted culture supernate. The discrepancy in the relative activity of the two enzymes is probably even greater than that indicated, since the desoxyribonucleic acid has a much higher molecular size than the ribonucleic acid and presumably more enzymatic action is required to reduce the former to acid-soluble products. Evidence for this view was obtained in an experiment in which partial breakdown products of desoxyribonucleic acid, more nearly comparable in molecular size to the ribonucleic acid, were used as substrate in the turbidimetric test. At the same enzyme concentration, the rate of decrease in acid precipitability of the degraded substrate was four times as rapid as that of the intact nucleic acid.

The results of determination of desoxyribonuclease activity by the more

sensitive viscosimetric method confirm the findings of the turbidimetric test. With the same culture supernate as that employed in the test recorded in Fig. 1,

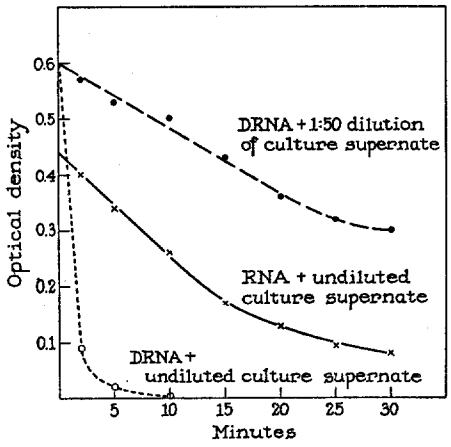

FIG. 1. Ribonuclease and desoxyribonuclease activity of culture supernate of strain C817 grown in neopeptone dialysate broth. DRNA indicates desoxyribonucleic acid, and RNA ribonucleic acid.

TABLE II

Relative Desoxyribonuclease Activity of Various Source Materials

Material	Units per cc.
Beef pancreas—0.25 N H_2SO_4 or aqueous extract*	3300–3500
Swine pancreas—aqueous extract*	1000–2000
Group A hemolytic streptococci—culture supernate	200–400
Type IIR pneumococcus—culture supernate	1–2
Beef spleen—aqueous extract*	<1
Calf thymus—aqueous extract*	<1
Escherichia coli‡—culture supernate	0
Bacillus subtilis‡—culture supernate	0

* Tissue extracted with 2 to 3 volumes of solvent.

‡ One strain only tested.

0.0025 cc. (0.5 cc. of a 1:200 dilution) contains one unit of enzyme in the terms defined for use with the pancreatic nuclease (6). Thus, the unconcentrated culture supernate contains 400 units per cc., and a final dilution in the viscosimeter of 1:2000 causes a rapid fall in viscosity. With the exception of pancreatic extracts, streptococcal cultures have proved to be the most potent crude preparations of desoxyribonuclease of the various tissue extracts and bacteriological preparations tested in this laboratory. A comparison of the desoxyribonuclease activity of representative source materials is given in Table II.

It will be seen that the activity of the initial extracts of beef pancreas is about eightfold greater than that of streptococcal cultures. On the other hand, extracts of organs such as thymus and spleen have relatively little activity and the cultures of certain other species of microorganisms appear to be wholly devoid of desoxyribonuclease.

Rate of Production of Desoxyribonuclease.—Desoxyribonuclease is released into the medium by streptococci early in the active growth phase. This is demonstrated in Fig. 2, which records the results of a series of quantitative viscosi-

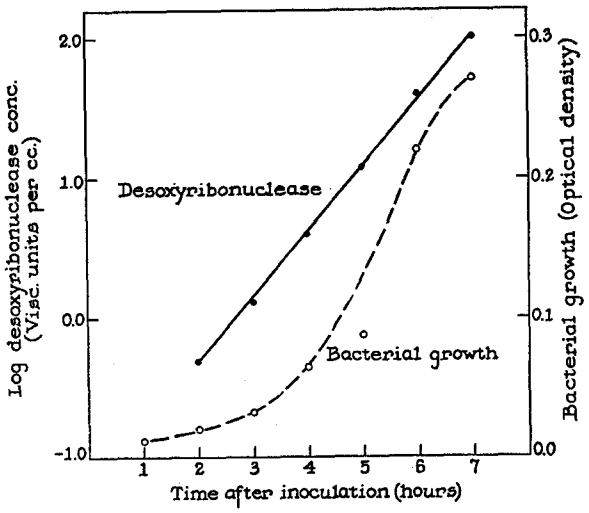

FIG. 2. Rate of increase in concentration of desoxyribonuclease in culture supernate during active growth of group A hemolytic streptococcus (strain H105).

metric determinations of desoxyribonuclease on samples of the supernate of the same culture at intervals after inoculation. A rather large inoculum of washed young cells (2×10^5 organisms per cc.) was used in order to avoid a long lag period. Desoxyribonuclease was detectable in the culture supernate at one hour and was present in accurately measurable amounts at 2 hours after inoculation. Bacterial growth was estimated turbidimetrically. As shown in Fig. 2, the concentration of enzyme increased logarithmically throughout the logarithmic growth phase of the organisms. It appears relatively certain, therefore, that the presence of the enzyme in the culture medium is not merely the result of the degeneration of aging cells.

Effect of Substrate on Nuclease Production.—An attempt was made to determine whether the presence of nucleic acids or enzymatic split products of nucleic acid influences the quantity of nuclease produced during growth. For this purpose it was necessary to use the partially defined medium of Adams and

Roe, since the more complex media contain unknown amounts of nucleotides. Most strains of group A hemolytic streptococci will not grow on repeated transfer in the Adams-Roe medium, but a few strains will give good growth provided moderately large inocula are used. A study of the growth of one strain (D24) by serial transfers in this medium revealed that both ribonuclease and desoxyribonuclease appear in the cultures, although in consistently smaller amounts than in the dialysate broth cultures.

The following substances were incorporated in the Adams-Roe medium at a final concentration of 0.1 to 1.0 mg. per cc.: desoxyribonucleic acid from calf thymus, ribonucleic acid from yeast, enzymatic split products of desoxyribonucleic acid prepared with pancreatic desoxyribonuclease, and enzymatic split products of ribonucleic acid prepared with crystalline ribonuclease. Strain D24 was grown in the presence of these various added substances, and in no case was there any measurable increase in nuclease content of the cultures as compared with control cultures in plain Adams-Roe medium. Furthermore, desoxyribonuclease production was not enhanced by the addition of large amounts of sodium desoxyribonucleate (up to 1 gm. per 100 cc. of culture) to cultures of streptococci in which heavy growth was obtained by continued neutralization of the acid formed in the presence of excess glucose. These experiments suggest that the nucleases, in contrast to streptococcal hyaluronidase (7), are not "adaptive" in character.

Relationship of Nuclease Production to Requirement for a Purine Growth Factor.—Wilson (1) has shown that certain strains of group A streptococci when inoculated into Adams-Roe medium supplemented with horse serum require the addition of a purine factor for growth. Viscous desoxyribonucleic acid did not serve as a source of the growth factor, although it was rendered active by digestion with pancreatic desoxyribonuclease. In a previous paper this finding was interpreted as indicating that these strains of group A streptococci lack the capacity to form an enzyme analogous to pancreatic desoxyribonuclease (6). However, the results of the present studies are not compatible with this interpretation, since several of the strains employed by Wilson have been tested and found not to differ from other strains with respect to desoxyribonuclease formation. Furthermore, a partially purified preparation of the enzyme was prepared from one of Wilson's strains (2884F, designated C811 in Table I) and was shown to be capable of converting calf thymus desoxyribonucleic acid into an active source of the growth factor, just as does pancreatic desoxyribonuclease. It is apparent, therefore, that the failure of the organisms to grow in the horse serum medium with added desoxyribonucleic acid is not referable to an inherent inability to form desoxyribonuclease. However, with the small inocula used (10^{-5} cc.), growth is probably not initiated in the absence of the purine factor and thus no enzyme is formed to convert the desoxyribonucleic acid into an available source of the factor.

DISCUSSION

The data recorded in the present paper indicate that both ribonuclease and desoxyribonuclease are produced by a wide variety of strains of group A streptococci during growth in fluid media. In relative terms, a large amount of desoxyribonuclease is formed; for example, 1 cc. of unconcentrated culture supernate is sufficient to cause depolymerization of several grams of desoxyribonucleic acid. Nothing is known at present concerning the possible rôle of the nucleases in the multiplication of streptococci in host tissues, and it has not been determined whether antibodies which inhibit the enzymes are formed following streptococcal infections. Because of the nature of their action, it seems unlikely that either of the nucleases is capable of damaging living cells, and their action *in vivo* is probably limited to the breakdown of nucleic acids released by tissue cells that have been destroyed by other agents.

The occurrence of the nucleases in culture supernates is not only of theoretical interest but has been shown to be of practical importance in the isolation and purification of other extracellular products, such as streptokinase and proteinase, since the nucleases must be separated from them in the course of purification procedures. Measurement of nuclease activity provides a delicate and simple test for the efficacy of the fractionation procedures employed. Furthermore, since streptococcal desoxyribonuclease is present in relatively large amounts, its preparation in purified form is feasible. In preliminary experiments fractions have been obtained which have a higher specific activity than pancreatic desoxyribonuclease made by the method previously described (6). These streptococcal desoxyribonuclease preparations have the additional advantage of being free of proteolytic activity.

The several enzymes which have been described as being released or "secreted" into the environment by group A hemolytic streptococci have in common the property of attacking substrates of large molecular size. Thus, streptokinase, streptococcal proteinase, hyaluronidase, ribonuclease, and desoxyribonuclease are involved directly or indirectly in the degradation of macromolecules. From one point of view, these enzymes may be interpreted as serving as a "digestive" function; that is, of preparing potential nutrient material so that it can be assimilated by the microorganism. Wilson's studies on the purine growth factor are in accord with this interpretation, since it seems apparent that the action of desoxyribonuclease on desoxyribonucleic acid results in the formation of certain products which the streptococcal cell is able to utilize. In connection with the possible "digestive" nature of these enzymes, it is of interest to compare the known enzymes of streptococcal supernates with those of mammalian pancreatic secretion. A single streptococcal culture supernate can contain a proteinase, a carbohydrase, and the two nucleases, enzymes which are representative of some of the main components of the pancreatic secretion. The analogy can be pursued further, since streptococcal proteinase,

like the pancreatic proteinases, has been shown to be released in the form of an inactive precursor (8).

SUMMARY

1. All of the thirty-six strains of group A hemolytic streptococci tested were found to elaborate ribonuclease and desoxyribonuclease during growth in liquid cultures. Both enzymes are released into the medium.

2. Desoxyribonuclease is consistently produced in greater amount than ribonuclease. The concentration of desoxyribonuclease in the culture increases logarithmically during active growth of the organisms.

3. Under the conditions employed, the presence of specific substrate or enzymatic split products of the substrate did not influence the production of either nuclease.

4. The failure of viscous desoxyribonucleic acid to serve as a source of the purine growth factor required by certain strains of group A streptococci was shown not to be referable to the inability of these strains to form desoxyribonuclease.

5. The determination of nuclease activity provides another criterion for evaluating purification procedures used in the attempted isolation of substances such as streptokinase and proteinase from the supernates of streptococcal cultures.

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