ELABORATION OF DESOXYRIBONUCLEASE BY STREPTOCOCCI IN THE RESTING STATE AND INHIBITION OF THE ENZYME BY A SUBSTANCE EXTRACTABLE FROM THE COCCI*

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Although many bacteria produce extracellular enzymes during growth, there appear to be no instances reported in which bacteria in the resting state have been demonstrated to form such enzymes. This communication records observations showing that desoxyribonuclease is produced by washed streptococcal cells suspended in a medium in which proliferation does not occur. Early in the course of the study, it was suspected that desoxyribonuclease was being synthesized in the resting cell system, and that its appearance was not due to release of preformed enzyme. The principal object of the investigation was to explore this possibility and to find the simplest conditions under which enzyme production occurs. In addition, experiments were carried out showing that desoxyribonuclease elaborated by group A streptococci is specifically inhibited by ribonucleic acid extractable from these and other streptococci.

Methods

Strains.—A variety of strains was employed. When no designation is given, the C203S strain of *Streptococcus pyogenes* (group A) was used.

Preparation of Cultures and of Resting Cocci.—One hundred cc. of infusion broth, prepared as described in an earlier publication (1), and containing 0.01 per cent freshly neutralized thioglycollic acid, was inoculated with 0.1 cc. broth culture and incubated at 37°. After 15 to 17 hours, the culture was centrifuged, and the supernate set aside. Resting[1] cell suspensions were prepared by washing the cocci in 50 cc. phosphate-buffered saline (M/15 phosphate at pH 7.0 and M/12.9 NaCl) and dispersing them in 7 cc. of suspending solution. In some experiments, one-tenth these volumes was employed.

Bacterial Growth.—Bacterial growth was expressed as optical density, measured at 650 mμ in a Beckman DU spectrophotometer carrying 10 mm. cells. Cultures were read against culture supernate as a blank.

Estimation of Desoxyribonuclease.—Desoxyribonucleic acid was prepared from calf thymus

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[1] The term "resting" is here used to denote viable cocci which are not undergoing proliferation.

by Mirsky and Pollister's method (2), slightly modified. Desoxyribonuclease was estimated by measuring the capacity of solutions of enzyme to decrease the viscosity of a solution of the substrate. McCarty's method (3) of estimating the enzyme was followed except that the volumes employed were reduced to one-half in order to save materials. The unit of desoxyribonuclease is the same as that defined by McCarty.

Desoxyribonuclease Diluent.—Dilutions of desoxyribonuclease and, in most instances, of inhibitor were prepared in a solution which contained 0.25 per cent gelatin, 0.075 M $MgSO_4$, and 0.025 M veronal buffer, pH 7.5 (McCarty (3)).

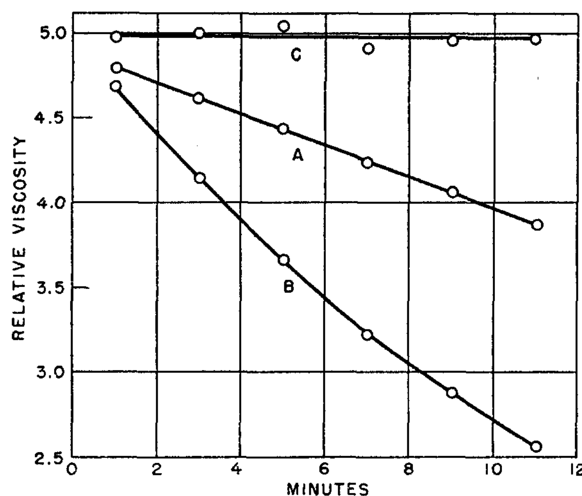

FIG. 1. Partial inhibition of desoxyribonuclease by extract of streptococci.

A. 2.4 cc. substrate solution + 0.1 cc. solution A. Solution A: 0.2 cc. desoxyribonuclease from strain C203S (30 units) + 0.2 cc. desoxyribonuclease diluent + 0.2 cc. supernate from sonically treated C203S.

B. 2.4 cc. substrate solution + 0.1 cc. solution B. Solution B: 0.2 cc. desoxyribonuclease from strain C203S (30 units) + 0.4 cc. desoxyribonuclease diluent.

C. 2.4 cc. substrate solution + 0.1 cc. desoxyribonuclease diluent.

Preparation of Inhibitor Solutions.—Two methods were employed: (*a*) The washed bacteria from 500 cc. of broth culture were suspended in 15 cc. of saline-phosphate, pH 6.9, and exposed for 30 minutes to the vibration of a 9000 cycle oscillator (Raytheon Corporation, Waltham, Massachusetts). The mixture was centrifuged, and the supernatant fluid served as a crude inhibitor solution. (*b*) A second method of preparing inhibitor solutions involved grinding for 24 hours 1 gm. of lyophilized or acetone-ether-dried cocci in a mill (4) of quarter-inch chrome steel balls. After the contents of the mill were washed out with portions of cold water totalling 100 cc., the pooled washings were centrifuged, and the supernate employed as inhibitor solution.

Estimation of Desoxyribonuclease Inhibition.—Two-tenth cc. of a desoxyribonuclease solution diluted to contain approximately 30 units (150 units per cc.) was mixed with 0.2 cc. of desoxyribonuclease diluent and 0.2 cc. of solution to be tested for inhibitory activity. The desoxyribonuclease activity of the mixture was then measured by mixing 0.1 cc. with 2.4 cc. of substrate solution and determining the viscosity as a function of time. A control solution

containing no inhibitor was always assayed at the same time. The results of a determination are illustrated in Fig. 1. Inhibition can be expressed as 1 minus the ratio of the initial rates of fall in viscosity of desoxyribonucleate in the presence (curve A) and absence (curve B) of inhibitor solution. In practice, however, initial rates of fall of viscosity were found to be a linear function of enzyme concentration only over a relatively narrow range of dilutions, and for this reason, enzyme activities of solutions A and B were always measured by using dilutions which produced a fall of 0.3 to 0.9 relative viscosity units in 10 minutes. Per cent inhibition was then computed by multiplying by 100 the ratio of enzyme activities in the presence and absence of inhibitor solution, and subtracting this figure from 100. All solutions except that of the substrate were kept in a bath of ice water prior to assay. The substrate solution was placed at 30° for at least 5 minutes before mixing with test solutions.

EXPERIMENTAL

Part I

Capacity of Various Strains of Streptococci to Produce Desoxyribonuclease.—McCarty (5) studied 36 strains of streptococci belonging to group A, and reported that all of them produced desoxyribonuclease. Of 8 group A strains studied by Tillett, Sherry, and Christensen (6) all were found to be positive for desoxyribonuclease. The latter authors found one group B strain and one group C strain positive, and two strains, one of group C and one of group E, negative. Brown (7) recently reported the results of a survey of 267 streptococcal cultures. All group A strains, about a quarter of the group B strains, and all but one group C strain were positive. In addition, some strains belonging to groups F, G, and L produced desoxyribonuclease. In the present study, a series of 34 strains of diverse origin, and belonging to various groups, were examined quantitatively for their capacity to produce desoxyribonuclease in growing cultures as well as in the resting state.

The washed cocci from 10 cc. of 17 hour broth cultures were suspended in 0.7 cc. of the following solution: 2 cc. M/40 maltose, 1 cc. 2 per cent KH_2PO_4 previously brought to pH 7 with NaOH, 2 cc. 0.2 per cent $MgSO_4 \cdot 7H_2O$, and 2 cc. water. After 3 hours at 37°, the suspensions were chilled and centrifuged. The desoxyribonuclease of the resting cell supernates as well as of the supernates of the original broth cultures was measured with the results shown in Table I.

The results show that, *in broth cultures*, all the strains of Lancefield group A produced some desoxyribonuclease, although the quantity formed varied considerably from strain to strain. Of the 4 group B strains, 2 produced small but significant amounts of enzyme while 2 produced none. Of the 8 group C strains, all but 3 yielded 10 or more units per cc. of culture. Among the 15 strains belonging to groups other than A, B, and C, only the group D strain, C3, produced more than traces of enzyme. The results indicate that strains elaborating large amounts of desoxyribonuclease may not be commonly encountered in groups other than A and C.

Of the same 34 strains examined for their capacity to elaborate desoxyribo-

TABLE I

Capacity of Various Streptococcal Strains to Produce Desoxyribonuclease in Cultures and in the Resting State

Strain designation*	Lancefield group	Cell mass as optical density	Desoxyribonuclease units per cc. of broth supernate	Desoxyribonuclease units per cc. of supernate of resting cell suspension
C203S	A	0.096	535	78
C203U	A	0.079	380	94
Blackmore	A	0.109	81	10
Witcher	A	0.097	71	0
1685M	A	0.065	12	0
Wilson	A	0.077	166	1
30M	A	0.092	352	48
V9	B	0.135	0	0
C245	B	0.126	0	0
H47C	B	0.145	8	2
D136A	B	0.143	12	0
K64	C	0.111	3	0
J148C	C	0.119	7	0
Angel 21	C	0.151	10	0
H46A	C	0.182	64	0
D181	C	0.150	41	1
GP11	C	0.132	0	3
Type 7	C	0.137	24	0
Type 20	C	0.161	19	0
C3	D	0.092	90	3
C1	D	0.086	0	0
D76	D	0.121	0	0
K131	E	0.143	0	0
K129	E	0.125	4	0
H127	F	0.088	0	0
C468	F	0.129	0	0
F68A	G	0.098	0	0
D166B	G	0.136	0	0
K208H	H	0.121	0	0
F90A	H	0.111	0	0
D167B	L	0.127	2	0
D167A	L	0.116	2	0
D168E‡	M	0.044	0	0
D168A‡	M	0.122	0	0

* Most of the strains were kindly supplied by Dr. Rebecca C. Lancefield.

‡ Cultures incubated 41 hours.

nuclease *in the resting state*, only 4 yielded 10 or more units of enzyme per cc. It is notable that all 4 belonged to group A. In addition, it is evident (*a*) that strains yielding desoxyribonuclease in cultures produced considerably less

enzyme per unit of cocci in the resting cell system, and (*b*) that 4 of the strains yielding appreciable amounts of enzyme in cultures produced little or none in the resting cell system. It is possible that these 4 strains are capable of yielding enzyme in the resting state and that they would do so if other conditions were used.

Capacity of Resting Cocci to Produce Desoxyribonuclease as a Function of Age of Culture from Which the Cocci Are Derived.—A considerable degree of variation in the quantity of enzyme formed by washed cocci (C203S) was observed from day to day. This variation is due to differences between successive lots of broth, to differences in rate of growth, and possibly to other factors. The

TABLE II

Capacity of Washed Cocci (C203S) to Produce Desoxyribonuclease as a Function of Age of Culture

Age of culture	Cell mass as optical density	Desoxyribonuclease units per cc. of supernate of resting cell suspension
hrs.		
13	0.11	156
14	0.11	198
15	0.13	207
16	0.13	237
17	0.12	170
18	0.13	160
19	0.13	132
20	0.13	99
21	0.12	83
22	0.13	76

Resting cell suspensions incubated 45 minutes at 37°. Conditions otherwise the same as in preceding experiment.

dependence of enzyme formation on age of the culture from which the cocci are derived is illustrated by the data of Table II. It can be seen that even though the growth was at or near a maximum in 13 hours, the cocci were not in an optimal state for desoxyribonuclease production until the 16th hour. Beyond the 16th hour, the capacity of cocci to yield the enzyme decreased markedly.

Substances Necessary for the Production of Desoxyribonuclease.—The simplest suspending solution in which desoxyribonuclease appeared was one containing an energy source, for which either glucose or maltose served, magnesium and phosphate ions. The requirement for these factors is demonstrated in experiments in which washed cocci were suspended in a solution of maltose, magnesium sulfate, and potassium phosphate, pH 7, and in solutions deficient in each of these components. The appearance of desoxyribonuclease at 37° was

followed by removing after various intervals of time 0.5 cc. aliquots of the resting cell suspensions, adding 0.05 cc. M/1000 mercuric chloride to stop metabolic processes, chilling, centrifuging, and estimating desoxyribonuclease in the supernates. The results (Table III) show that no enzyme appeared unless all three components of the suspending solution were present. It was further observed that potassium could be replaced by sodium without change in the amount of enzyme formed.

The addition to the resting cell medium of a considerable number of substances including various amino acids and vitamins did not alter greatly the

TABLE III

Requirement (Strain C203S) for Energy Source, Magnesium and Phosphate Ions

	Composition of suspending solution					
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
M/40 maltose	2	2	2	0	2	2
0.2 per cent $MgSO_4 \cdot 7H_2O$	2	2	2	2	0	2
2 per cent KH_2PO_4, pH 7.0	1	1	1	1	1	0
H_2O	2	2	2	4	4	3
Length of time at 37°	Desoxyribonuclease units per cc. of supernate					
	a	*b*	*c*			
min.						
5	14	11	17	0	0	0
15	17	23	26	0	0	—
20	25	38	25	0	0	1
30	28	44	29	0	0	0
45	30	49	52	—	0	—
60	36	53	65	0	0	0

Columns *a*, *b*, and *c* indicate results obtained on different days using the complete suspending medium.

—, not measured.

amount of desoxyribonuclease which appeared. Likewise inclusion of desoxyribonucleate, either in the resting cell medium or in the broth used for growth of the cocci, failed to increase the amount of enzyme elaborated, indicating that the formation of streptococcal desoxyribonuclease is not an adaptive process. A similar conclusion was reached by McCarty (5).

Estimation of Preformed Desoxyribonuclease in Washed Cocci.—The foregoing results show that under the conditions used, desoxyribonuclease appears in the supernate of the resting cell system. They do not indicate, however, whether the desoxyribonuclease found in the supernate is actually synthesized in the resting cell system or whether it is synthesized by the cocci during the time they are growing in broth and subsequently released into the suspending

solution of the resting cell system. If the enzyme is synthesized and retained during growth, it should be possible to demonstrate its presence in the cocci.

The cocci (approximately 20 mg. dry weight) from 500 cc. of broth culture were centrifuged, washed in 250 cc. saline-phosphate, and suspended in 30 cc. saline-phosphate. The suspended cocci were exposed for 30 minutes to the vibration of a 9000 cycle oscillator and, after centrifugation, the supernate was assayed for desoxyribonuclease.

As shown in the upper portion of Table IV, no enzyme was found, and when the experiment was repeated in the presence of 0.1 per cent bovine serum albumin, no more than a trace of desoxyribonuclease was obtained. Similar results were obtained when very thick suspensions of cocci were sonically

TABLE IV

Apparent Absence of Preformed Desoxyribonuclease in Streptococci, and Effect of Sonic Vibration on Solutions of the Enzyme

Material treated sonically		Desoxyribonuclease before sonic treatment	Desoxyribonuclease after sonic treatment	Recovery of added desoxyribonuclease
		units/cc.	*units/cc.*	*per cent*
30 cc. cocci from 500 cc. of culture, in buffered saline	Trial I	—	0	—
" " " " " " " " " " "	" II	—	0	—
" " " " " " " " " " "	" III	—	0	—
" " " " " " " " " " " *	" I	—	1	—
" " " " " " " " " " " *	" II	—	0	—
30 cc. desoxyribonuclease, C203S (group A), in resting cell medium		146	150	103
Same plus cocci from 500 cc. of culture	Trial I	92	37	40
" " " " " " " "	" II	150	41	27
" " " " " " " " *	" I	108	53	49
" " " " " " " " *	" II	106	62	59
30 cc. desoxyribonuclease, H46A (group C), in resting cell medium*		113	121	107
Same plus cocci from 500 cc. of culture*		113	120	106

* Contained 0.1 per cent serum albumin.

disrupted. Further experiments designed to reveal the effect of sonic treatment on preformed desoxyribonuclease (Table IV) showed that the enzyme is stable to sonic treatment in the absence of cocci. When cocci were present, however, partial inactivation occurred. The results suggest, but do not prove, that appreciable amounts of preformed desoxyribonuclease are not present in the cocci.

Demonstration of a Desoxyribonuclease Inhibitor in Cocci.—The fact that desoxyribonuclease is stable to sonic treatment in the absence of cocci, but shows partial loss of activity in their presence, can be explained by assuming that disruption of the cocci liberates a substance which partially inactivates the enzyme. The correctness of this assumption is demonstrated by the following experiment:

The washed cocci derived from 500 cc. of broth culture were suspended in 35 cc. of saline-phosphate and then treated sonically for 30 minutes. After centrifugation, the supernate was removed and designated "sonic extract." The insoluble sediment was suspended in 35 cc. of saline and designated "sonic residue." Inhibitory activity was estimated by mixing 0.2 cc. of solution to be tested with 0.4 cc. of desoxyribonuclease and measuring viscosimetrically the enzyme activity of the resulting mixture. The desoxyribonuclease was prepared from strain C203S and previously diluted in gelatin-veronal diluent (3) to contain approximately 30 units per cc.

The results presented in Table V show that sonic treatment of cocci liberates a soluble substance which partially inhibits the desoxyribonuclease produced by strain C203S. The degree of inhibition observed in the course of this and

TABLE V

Inhibitory Effect of Sonic Extract of Streptococci (C203S) on Desoxyribonuclease of Strain C203S (Group A) and Lack of Inhibitory Effect of the Same Extract on Desoxyribonuclease of Strain H46A (Group C)

0.4 cc. desoxyribonuclease derived from:	Desoxyribo-nuclease
	units/cc.
Group A (C203S) plus 0.2 cc. saline-phosphate	54
" " " " " sonic extract group A (C203S)	23
" " " " " " residue " " "	51
Group C (H46A) plus 0.2 cc. saline-phosphate	50
" " " " " sonic extract group A (C203S)	56

many other experiments was always approximately 50 per cent. In addition, it is of interest to note that the desoxyribonuclease derived from H46A, a group C strain, tested under the same conditions, was not inhibited by the sonic extract of C203S, a group A strain.

The results just described are consistent with the observation that approximately half of the added desoxyribonuclease was recovered when preformed enzyme was sonically treated in the presence of cocci (Table IV). If the 50 per cent recovery were due to inactivation of half the added enzyme by inhibitor, it follows that substitution of desoxyribonuclease derived from strain H46A for C203S-desoxyribonuclease should yield complete recovery after sonic treatment. The results presented in the last two lines of Table IV show that this is the case.

Effect of Enzyme Poisons on Desoxyribonuclease Formation.—In order to obtain some insight into the metabolic processes involved in enzyme production, the effects of various poisons on the resting cell system were studied.

The substances to be tested were incorporated into resting cell suspensions similar to those previously described, and in most instances, twofold increments in concentration were used.

After incubating for 45 minutes at 37°, the supernates of the resting cell suspensions were assayed for desoxyribonuclease, and the difference in desoxyribonuclease activity in the presence and absence of poison was expressed as per cent inhibition.

As shown in Table VI, all the substances tested, with the exception of malonate and azide, inhibited desoxyribonuclease formation. The most potent poisons were the sulfhydryl inhibitors, mercuric ion and iodoacetate, which were effective in concentrations of 4×10^{-5} and 2.5×10^{-5} M, respectively. Dinitrophenol, selenite, arsenate, fluoride, cyanide, and arsenite all inhibited, the last only in very high concentration. None of these substances, in the concentrations tested, was found to inhibit preformed desoxyribonuclease, indicating that the poisons interfere with the formation or release of enzyme rather than with its assay.

TABLE VI

Effect of Enzyme Poisons on Elaboration of Desoxyribonuclease

	Molar concentration producing approximately 50 per cent inhibition of desoxyribonuclease formation
Sodium malonate	$>1.5 \times 10^{-1}$
Potassium cyanide	2×10^{-2}
Sodium fluoride	1×10^{-2}
" azide	$>1.5 \times 10^{-1}$
" arsenate	1×10^{-2}
" selenite	3×10^{-2}
Dinitrophenol	2×10^{-3}
Sodium iodoacetate	2.5×10^{-5}
" arsenite	1×10^{-1}
Mercuric chloride	4×10^{-5}

It is of some interest to compare the concentrations of poisons necessary to inhibit desoxyribonuclease formation with those found (8) necessary to inhibit formation of the toxin, streptolysin S, by resting cocci of the same streptococcal strain. Toxin formation was effectively inhibited by malonate and azide while desoxyribonuclease formation was not. Formation of both enzyme and toxin was inhibited by approximately the same concentration of iodoacetate, and the same was true for arsenate, fluoride, and cyanide. Inhibition of enzyme formation required 10 to 60 times as much mercuric chloride, dinitrophenol, or selenite as was necessary for inhibition of toxin formation. The most striking difference, however, was encountered in the effects of arsenite. The concentration necessary to inhibit desoxyribonuclease formation (1×10^{-1} M) was 3300 times as great as that necessary to prevent the formation of streptolysin S (3×10^{-5} M). Moreover, arsenite, in concentrations inhibitory to toxin formaion was found unexpectedly to potentiate desoxyribonuclease formation.

Part II

Experiments described in Part I show that the depolymerase activity of streptococcal desoxyribonuclease is partially inhibited by extracts prepared from sonically disrupted cocci. In view of the recent finding by Zamenhof and Chargaff (9) that yeast desoxyribonuclease is specifically inhibited by a protein extractable from yeast it was of interest to investigate further the streptococcal system, especially in regard to the nature and specificity of the inhibitory substance present in these organisms.

Specificity of Desoxyribonuclease Inhibition.—Sonic extracts (see Methods) of 9 strains of hemolytic streptococci were tested for inhibition of desoxyribonuclease prepared from 8 of these strains and from barley and pancreas as well. In addition, the sonic extracts alone were tested for desoxyribonuclease, and in each instance were found to be negative. The results of the 90 combinations of extracts and enzymes are presented in Table VII. They show that the desoxyribonuclease of three of the strains, all of them group A, was partially inhibited by extracts of group A streptococci and also by extracts of cocci belonging to groups B, C, and D. In contrast to this finding, the desoxyribonucleases of streptococci belonging to groups B and C were inhibited neither by extracts of the homologous strains nor by extracts of strains belonging to other groups. In no instance was pancreatic desoxyribonuclease inhibited by the bacterial extracts.

In regard to desoxyribonuclease prepared from barley, 7 of the 9 streptococcal extracts failed to produce a significant amount of inhibition, but two of the strains, H47C and C20, inhibited 32 and 24 per cent, respectively. The reason for the exceptional results obtained with extracts of these two strains is not apparent.

Beside the data presented in Table VII, an experiment carried out in collaboration with Dr. S. Zamenhof and Dr. E. Chargaff provided additional information concerning the specificity of the inhibition. It was found that yeast desoxyribonuclease inhibitor (prepared from yeast (9)) failed to affect streptococcal desoxyribonuclease, and conversely, streptococcal desoxyribonuclease inhibitor (C203S) failed to inhibit the yeast enzyme (9).

Nature of the Inhibitor Prepared from Streptococci (Strain C203S).—In no instance was the inhibition observed to be complete. It was found usually to be between 50 and 70 per cent, and even concentrated solutions did not produce more than 85 per cent inhibition. Combination of enzyme with inhibitor occurred within 2 or 3 minutes and the degree of inhibition was not increased by allowing mixtures to stand for longer times before estimating enzyme activity. Mixtures of enzyme and inhibitor allowed to stand in the cold exhibited the same amount of inhibition as at room temperature, indicating that the inhibitor probably is not an enzyme.

The inhibitor was found to be relatively thermostable as shown by the fact

that little or no loss of activity was caused by exposure to 55° for 30 minutes at pH values between 5.0 and 9.0. It was completely destroyed by acid (N/10 HCl at 55° for 30 minutes) and also by alkali (N/10 NaOH at 55° for 30 minutes). In additional experiments, the lability to alkali was seen to be especially pronounced: Most of the inhibitory activity disappeared in 5 minutes at room temperature in the presence of N NaOH. The inhibitor failed to dialyze through cellophane, and it was neither inactivated nor removed by shaking with $CHCl_3$.

TABLE VII

Inhibition Produced by Sonic Extracts of Various Streptococcal Strains When Tested against Desoxyribonuclease Derived from Streptococci of Groups A, B, and C and from Barley and Pancreas

Source of desoxyribonuclease	Source of inhibitor								
	A	A	A	B	B	C	C	C	D
	C203S	Wilson	30M	H47C	D138	H46A	C7	C20	C3
Strep. group A, C203S	++	+++	+++	++	++	+++	++	++	+++
" " " Wilson	+++	+++	+++	++	+	++	++	++	+++
" " " 30M	+	++	++	+	+	++	+	++	++
Strep. group B, H47C	–	–	–	–	–	–	–	–	–
" " " D138A	–	–	–	–	–	–	–	–	–
Strep. group C, H46A*	–	–	–	–	–	–	–	–	–
" " " C7	–	–	–	–	–	–	–	–	–
" " " C20	–	–	–	–	–	–	–	–	–
Barley‡	–	–	–	+	–	–	–	+	–
Pancreas§	–	–	–	–	–	–	–	–	–

– indicates –20 to +19 per cent inhibition.
+ indicates 20 to 39 per cent inhibition.
++ indicates 40 to 59 per cent inhibition.
+++ indicates 60 to 85 per cent inhibition.
* Partially purified preparation kindly supplied by Lederle.
‡ Prepared from commercial malt diastase according to Zamenhof and Chargaff (9).
§ Kindly supplied by Dr. M. McCarty.

By making use of these and other properties, the inhibitor could be partially purified.

The effect on the inhibitor of a series of enzymes was studied. In short, proteolytic enzymes such as trypsin, chymotrypsin, papain, and ficin failed to affect its activity. In contrast to these results, minute amounts of pancreatic ribonuclease were found to be very effective in destroying the inhibitor. As shown in Table VIII, ribonuclease, in a final concentration as low as 0.10 μg. per cc., brought about partial inactivation of the inhibitor, while higher concentrations caused essentially complete inactivation. The failure of proteolytic enzymes to produce a similar effect is illustrated in the table by data for chymo-

trypsin. In view of the effect of ribonuclease, the action of intestinal phosphatase on inhibitor was examined. A preparation (10) of this enzyme was also found to be capable of destroying the inhibitor.

Failure of Yeast Ribonucleic Acid to Inhibit Group A Desoxyribonuclease.—The foregoing results are interpreted as identifying the inhibitor as streptococcal

TABLE VIII

A. Failure of Chymotrypsin to Affect Desoxyribonuclease Inhibitor
B. Capacity of Pancreatic Ribonuclease to Destroy Desoxyribonuclease Inhibitor

Solution tested for inhibitory activity*	Desoxyribonuclease found in test system	Inhibition of desoxyribonuclease
	units/cc.	*per cent*
Experiment A		
1 cc. water	58	0
1 " inhibitor solution‡ + 0.2 cc. water	25	57
1 " " " + 1.0 mg. cryst. chymotrypsin§ (0.1 cc.)	28	52
1 " " " + 0.1 " " " " "	28	52
1 " water + 1.0 mg. cryst. chymotrypsin (0.1 cc.)	57	2
1 " " + 0.1 " " " " "	57	2
Experiment B		
1 cc. water	52	0
1 " inhibitor solution + 0.1 cc. water	21	60
1 " " " + 1000 μg. cryst. ribonuclease (0.1 cc.)‖	55	−6
1 " " " + 100 " " " " "	48	8
1 " " " + 10 " " " " "	49	6
1 " " " + 1 " " " " "	42	19
1 " " " + 0.1 " " " " "	34	35
1 " " " + 0.01 " " " " "	24	54
1 " water + 1000 μg. cryst. ribonuclease (0.1 cc.)	48	8

* After incubating for 4 hours at 37°, 0.2 cc. of each solution was mixed with 0.2 cc. of broth supernate of streptococcal culture (strain C203S) diluted 1:5, as a source of desoxyribonuclease, and 0.2 cc. of desoxyribonuclease diluent. After standing a short time in an ice water bath, the mixtures were assayed for desoxyribonuclease. Controls not included in the table showed that the chymotrypsin and the ribonuclease, alone or mixed with inhibitor, possessed no desoxyribonuclease activity.

‡ Sonic extract of streptococci, strain Wilson.

§ Armour.

‖ Kindly supplied by Dr. M. Kunitz.

ribonucleic acid. There arises the question of whether ribonucleic acids from other sources also have the capacity to inhibit desoxyribonuclease from group A streptococci. Commercial yeast nucleic acid was tested in a concentration of 3.3 mg. per cc. of test system, and it was found to cause no inhibition. As a control, streptococcal ribonucleic acid, prepared as described in reference 1, effectively inhibited in a concentration of 1.7 mg. per cc. of test system.

On the assumption that freshly prepared yeast nucleic acid is in a less degraded state than the commercial product, dried bakers' yeast was ground in a ball mill and the ground yeast was extracted under the same conditions as were used in the preparation of inhibitor from streptococci. The resulting crude solution of yeast ribonucleic acid resembled the commercial nucleic acid in failing to cause inhibition.

Inhibition Apparently Not Due to Binding of Mg^{++}.—Since streptococcal desoxyribonuclease, like pancreatic desoxyribonuclease, is known (6) to be activated by Mg^{++}, it seemed possible that the inhibition of group A desoxyribonuclease by streptococcal ribonucleic acid might involve the binding of Mg^{++} by the latter substance. If this hypothesis is correct, one would expect to find a quantitative difference between the dependence on Mg^{++} of group A and group C desoxyribonucleases. Measurements of depolymerase activity of the two enzymes in the presence of various concentrations of Mg^{++} revealed no differences between the two desoxyribonucleases. In other experiments, the addition of an excess of Mg^{++} failed to abolish the inhibition of group A desoxyribonuclease by streptococcal ribonucleic acid. These findings indicate that inhibition does not involve the binding of Mg^{++} by inhibitor and they suggest that the enzyme is inhibited by direct combination with streptococcal ribonucleic acid.

DISCUSSION

The observations recorded in Part I of this paper show that when washed cocci of certain strains of *Streptococcus pyogenes* are suspended in a non-nutrient solution, desoxyribonuclease is elaborated into the suspending medium. So far as we are aware, this is the only example known of the elaboration of an extracellular enzyme by resting bacteria. Appearance of the enzyme is dependent upon the presence of an energy source, phosphate and magnesium ions, and if any one of these factors is missing no enzyme is found in the supernate of the resting cell system. It is notable that elaboration of desoxyribonuclease is inhibited by a variety of enzyme poisons, suggesting that metabolic processes in addition to glycolysis are a functional part of the system.

Of primary interest is the question concerning the origin of the enzyme: Is the observed desoxyribonuclease the result of synthesis in the resting cells or is the desoxyribonuclease preformed within the cocci during growth, and subsequently released into the suspending solution? A definitive answer to this question cannot be given, but the evidence obtained indicates that little or no desoxyribonuclease is present in a preformed state within the cells. It therefore appears that enzyme synthesis occurs in the resting cell system. It should be noted, however, that no external source of nitrogen is present, and that "enzyme synthesis" may represent anything from the formation of desoxyribonuclease molecules from an endogenous pool of amino acids to a one-step conversion of a closely related precursor to active desoxyribonuclease. Further experiments

employing cell-free extracts of streptococci may perhaps provide additional information bearing on the general problem of protein synthesis.

The studies described in Part II show that the extracellular desoxyribonuclease formed by group A streptococci is partially inhibited by extracts of strains belonging to other Lancefield groups. In contrast to this, the extracellular desoxyribonucleases formed by strains belonging to groups B and C were, in no instance, inhibited by the extracts. Likewise, the desoxyribonucleases derived from other sources, as yeast, barley, and pancreas, failed in general to be inhibited by the streptococcal extracts. The results indicate that the group A desoxyribonuclease differs from all the others tested in being specifically inhibited by a substance which is extractable from streptococcal cells.

It has been shown by Zamenhof and Chargaff (9) that there is present in yeasts a substance which specifically inhibits the desoxyribonuclease formed in these organisms. It is evident that the yeast inhibitor differs from the streptococcal inhibitor not only in specificity but also in chemical nature, for the former is a protein while the latter is a ribonucleic acid. A specific desoxyribonuclease inhibitor has also been discovered in the crop gland tissue of the pigeon (11) and in other tissues (12). This inhibitor likewise appears to be a protein.

It would be of considerable interest to know whether the inhibition of group A streptococcal desoxyribonuclease is a property specifically associated with streptococcal ribonucleic acid or whether ribonucleic acids from other sources can also inhibit the enzyme. Experiments in which preparations of yeast ribonucleic acid were tested revealed lack of inhibitory power, but the results must be interpreted with caution because it is not known to what extent yeast ribonucleic acid may be degraded in the course of its preparation even when gentle methods are employed. Bearing in mind this reservation, it appears that ribonucleic acid from yeast differs from that of streptococci in failing to inhibit the group A desoxyribonuclease.

SUMMARY

As a preliminary to the study of desoxyribonuclease elaboration by resting cells, 34 strains of streptococci were examined for their capacity to produce desoxyribonuclease in broth cultures. The largest amounts of enzyme were found to be produced by strains belonging to Lancefield group A and by certain strains belonging to groups C and D. Many strains, especially those belonging to other groups, produced little or no enzyme.

Washed cocci of certain strains elaborated desoxyribonuclease extracellularly upon suspending them in a solution containing an energy source, phosphate and magnesium ions. When any one of these factors was omitted, no enzyme was produced. The appearance of extracellular desoxyribonuclease was found to be inhibited by a variety of enzyme poisons, and this and other findings indicate that desoxyribonuclease is synthesized in the resting cell system.

Cocci were found to contain a substance which partially inhibits the desoxyribonuclease formed by group A strains but which fails to inhibit the desoxyribonuclease formed by strains belonging to groups B and C or to inhibit the desoxyribonuclease derived from yeast, barley, and pancreas.

The inhibitor of group A desoxyribonuclease has been identified as streptococcal ribonucleic acid. Preparations of ribonucleic acid derived from yeast differed from streptococcal ribonucleic acid in failing to inhibit the group A desoxyribonuclease.

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