Differentiation of Ribonucleic Acids by Inhibition of Streptococcal Deoxyribonuclease

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It has been shown (Bernheimer & Ruffier, 1951) that physical disruption of streptococci belonging to Lancefield groups A, B, C and D results in the liberation of a substance which partially inhibits the depolymerizing action of group A streptococcal deoxyribonuclease. One of the striking characteristics of the inhibitory substance is its specificity, for although it inhibits the deoxyribonuclease produced by group A streptococci, it fails to inhibit deoxyribonucleases prepared from pancreas, barley, yeast or even those of streptococci belonging to groups B and C. The inhibitor has been identified as streptococcal ribonucleic acid. There arises the question of whether the inhibition of group A streptococcal deoxyribonuclease is an effect associated specifically with ribonucleic acid of streptococcal origin or whether ribonucleic acids, regardless of source, will inhibit the enzyme. The only information bearing on this point is the result of an experiment in which yeast nucleic acid was found not to inhibit (Bernheimer & Ruffier, 1951). The present study is concerned with the capacity of ribonucleic acids, prepared from diverse sources, to inhibit the streptococcal enzyme.

METHODS

The methods of estimating deoxyribonuclease activity and inhibition were identical with those used by Bernheimer & Ruffier (1951). Inhibition of depolymerizing action of

deoxyribonuclease was estimated by measuring the rate of decrease in viscosity of sodium deoxyribonuclease solution by streptococcal deoxyribonuclease (*a*) in the presence, and (*b*) in the absence of solution to be tested for inhibitory activity. The sodium deoxyribonuclease was prepared from calf thymus (Mirsky & Pollister, 1942) and served as substrate in the presence of veronal buffer and Mg ions (McCarty, 1946). The supernatant fluid of broth cultures of streptococci, strain C203S, with or without preliminary fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$, was used as a source of deoxyribonuclease. There was no indication that the degree of inhibition was affected by the state of purity of the enzyme. In some experiments, deoxyribonucleases produced by other strains of streptococci (Wilson, group A; C7, group C) were employed in lieu of that formed by strain C203S.

RESULTS

Inhibition of deoxyribonuclease by crude bacterial extracts

Before attempting to isolate ribonucleic acid from bacterial and other sources, crude extracts of bacteria were prepared with a view to testing them for inhibitory action against group A streptococcal deoxyribonuclease.

(*a*) *Sonic extracts.* Neopeptone infusion broth (500 ml.) was inoculated with 0.5 ml. of broth culture and incubated at 37°. After 17 hr. the amount of growth was estimated by measuring against a broth blank the optical density of the culture in a Beckman model DU spectrophotometer (wavelength, 650 m μ ; light path, 10 mm.). The bacteria were

Table 1. *Inhibition of group A streptococcal deoxyribonuclease by extracts of various bacterial species*

Bacterial species	Method of disruption	Ratio of dry wt. of bacteria disrupted to vol. of extraction solution (mg./ml.)	Inhibition of group A streptococcal deoxyribonuclease (strain C203S) (%)	Inhibition of group A streptococcal deoxyribonuclease (strain Wilson) (%)	Inhibition of group C streptococcal deoxyribonuclease (strain C7) (%)
<i>Escherichia coli</i> (S3)	Sonic	5.8	88	64	6
<i>Clostridium welchii</i> (NYS)	Sonic	9.6	92	67	10
<i>Pasteurella avicida</i> (Hop)	Sonic	4.2	81	78	-2
<i>Neisseria catarrhalis</i> (Cor)	Sonic	1.2	41	33	-2
<i>Bacillus subtilis</i>	Sonic	2.1	72	67	4
<i>Micrococcus pyogenes</i> , var. <i>aureus</i> (209)	Sonic	5.4	77	72	-12
<i>Corynebacterium xerose</i> (NYS 155)	Sonic	1.9	10	-8	14
<i>Escherichia coli</i> (S3)	Ball mill	10	60	—	—
<i>Clostridium welchii</i> (NYS)	Ball mill	10	62	—	—
<i>Pasteurella avicida</i> (Hop)	Ball mill	7.2	54	—	—
<i>Bacillus subtilis</i>	Ball mill	10	63	—	—
<i>Micrococcus pyogenes</i> , var. <i>aureus</i> (209)	Ball mill	10	45	—	—
<i>Corynebacterium xerose</i>	Ball mill	9.2	25	—	—
<i>Streptococcus pyogenes</i> (C203S)	Ball mill	10	74	—	—

sedimented by centrifugation, the supernatant fluid discarded, and the bacteria washed in 250 ml. of saline buffered with phosphate at pH 7.0. The washed bacteria were suspended in 15 ml. of buffered saline and disrupted by exposing for 30 min. to the vibration of a 9000-cycle oscillator (Raytheon Corporation, Waltham, Massachusetts). The sonically treated material was centrifuged, and the supernatant fluid tested for inhibition by adding 0.2-0.4 ml. of deoxyribonuclease diluted in diluent (Bernheimer & Ruffier, 1951) to contain 30 units, and assaying the mixture. The inhibition produced by extracts of various bacterial species, tested against deoxyribonucleases formed by three streptococcal strains (C203S, group A; Wilson, group A; C7, group C), is shown in Table 1.

(b) *Extracts of ground bacteria.* The bacteria from 5 to 10 l. of a 17 hr. broth culture were washed in saline and freeze-dried. The dried bacteria, weighing about 1 g., were ground in a ball mill (Swift & Hirst, 1937; Bernheimer & Rodbart, 1948) for approximately 24 hr. and washed from the mill with small portions of cold distilled water totalling 100 ml. The pooled mill washings were centrifuged, and the clear supernatant fluid tested for inhibition of group A (strain C203S) deoxyribonuclease (Table 1).

The results show that sonic extracts of all bacterial species studied, with the exception of *Corynebacterium xerose*, partially inhibited the streptococcal deoxyribonuclease formed by group A streptococci (strains C 203S and Wilson) but failed to inhibit the deoxyribonuclease of the group C strain (C7). It is evident, therefore, that many bacterial species contain a substance which resembles streptococcal ribonucleic acid in its inhibitory effect on streptococcal deoxyribonucleases. The specificity of the inhibition suggests that the substance may be ribonucleic acid.

Extracts prepared from ground bacteria inhibited group A deoxyribonuclease, but were quantitatively somewhat less effective than the sonic extracts. It may be noted that the extract of ground *C. xerose* showed slight, but significant, inhibition as contrasted to the insignificant degree of inhibition shown by the sonic extract of the same organism.

In some instances, the inhibitory power of the extracts decreased during storage in the refrigerator for periods of a few days to a week. The loss appeared to be due to small amounts of bacterial ribonucleases, the presence of which was revealed by finding that some of the extracts were able to depolymerize commercial yeast nucleic acid.

Inhibition of deoxyribonuclease by purified ribonucleic acids

Ribonucleic acids were prepared from *Streptococcus pyogenes*, *Clostridium welchii*, *Escherichia coli*, wheat germ, beef liver and beef pancreas, according to the methods indicated in Table 2. The sources of additional preparations derived from tobacco leaves, tobacco mosaic virus and pancreas are also indicated in the table. The specific absorption at 260 m μ . and the phosphorus content of the preparations indicated that they had a purity of at least 50%. Some of them were known to be contaminated with deoxyribonucleic acid, but it is unlikely that the presence of this substance affected the results because authentic samples of deoxyribonucleic acid and its hydrolysis products failed to cause inhibition under the conditions of the test. In addition, all the preparations, including the crude extracts, were examined, with negative results, for the presence of contaminating deoxyribonuclease, which, if present, would have made the preparations unsuitable for the purpose at hand.

Table 2. *Capacity of various preparations of ribonucleic acids to inhibit group A streptococcal deoxyribonuclease*

Source of ribonucleic acid	Method of preparation	Concentration of ribonucleic acid preparation required to produce approx. 30% inhibition of deoxyribonuclease in test mixture (mg./ml.)
Liver (beef)	Mirsky & Pollister (1942) modified	>5.0
Liver (beef)*	Davidson & Waymouth (1944)	>5.0
Liver (beef)	Grinnan & Mosher (1951)	>5.0
Pancreas (beef)†	Johnson (unpublished observations)	>5.0
Pancreas (beef)*	Kerr & Seraidarian (1949)	>5.0
Wheat germ	Clarke & Schryver (1917) modified	>5.0
Yeast	Commerical	>5.0
Tobacco leaf‡	Pirie (1950)	>5.0
Tobacco mosaic virus, prep. 239 G‡	Bawden & Pirie (1937)	5.0
Tobacco mosaic virus, prep. 339 E‡	Bawden & Pirie (1937)	5.0
Tobacco mosaic virus, prep. 339 D‡	Bawden & Pirie (1937)	>5.0
Tobacco mosaic virus, prep. 335 A‡	Pirie (unpublished observations)	>5.0
<i>Strep. pyogenes</i>	Henry & Stacey (1946)	0.10
<i>Cl. welchii</i>	Henry & Stacey (1946)	0.23
<i>Esch. coli</i>	Sevag, Lackman & Smolens (1938)	0.31

* Prepared by Mr Clyde C. Doughty.

† We are greatly indebted to Dr Edward Johnson for supplying this preparation.

‡ For these nucleic acids we are greatly indebted to Mr N. W. Pirie who informs us that preparations 239 G and 339 E were isolated after splitting the virus nucleoprotein by the acetic acid method, while preparation 339 D was isolated following fission by boiling and 335 A by fission with Sr(NO₃)₂.

The ribonucleic acids were used in the form of their sodium or potassium salts. Each preparation was tested by mixing 0.2 ml. of solution containing 15 mg. ribonucleate/ml. with 0.4 ml. of group A streptococcal deoxyribonuclease containing approximately 75 units/ml., and assaying the mixture for depolymerase activity. If inhibition of more than 30% was observed, lower concentrations of ribonucleate were tested until the concentration producing approximately 30% inhibition was found.

The results (Table 2) show that the three ribonucleic acids of bacterial origin were effective in inhibiting the test deoxyribonuclease in relatively low concentration. In contrast, the ribonucleic acids derived from mammalian tissues as well as those obtained from yeast, wheat germ and tobacco leaves failed to inhibit the test enzyme in concentrations as high as 5 mg./ml. Several of the latter group of preparations tested at a level of 15 mg./ml. likewise failed to show significant inhibition. Two of the viral nucleic acids showed a qualitative resemblance to those of bacteria, but differed from them in the relatively high concentration needed to produce a significant degree of inhibition; the other two viral preparations did not inhibit in the concentrations tested.

Destruction of inhibitory activity by ribonuclease

It has been shown (Bernheimer & Ruffier, 1951) that the deoxyribonuclease-inhibiting property of a crude ribonucleate-containing streptococcal extract is destroyed by pancreatic ribonuclease. If the inhibition described in the previous section is due to ribonucleic acid, rather than to a contaminating substance in the preparations tested, it follows that the inhibitory power of the bacterial ribonucleic acids should be destroyed by treatment with ribonuclease. An experiment, the conditions and results of which are presented in Table 3, shows that the deoxyribonuclease inhibition is prevented by ribonuclease.

DISCUSSION

Recent studies (Chargaff *et al.* 1950) utilizing paper-chromatographic techniques have demonstrated differences in the quantities of purine and pyrimidine bases present in ribonucleic acids prepared from various animal and plant materials. There appear, however, to be no methods which permit differentiation of isolated ribonucleic acids without preliminary hydrolysis of the large molecules. A partial exception to this generalization depends upon the capacity of ribonucleic acid, or its ribonuclease-resistant fraction, to induce the formation of streptolysin S, ribonucleic acid derived from tobacco mosaic virus proving inactive in contrast to ribonucleic acids prepared from other sources, all of which were active in inducing streptolysin S formation (Bernheimer & Rodbart, 1948).

A second indication of individuality in unhydrolysed ribonucleic acids is provided by the data of the present report. It is shown that relatively low concentrations of bacterial ribonucleic acids partially inhibit group A streptococcal deoxyribonuclease, while the ribonucleic acids derived from mammalian tissues and from plants fail to exhibit a comparable effect even in concentrations 15–50 times greater than those of the bacterial preparations. The results are interpreted as pointing to the existence of at least two qualitatively different, naturally occurring types of ribonucleic acid.

Other interpretations of the findings can, however, be entertained. The methods used for the isolation of the bacterial ribonucleic acids differ from those employed for isolating ribonucleic acids from mammalian tissues, wheat germ, etc. and it is possible that the results reflect effects of the procedures used in extraction and purification rather than differences in the native nucleic acids. Attempts to test this possibility were made, but they failed because the methods used for the extraction of bacterial ribonucleic acids were found not to be

Table 3. *Prevention of deoxyribonuclease inhibition by pancreatic ribonuclease*

Solution tested for inhibitory activity*		Deoxyribo- nuclease found in test system (units/ml.)	Inhibition of deoxy- ribonuclease (%)
Deoxyribonuclease diluent (0.2 ml.)	+ 0.2 ml. water	57	0
<i>Esch. coli</i> ribonucleic acid (RNA) (0.4 mg. in 0.2 ml.)	+ 0.2 ml. water	25	60
<i>Strep. pyogenes</i> RNA (0.4 mg. in 0.2 ml.)	+ 0.2 ml. water	28	51
<i>Esch. coli</i> RNA (0.4 mg. in 0.2 ml.)	+ 2.0 µg. cryst. ribonuclease (0.2 ml.)	60	– 5
<i>Strep. pyogenes</i> RNA (0.4 mg. in 0.2 ml.)	+ 2.0 µg. cryst. ribonuclease (0.2 ml.)	60	– 5
Deoxyribonuclease diluent (0.2 ml.)	+ 2.0 µg. cryst. ribonuclease (0.2 ml.)	55	3

* The nucleic acids were dissolved in 'deoxyribonuclease diluent'; the ribonuclease in water. After 4 hr. at 37°, 0.2 ml. of each mixture was mixed with 0.4 ml. of a solution of partially purified group A streptococcal deoxyribonuclease diluted in 'deoxyribonuclease diluent' to contain approx. 75 units/ml. After standing a few minutes in an ice-water bath the mixtures were assayed for deoxyribonuclease. Controls not included in the table showed that ribonuclease, alone or mixed with ribonucleic acid, possessed no deoxyribonuclease activity.

applicable to tissue ribonucleic acid, and conversely. It may be noted, however, that a crude ribonuclease-containing extract of yeast was non-inhibitory (Bernheimer & Ruffier, 1951) even though prepared under the same conditions as those which yield inhibitory bacterial extracts.

It has been shown that the capacity of bacterial ribonucleic acids to inhibit streptococcal deoxyribonuclease is destroyed by minute amounts of pancreatic ribonuclease, and perhaps the non-inhibiting ribonucleic acids isolated from liver, pancreas, etc. have been altered by ribonuclease during the course of their extraction and purification. It seems probable that the testing of this hypothesis will have to await the discovery and application of effective inhibitors of ribonuclease. Likewise, it appears desirable to await the development of improved methods of isolation before attempting to extend the present observations to include ribonucleic acids of numerous other plant and animal species.

SUMMARY

1. A study has been made of the depolymerizing action of group A streptococcal deoxyribonuclease on thymus deoxyribonucleic acid as affected by

ribonucleic acids of diverse origin. Crude and purified preparations of ribonucleic acids derived from a variety of bacteria partially inhibited the deoxyribonuclease. In contrast, ribonucleic acids obtained from mammalian pancreas and liver, tobacco leaves, wheat germ and yeast failed to show inhibition. Two preparations of tobacco mosaic virus nucleic acid resembled the bacterial ribonucleic acids in causing inhibition, but they were effective only in relatively high concentration.

2. The results are interpreted as indicating the possible existence in living matter of two qualitatively different types of ribonucleic acid: (a) an inhibitory form which is present in bacteria and possibly in some viruses, and (b) a non-inhibitory form which is present in mammalian tissue and in plants.

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A Biological Method of Assay of Hyaluronidase

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Hyaluronidase activity may be determined by various biological and physicochemical methods (Meyer, 1947) and theoretically any method measuring the depolymerizing activity of hyaluronidase may be considered suitable. The clinical efficacy of hyaluronidase, however, depends mainly on its power to split the highly polymerized mucopolysaccharides in the dermis, thus breaking down the

barrier which hinders the inflow or the absorption of injected fluid. Hyaluronidase preparations, although purified during manufacture, may contain polysaccharidases which do not take part in the early stages of depolymerization. It is generally agreed that, if possible, the ultimate reference method of assay of hyaluronidase for therapeutic use should be a direct biological test based on de-