THE EFFECT OF NUCLEIC ACIDS AND OF CARBOHYDRATES ON THE FORMATION OF STREPTOLYSIN*

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Yeast nucleic acid has been shown by Okamoto (1) to induce the formation of a potent hemolysin in cultures of *Streptococcus pyogenes*. As reported by Okamoto, and confirmed in the present paper, the effect of nucleic acid is readily demonstrable since the hemolytic activity of filtrates or supernates of nucleic acid–broth cultures is at least 20 to 100 times greater than the activity of cultures prepared in broth to which no nucleic acid has been added. On blood agar containing yeast nucleic acid, the effect is manifest by the appearance of very large zones of beta hemolysis around the colonies.

The nucleic acid effect has been investigated further in order to define the conditions necessary for producing the hemolysin on a large scale and in a manner which will facilitate its purification. In addition, although some of the properties of the nucleic acid–induced hemolysin have been described by Itô (2), the relationship of this hemolysin to the better characterized streptococcal hemolysins, streptolysin O and streptolysin S, requires clarification. Finally, an opportunity is afforded of studying an almost unknown biological effect of nucleic acid.

Methods, Materials, and Terminology

Preparation of Peptone-Infusion Broth.—Fresh beef hearts, freed of gross fat, were ground and then mixed well with tap water, 1 liter of water for each pound of ground heart. After being brought to 85°C. and infused at that temperature for 45 minutes, the mixture was filtered while hot, through paper. To each liter of infusion was added 10 gm. peptone (neopeptone Difco) and 5 gm. reagent sodium chloride. After the ingredients were completely dissolved by bringing to a boil, the pH was brought to 7.9, the medium was boiled 1 to 2 minutes, and then filtered immediately through paper. The medium was sterilized in the autoclave at 121°C. for 15 to 30 minutes. Immediately before inoculating, a freshly prepared solution of sodium thioglycollate was added to give a final concentration of 0.01 per cent.

Culture Technique.—A 6 to 8 hour peptone-infusion broth culture of *Streptococcus pyogenes*, strain C203S, was distributed in 0.5 cc. amounts among a large number of sterile tubes, and then rapidly frozen solid in an alcohol–carbon dioxide freezing mixture. The tubes were stored in a dry-ice chest until needed. When required, a tube was thawed at room temperature, the contents diluted 10 times in sterile saline, and 0.1 cc. inoculated into each 10 cc. of

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culture medium. Cultures were 10 cc. in volume, and were contained in 16 × 150 mm. culture tubes. They were incubated at 37°C. for 18 to 24 hours. Unless otherwise indicated, the strain of streptococcus employed was C203S.

Estimation of Hemolytic Activity.—The method is the same as that employed in previous studies (3). The hemolytic unit is the amount of hemolysin which will lyse half the erythrocytes (human) contained in 2 cc. of phosphate-buffered saline, pH 7, in 30 minutes at 37°C. It has been observed that the stock suspension of washed erythrocytes slowly increases in sensitivity to the nucleic acid–induced hemolysin even though the suspension is stored at 3–5°C. An erythrocyte suspension which is kept in the cold for 5 days is lysed by about half as much nucleic acid–induced streptolysin as that required to lyse suspensions prepared from fresh cells. The age of the erythrocyte suspension must therefore be taken into account when the same suspension is used for experiments performed over a period of several days.

Preparation of Streptococcal Nucleic Acid.—Streptococcus pyogenes, strain C203S, was grown in 30 liters of casein hydrolysate medium according to the method described earlier (4). After centrifugation of the cultures, the cells were washed in saline, and dried *in vacuo* from the frozen state. Yield: 25 gm.

8 gm. dried cocci was ground in a mill of quarter-inch steel balls. The mill, one designed by Swift and Hirst (5), was charged with 2 gm. of cocci at a time, and operated for 20 hours at room temperature for each charge. Each 2 gm. of ground cocci was washed from the mill with portions of demineralized water totalling 100 cc. The pooled mill-washings were centrifuged, the insoluble "cell fragments" were washed once in water, and the washing added to the supernatant aqueous extract. The aqueous extract contained 70 per cent of the total phosphorus present in the intact cocci. Nucleic acid, chiefly of the ribose type, was isolated from the acid-precipitated nucleoprotein contained in the aqueous extract, according to the procedure of Sevag, Lackman, and Smolens (6). Yield: 0.82 gm.

Analytical Methods.—Total phosphorus was determined by the method of Lohmann and Jendrassick (7). Nitrogen was estimated by a modified micro Kjeldahl procedure. Purine ribose was estimated by the orcinol-pentose reaction, employing conditions essentially the same as those of Mejbaum (8). Under these conditions, the reaction measures purine ribose to the exclusion of pyrimidine ribose (9).

Terminology.—The terms, ribonucleic acid and desoxyribonucleic acid, are employed with the knowledge that the pentose and desoxypentose of nucleic acids other than those of yeast and thymus, in most cases, have not been identified.

EXPERIMENTAL

I. Nucleic Acids

Streptolysin Production as a Function of Concentration of Yeast Sodium Nucleate.—A series of tubes of broth containing different concentrations of commercial yeast sodium nucleate were inoculated and incubated at 37°C. After 18 hours, the hemolytic activity of the cultures was measured and plotted as a function of sodium nucleate concentration. As can be seen in Fig. 1, nucleate concentrations of less than 1 mg. per cc. had little effect, while concentrations between 1 and 4 mg. per cc. caused increasing amounts of hemolysin to appear. In this experiment, concentrations greater than 4 mg. per cc. caused no further increase in the amount of hemolysin. The slope of the linear portion of the curve, and of curves obtained in similar experiments, showed that on the average, doubling the concentration of sodium nucleate

approximately tripled the hemolytic activity. The maximum hemolytic activity varied with different lots of broth, from 2000 hemolytic units per cc., as in the present experiment, to 4000 hemolytic units per cc. in others.

When streptococci which had grown in nucleic acid–broth were transferred to plain broth, the amount of streptolysin formed was the same as that usually found in plain broth, namely 30 to 100 hemolytic units per cc. It is evident, therefore, that nucleic acid does not induce a permanent change in the capacity of streptococci to form hemolysin. Maintenance of the effect in serial cultures requires the continued presence of nucleic acid.

Sodium nucleate had no effect on the amount of bacterial growth as measured turbidimetrically. The final pH of cultures containing high concentrations of

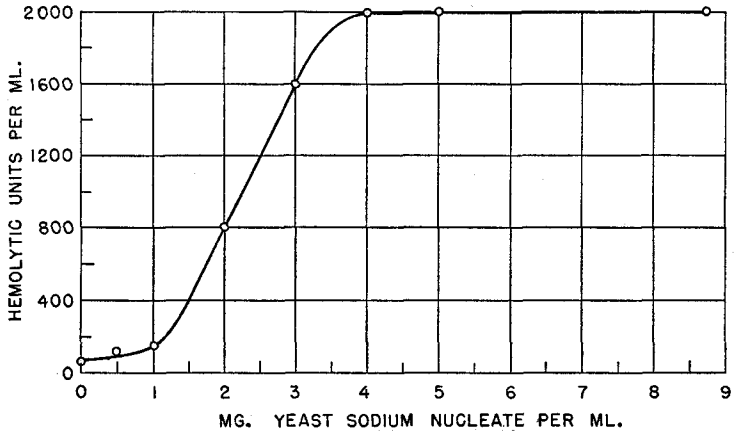

FIG. 1. Hemolytic titer of broth cultures as a function of concentration of yeast sodium nucleate.

sodium nucleate did not differ by more than 0.17 unit from that of cultures grown in the absence of sodium nucleate.

Probable Identity of Nucleic Acid–Induced Streptolysin with Streptolysin S.— Streptococci of the Lancefield group A (*Streptococcus pyogenes*) have been shown by Todd (10) to produce two distinct hemolysins designated streptolysin O and streptolysin S. Either or both hemolysins are formed *in vitro* depending upon the strain, the culture medium, and the conditions of incubation (11). The properties of these hemolysins are described in the papers of Todd (10) and Herbert and Todd (11, 12).

Study of the nucleic acid–induced hemolysin has shown that it is not activated by reducing agents nor is it neutralized by appropriately diluted sera containing antistreptolysin O. It cannot be streptolysin O, therefore. The nucleic acid–induced hemolysin has the following additional properties: (*a*) Unlike certain other hemolysins, including streptolysin O, it is not inhibited

by cholesterol (2, 13). (*b*) It is irreversibly inactivated by dilute acid and by brief heating. (*c*) Its production in infusion broth is suppressed by glucose (14). (*d*) Initiation of hemolysis is preceded by a relatively long latent period (13). In these respects, the nucleic acid–induced streptolysin is identical with streptolysin S. There is, moreover, no evidence that the two hemolysins are not the same.

If the nucleic acid–induced hemolysin and streptolysin S are identical, it follows that yeast nucleic acid should cause increased hemolysin production in strains known to produce streptolysin S. It follows, also, that yeast nucleic acid should not increase the formation of hemolysin by strains which produce only streptolysin O. A series of strains of group A streptococci were investi-

TABLE I

Nucleic Acid Effect in Relation to Strain of Group A Streptococcus

Strain	Produces streptolysin S	Produces streptolysin O	Hemolytic titer of plain broth culture			Hemolytic titer of broth culture containing 0.2 mg. ribonuclease-resistant fraction of yeast nucleic acid per cc.			
			Hrs. of incubation at 37°C.			Hrs. of incubation at 37°C.			
			23	48	96	23	48	96	168
C203S	+	+	<100	<100	<100	2650	2260	2000	1200
1685M	+	?	<100	<100	<100	100	600	2000	600
Blackmore	+	−	<100	<100	<100	2000	2000	2650	750
Lang	+	−	<100	<100	<100	2000	2000	2650	750
C203U	−	+	<100	<100	<100	<100	<100	<100	<100
Wilson 868	−	+	<100	<100	<100	<100	<100	<100	<100
Hodder 872	−	+	<100	<100	<100	<100	<100	<100	<100
Tucker 873	−	+	<100	<100	<100	<100	<100	<100	<100

gated from the point of view of the nucleic acid effect. The strains studied included several (Blackmore, Lang, Wilson 868, Hodder 872, and Tucker 873) which had been previously analyzed by Colebrook *et al.* (15) and by Herbert and Todd (11) for their capacity to produce streptolysins O and S.

The results, presented in Table I, show that the nucleic acid effect is readily demonstrable when strains known to produce streptolysin S are employed, and that a comparable effect is not observed in strains which lack this capacity. These results constitute strong evidence for the identity of nucleic acid–induced streptolysin with streptolysin S, particularly when considered together with the fact that the properties of the two hemolysins do not allow them to be differentiated.

Streptolysin-Inducing Activity of Various Nucleic Acid Preparations.—The usual methods of purifying nucleic acids did not alter appreciably the activity of commercial yeast nucleic acid. These methods included deproteinization

with chloroform and amyl alcohol (6), precipitation with glacial acetic acid (16), ethanol or barium (17), dialysis, and combinations of these procedures. In

TABLE II

Streptolysin-Inducing Activity of Different Preparations of Ribonucleic and Desoxyribonucleic Acids. Effect of Ribonuclease on Streptolysin-Inducing Activity

	Type and source of nucleic acid	Method of preparation	Amount required to induce formation of 1000 hemolytic units
			mg.
	1. Ribonucleic acid, yeast	Commercial (Ma)	4.0
	2. Ribonucleic acid, yeast	Commerical (Pf)	2.3
	3. Ribonucleic acid, yeast	Commercial (Scl)	5.0
	4. Ribonucleic acid, yeast	Commercial (Sc2)	4.0
	5. Ribonucleic acid, yeast	Commerical (El)	6.0
A	6. Ribonucleic acid, yeast	Commercial (E2)	6.0
	7. Ribonucleic acid, beef liver	Mirsky and Pollister (32), modified	0.6
	8. Ribonucleic acid, wheat germ	Clarke and Schryver (33), modified	2.5
	9. Desoxyribonucleic acid, thymus*	Hammarsten (34)	>10.0‡
	10. Desoxyribonucleic acid, thymus (?)	Commercial	>10.0§
	11. Desoxyribonucleic acid, human placenta	Hammarsten, modified	>10.0
	12. No. 3 treated with ribonuclease		0.5
	13. No. 7 treated with ribonuclease		0.6
	14. No. 8 treated with ribonuclease		0.7
	15. Nucleic acid, streptococcal,‖ strain	Sevag, Lackman, and	1.0
B	1685M, treated with ribonuclease	Smolens	
	16. Nucleic acid, streptococcal,¶ strain C203S, treated with ribonuclease	(See Methods)	0.8
	17. Ribonucleic acid, tobacco mosaic virus** treated with ribonuclease		>2.0

* Kindly supplied by Dr. Milton Levy, New York.

‡ 5 mg. per cc. induced formation of 150 hemolytic units per cc.; control: 60 hemolytic units per cc.

§ 5 mg. per cc. induced formation of 125 hemolytic units per cc.; control: 125 hemolytic units per cc.

‖ Kindly supplied by Dr. M. G. Sevag, Philadelphia. Chiefly ribonucleic acid, but stated to contain 10 to 30 per cent desoxyribonucleic acid.

¶ Chiefly ribonucleic acid.

** Kindly supplied by Dr. H. S. Loring of Stanford University, California.

view of these findings, it seemed worth while to investigate the activity of nucleic acids from different sources.

The sodium salts of eight preparations of ribonucleic acid and three of

desoxyribonucleic acid were tested for streptolysin-inducing activity. The sources of these preparations and the quantity of each required to cause the formation of 1000 hemolytic units are shown in Table II, A. All the preparations of ribonucleic acid including those from liver and from wheat as well as those from yeast were active. Not all of the preparations, however, were equally effective in causing streptolysin formation, and it is particularly notable that the ribonucleic acid from liver and that from wheat were more active than most of the preparations derived from yeast. The three specimens of desoxyribonucleic acid contrasted strongly with ribonucleic acid in failing to stimulate streptolysin formation.

Specificity of Ribonucleic Acid in Inducing Streptolysin Formation.—A number of naturally occurring materials were tested in order to determine whether any of them produced the same effect as ribonucleic acid when added to broth. These included yeast extract (20 mg. per cc.), Witte's peptone (100 mg. per cc.) acid hydrolysate of casein (0.6 per cent by volume), crystalline hemoglobin (2 mg. per cc.), horse serum (10 per cent by volume), protamine (1 γ per cc.), lecithin 3.5 mg. per cc.), brain phosphatides (4.3 mg. per cc.), lipovitellin (1 mg. per cc.), glycogen (0.5 mg. per cc.), and a considerable number of other polysaccharides. With the exception of lecithin and rabbit serum, none of these materials was active. Lecithin and rabbit serum caused slightly greater hemolysin formation than that which occurred in broth to which nothing had been added. The effect of serum and lecithin was less than one-twentieth of that of comparable concentrations of ribonucleic acid.

Adenosine triphosphate and diphosphopyridine nucleotide in concentrations of 0.5 and 1.0 mg. per cc., respectively, were found to be inactive. In addition, the following substances were found to have no activity: yeast adenylic and guanylic acids (0.4 mg. per cc.); adenosine and guanosine (0.4 mg. per cc.); adenine, guanine, uracil, and cytosine (0.1 mg. per cc.); *d*-ribose (0.4 mg. per cc.). In one experiment, however, adenosine appeared to be active in stimulating streptolysin formation. Subsequent experiments failed to confirm this finding.

The following hydrolysates of yeast nucleic acid were prepared:

(*a*) 5 gm. yeast nucleic acid was refluxed with 50 cc. 2 per cent sulfuric acid for 2 hours. The mixture was neutralized with sodium hydroxide. (*b*) 2.5 gm. of yeast nucleic acid was heated with 25 cc. of 2.5 per cent ammonium hydroxide in a sealed tube for 60 minutes at 115°C. After removing a gelatinous precipitate, sodium hydroxide was added, and ammonia removed under reduced pressure in a stream of air. (*c*) 2 gm. yeast nucleic acid was allowed to stand at 28°C. in 0.5 N sodium hydroxide. After 3 hours, the pH was adjusted to 7.5.

Each of the three hydrolysates was tested for streptolysin-inducing activity; none was found to be active.

Effect of Ribonuclease on Activity of Ribonucleic Acid.—Since the streptolysin-

inducing activity of yeast ribonucleic acid was found to be destroyed by either acid or alkaline hydrolysis, it was of interest to study the effect of enzymatic degradation. For this purpose, crystalline ribonuclease prepared from beef pancreas (16) was employed.

To 100 mg. sodium nucleate (yeast) dissolved in 4 cc. water was added 1 cc. 0.1 per cent solution of ribonuclease. After adjusting the pH to 7.6 and allowing digestion to proceed for 4 hours at room temperature, the solution was passed through a Seitz filter and tested for streptolysin-inducing activity. The results, together with those obtained on testing sodium nucleate which had not been treated with ribonuclease, are shown in Table III.

TABLE III

Activation of Yeast Ribonucleic Acid by Ribonuclease

Material added to broth	Final concentration	Hemolytic units per cc. culture
	mg./cc.	
Sodium nucleate	4.0	500
	2.0	125
	1.0	<30
	0.5	<30
Sodium nucleate plus ribonuclease	0.5	1000
	0.25	400
None	0.0	<30
Ribonuclease	0.05	<30

It can be seen that as little as 0.5 mg. of digested nucleic acid stimulated the formation of 1000 hemolytic units while 8 times as much of the undigested nucleic acid stimulated the formation of only 500 hemolytic units. Numerous experiments, in which time and concentrations were varied, showed that, on the average, ribonuclease digestion caused a tenfold activation of yeast ribonucleic acid. Ribonuclease itself was found not to affect hemolysin production. Since the ribonuclease was known to be contaminated with trypsin and since its activating effect could conceivably be attributed to the latter, nucleic acid was incubated with a solution of crystalline trypsin, and then tested for streptolysin-inducing activity. No change in activity occurred.

When ribonucleic acid derived from wheat germ was treated with ribonuclease, its activity increased 3½ times (Table II, B). Liver ribonucleic acid, however, showed no change in activity when treated with the enzyme. It is notable that the activity of enzyme-treated ribonucleic acid is approximately the same regardless of whether the ribonucleic acid derives from yeast, mammallian liver, or wheat germ (Table II, B).

Three additional preparations of nucleic acid were tested for streptolysin-inducing activity. Two of these were derived from group A streptococci, strains 1685M and C203S, respectively, while the third was ribonucleic acid of tobacco mosaic virus. Since these materials were available only in very small quantities, they were treated with ribonuclease prior to testing, on the assumption that activity could be detected if it were of the same order as that of enzyme-treated ribonucleic acid of wheat, liver, or yeast. As can be seen in Table II, B, nucleic acid from each of the two streptococcal strains was active, while that of tobacco mosaic virus was not. It is evident, therefore, that the nucleic acid derived from tobacco mosaic virus differs from all the other ribonucleic acid preparations in failing to cause lysin formation.

TABLE IV

Stability of Streptolysin-Inducing Activity of Ribonuclease-Treated Yeast Ribonucleic Acid

pH of solution	Treatment	Residual streptolysin-inducing activity compared to that (1.00) of solution heated at 100°C. for 10 min. at pH 7.1
2.7	Boiling water bath for 10 min.	0.25
4.1	Boiling water bath for 10 min.	0.50
7.1	Boiling water bath for 10 min.	1.00
7.5	Autoclave at 120°C. for 20 min.	0.65
8.5	Boiling water bath for 10 min.	0.27
9.5	Boiling water bath for 10 min.	0.17

In view of the effect of ribonuclease on the activity of yeast nucleic acid, there arose the question of whether desoxyribonucleic acid might not become active when treated enzymatically. Experiments in which desoxyribonucleic acid was treated with desoxyribonuclease, or with ribonuclease, showed that desoxyribonucleic acid is not activated by either of these enzymes.

Stability of Streptolysin-Inducing Activity of Ribonucleic Acid.—After adjusting the pH of a series of aliquots of a 5 per cent solution of ribonuclease-treated yeast sodium nucleate, and heating either in a boiling water bath or in the autoclave, the pH was adjusted to neutrality, and the solutions tested for streptolysin-forming activity. All solutions were sterilized prior to testing by immersing them in a boiling water bath for 10 minutes at neutrality. The residual activity of these solutions is shown in Table IV. It can be seen that the stability is greatest at or near neutrality, and that heating at a pH as low as 4.1 or as high as 8.5 caused a considerable amount of inactivation.

Preparation and Properties of Active Fraction (AF) of Yeast Nucleic Acid.—When the digestion mixture resulting from the action of ribonuclease on yeast nucleic acid was dialyzed against water, the streptolysin-inducing activity

failed to dialyze. Making use of this and other properties, a method was developed for the separation of the active portion of yeast nucleic acid:

100 gm. of commercial yeast nucleic acid (Schwarz) is dissolved in 750 cc. water by slowly adding 20 per cent sodium hydroxide, keeping the solution yellow to phenol red. The pH is then adjusted to 7.5, and the volume to 1 liter. The solution is now deproteinized by repeated shaking with chloroform and amyl alcohol according to the procedure of Sevag (6). To each 100 cc. of sodium nucleate solution, 10 mg. crystalline ribonuclease is added. After allowing digestion to proceed at room temperature for 18 hours, the pH is adjusted to 8.0 with 20 per cent sodium hydroxide.

To each 100 cc. of digestion mixture, 10 gm. sodium acetate is added with stirring. Sufficient ethanol is added, with stirring, to bring the concentration to 29 per cent. After 15 minutes, the mixture is centrifuged, and the supernate decanted. Additional ethanol is stirred into the supernate to a concentration of 40 per cent. After centrifugation and removal of the precipitate, the supernate is brought to an ethanol concentration of 50 per cent, recentrifuged, adjusted to 66 per cent ethanol, and again centrifuged. Each of the four precipitates is dissolved in 50 cc. water, and the solutions tested for streptolysin-forming activity. Most of the activity is present in the fraction precipitating between 29 and 40 per cent ethanol. This fraction is dialyzed in cellophane sacs against running tap water for 18 hours, during which time an increase in volume of approximately tenfold occurs. If a precipitate forms during or after dialysis, it is removed by filtration and discarded. The active material can be preserved in solution in the refrigerator, or, if desired, it can be precipitated with 2 to 3 volumes of ethanol, and dried with ether or acetone. The average yield was 4 to 8 gm. Approximately 0.05 mg. of the product is required to cause the formation of 1000 hemolytic units. The activity per unit weight is about 100 times as great as that of the untreated nucleic acid.

Several lots of yeast nucleic acid were fractionated in the manner described, but with some modifications in the order of the individual steps. Although the active fractions so obtained were not quantitatively identical in regard to content of nitrogen, phosphorus, and activity, certain differences between the active fraction and the parent nucleic acid were consistently observed. These differences are as follows: (*a*) The nitrogen:phosphorus ratio increased from 1.65 to 1.90–2.30. (*b*) The purine ribose increased from 23.5 per cent to 26–29.6 per cent. (*c*) The ultraviolet absorption curve differed significantly from that of ribonucleic acid (Fig. 2). Particularly notable are the shift in absorption maximum from 260 mμ to 256 mμ, the shift in absorption minimum from 231 mμ to 226 mμ, and the marked increase in maximum absorption per unit weight of phosphorus. It is evident that AF is a polynucleotide whose constitution differs from that of classical yeast nucleic acid.

Effect of Intestinal Phosphatase on Active Fraction of Yeast Nucleic Acid.— Yeast nucleic acid has been found (18, 19) to be completely dephosphorylated by phosphatase preparations from calf intestinal mucosa. For this reason, the effect of this enzyme on the streptolysin-forming activity of the active fraction of yeast nucleic acid was studied.

Under conditions that were essentially the same as those employed by Zittle (19) in studying yeast nucleic acid, 100 mg. of active fraction of nucleic acid

was incubated at 25°C. with 33 mg. phosphatase in 63 cc. 0.025 M sodium bicarbonate. At the end of 7 hours, it was found that 90 to 95 per cent of the total phosphorus had been converted to inorganic phosphorus. Assay of the digestion mixture for streptolysin-inducing activity showed that 2 to 3 times as much digestion mixture was required to stimulate the same amount of hemolysin formation as was induced by an undigested control solution con-

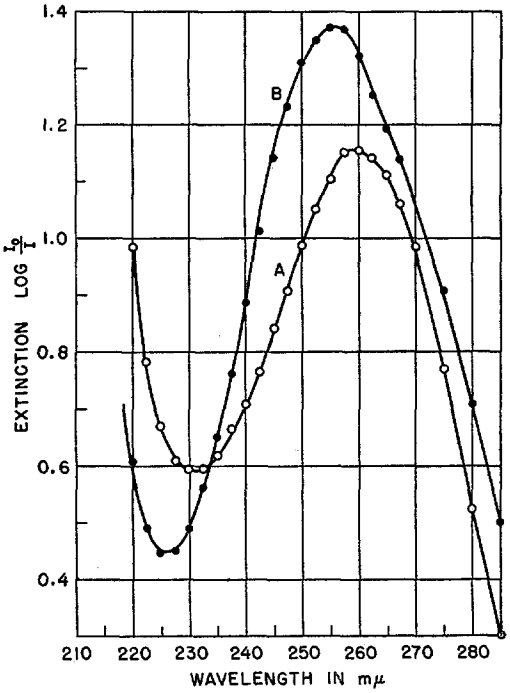

FIG. 2. Ultraviolet absorption spectra of yeast nucleic acid (*A*) and streptolysin-forming fraction (AF) of yeast nucleic acid (*B*). Both solutions diluted to contain 3.5γ phosphorus per cc. Absorption cells, 10 mm.

taining active fraction plus heat-inactivated phosphatase. It is evident that dephosphorylation resulted in a partial loss of activity.

Does Ribonucleic Acid, Regardless of Source, Possess a Ribonuclease-Resistant Fraction?—It has been repeatedly observed (16, 20–22) that yeast nucleic acid is incompletely digested by pancreatic ribonuclease, and that after treatment with the enzyme, there remains a ribonuclease-resistant, non-diffusible portion representing, according to Loring, Carpenter, and Roll (22), about 50 per cent of the original nucleic acid. The present studies show that the streptolysin-inducing activity is associated with the ribonuclease-resistant fraction. Since

the activity of ribonucleic acid was found to depend upon the source (Table II), those preparations from yeast, wheat germ, and streptococci being active, while that of tobacco mosaic virus being inactive, it was of interest to determine whether all of these preparations possessed ribonuclease-resistant fractions.

To approximately 20 mg. yeast sodium nucleate in 2 cc. 0.05 M borate buffer of pH 7.0, was added 1.6 mg. crystalline ribonuclease. The mixture, contained in a cellophane sac, was dialyzed against 1 liter of 0.05 M borate buffer at 22°C., with constant agitation. After 20 hours, the degree of hydrolysis was estimated from the quantity of purine and pyrimidine (absorption at 260 mμ) found in the residue and dialysate, and from analysis of the residue for total phosphorus. Preparations of nucleic acid derived from wheat germ, streptococcus, strain C203S, and tobacco mosaic virus, were treated with ribonuclease under conditions identical with those employed with yeast nucleic acid. In addition, the nucleate solutions were analyzed for desoxyribonucleic acid according to the method of Stumpf (23).

TABLE V

Diffusibility of Products of Ribonuclease-Treated Ribonucleic Acid Derived from Streptococci, Yeast, Wheat Germ, and Tobacco Mosaic Virus

Source of ribonucleic acid	Desoxy-ribonucleic acid content	(A) Proportion of 260 mμ absorbing substance which appeared in dialysate*	(B) Proportion of total P which appeared in dialysate‡	A, corrected for content of desoxy-ribonucleic acid§	B, corrected for content of desoxy-ribonucleic acid§
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Streptococcus C203S	8.5	69	62	75	68
Yeast	0.0	62	70	62	70
Wheat germ	11.5	53	59	60	67
Tobacco mosaic virus	—‖	62	—	62	—

* Determined in Beckman quartz spectrophotometer.

‡ Computed by subtracting total P inside of sac after dialysis, from total P before dialysis.

§ Calculation based on assumption that no desoxyribonucleic acid dialyzed.

‖ Not determined.

The results presented in Table V show that enzymatic treatment of the four ribonucleic acid preparations converted to diffusible form 75, 62, 60, and 62 per cent, respectively, of the 260 mμ-absorbing substances, and 68, 70, and 67 per cent of the phosphorus of the first three preparations. It therefore appears that preparations of nucleic acid derived from streptococci, yeast, wheat germ, and tobacco mosaic virus, resemble each other in possessing a ribonuclease-resistant fraction, and one which is present in about equal amount in all of them. It is evident, also, that the presence of a ribonuclease-resistant fraction does not mean that a particular nucleic acid will stimulate the production of hemolysin, since ribonucleic acid of tobacco mosaic virus, although possessing a ribonuclease-resistant fraction, failed to cause hemolysin formation.

II. Carbohydrates

The medium used in the experiments described in the preceding sections was peptone-infusion broth. As has already been stated, it was desired to produce the streptolysin under conditions which are better defined than those provided by this medium. For this purpose, a medium whose chemical composition is essentially defined (4) was substituted for peptone-infusion broth. In the defined medium, however, the streptococci produced relatively little streptolysin in the presence of an excess of AF, even though bacterial growth was comparable to that in peptone-infusion broth. Occasionally, cultures grown in defined medium containing AF produced 500 or more units of streptolysin

TABLE VI

Streptolysin-Promoting Effect of Peptone-Infusion Broth

Additions to defined culture medium	Hemolytic units per cc. of culture
None	<100
1 cc. YNA digest	<100
1 cc. YNA digest + 1 cc. peptone-infusion broth	1750
3 cc. peptone infusion broth	<100
1 cc. YNA digest + 1 cc. 1 per cent peptone	1000
1 cc. YNA digest + 1 cc. meat infusion	2000
1 cc. YNA digest + 0.5 cc. dialyzable fraction of meat infusion	1000
1 cc. YNA digest + 1 cc. non-dialyzable fraction of meat infusion	1000

Each tube contained 9 cc. defined medium (4), 0.05 per cent glucose, and 0.01 cc. inoculum.
YNA digest: 1 per cent solution of yeast nucleic acid containing 0.01 per cent ribonuclease.
Peptone-infusion broth prepared as described in Methods.
All solutions were sterilized at 15 pounds steam pressure for 15 minutes, excepting dialysis fractions which were sterilized at 100°C. for 10 minutes.

per cc., but the formation of this much streptolysin was unusual and not reproducible. When, however, a sufficient amount of peptone-infusion broth was added to the defined medium, the effect of AF was consistently demonstrable. It also was clear that, in the absence of AF, no amount of peptone-infusion broth was effective in inducing a significant degree of streptolysin formation. These observations suggested that peptone-infusion broth contains a factor, or factors, other than ribonucleic acid, necessary for the formation of streptolysin. When peptone and meat infusion were tested separately, it was found that either supplied the missing factor(s). The factor(s) in both materials withstood autoclaving at or near neutrality. Approximately 70 per cent of the activity of meat infusion was found to be dialyzable and was recoverable from the dialysate. Experimental data illustrating most of these results are summarized in Table VI.

Additional experiments showed that: (1) heating meat infusion at 100°C. for 60 minutes at pH 12 destroyed approximately three-fourths of the streptolysin-promoting activity; (2) heating the infusion at the same temperature for the same length of time at pH 1 resulted in little or no change in activity; (3) incineration of meat infusion destroyed completely the streptolysin-promoting activity.

Streptolysin-Promoting Effect of Maltose.—In order to find out whether they could replace meat infusion, a number of substances believed to be present in meat infusion, and having properties similar to those just described, were tested for streptolysin-promoting activity. It was found that very low concentrations of maltose consistently induced the formation of streptolysin in defined medium containing AF. The effect of maltose on streptolysin production is shown in Table VII. In studying streptolysin formation as a function

TABLE VII

Streptolysin-Promoting Effect of Maltose

Final concentration of maltose in medium	AF	Hemolytic units per cc. of culture
	cc.	
M/4000	0.1	2200
M/8000	0.1	2200
M/32,000	0.1	900
M/64,000	0.1	150
None	0.1	<100
M/4000	None	<100

Medium as described in reference 4 but containing 0.05 per cent glucose and 0.2 per cent potassium bicarbonate.

of maltose concentration, the amounts of streptolysin formed in response to the very lowest maltose concentrations were found not to be quantitatively reproducible. However, as little as M/64,000 maltose usually had a detectable effect, provided the initial concentration of glucose was M/360 and an excess of AF was present. The maximum yield of streptolysin, 1500 to 3000 units per cc. of culture, was obtained when the initial maltose concentration was approximately M/20,000. Higher concentrations sometimes depressed the yield. It was observed, also, that maltose was not effective in promoting streptolysin formation when the medium was deficient in AF (Table VII).

Effect of α-Glucosidase on Streptolysin-Promoting Action of Maltose.—Unless the effect of maltose is due to an impurity, it follows that enzymatic hydrolysis of maltose will destroy its streptolysin-promoting action.

To 20 cc. M/80 maltose in M/60 phosphate buffer, pH 7, was added 10 cc. water containing 10 mg. of a preparation of α-glucosidase. After setting the mixture at 30°C., 1.5 cc. aliquots

were removed at intervals, placed in boiling water for 5 minutes, and chilled. After bringing the volume of each aliquot to 5 cc., 0.1 cc. was tested for streptolysin-inducing activity, employing defined medium deficient in maltose but containing AF. In addition, each aliquot was tested for reducing sugar according to the method of Nelson (24), and from the reducing values, the extent of maltose hydrolysis was computed. Curves showing streptolysin-inducing action as a function of time, and maltose hydrolysis as a function of time are given in Fig. 3.

It can be seen that in 10 minutes, when approximately 45 per cent of the maltose had disappeared, there was an appreciable reduction in streptolysin-inducing activity, while at 60 minutes, when approximately 75 per cent of the maltose had disappeared, there no longer was stimulation of streptolysin formation. It is clear that hydrolysis of maltose was accompanied by disappearance of streptolysin-inducing capacity.

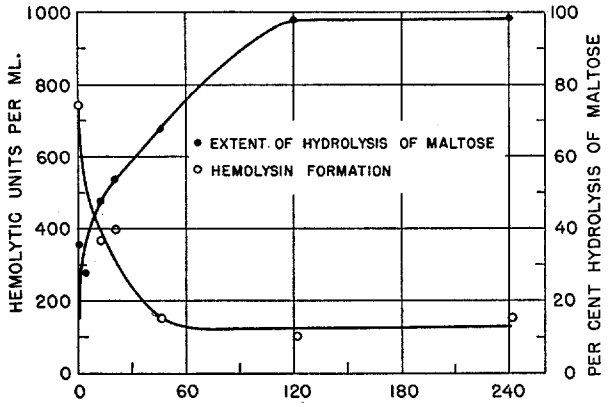

FIG. 3. Effect of α-glucosidase on streptolysin-promoting capacity of maltose. Abscissa: Time of hydrolysis of maltose in minutes at 37°C., pH 7.0. Zero time corresponds to M/40,000 maltose.

Specificity of Maltose.—In order to investigate the specificity of maltose in causing streptolysin formation, a considerable number of carbohydrates were tested.

After sterilizing by immersion in boiling water for 10 minutes, solutions of carbohydrates were added singly to defined medium containing AF and M/360 glucose. Most of the sugars and simple sugar derivatives were tested in final concentrations of M/4000 and M/40,000, while most of the polysaccharides were tested in final concentrations of 1 mg. and 0.1 mg. per cc. Additional concentrations were tested when the results indicated this to be desirable. Maltose, in a concentration of M/30,000, produced approximately 1000 units of streptolysin per cc., and it was arbitrarily assigned a "streptolysin-producing value" of 100. The relative activities of other carbohydrates were computed from the concentrations required for the formation of 1000 units of streptolysin. When the molecular weights were known, the relative activities were reckoned on a molar basis; otherwise on a weight basis. The streptolysin-inducing capacity of the substances tested is shown in Table VIII.

TABLE VIII

Streptolysin-Inducing Capacity of Carbohydrates and Related Substances

	Activity relative to maltose
Monosaccharides	
d-Glucose	<5
d-Mannose	10
l-Rhamnose	<5
d-Fructose	<5
d-Galactose	<5
d-Arabinose	<10
d-Ribose	<2
d-Xylose	<5
Disaccharides	
Maltose (4-*d*-glucopyranosyl-α-*d*-glucopyranoside)	100
Lactose (4-*d*-glucopyranosyl-β-*d*-galactopyranoside)	<5
Sucrose (1-α-*d*-glucopyranosyl-β-*d*-fructofuranoside)	<5
Trehalose (1-α-*d*-glucopyranosyl-α-*d*-glucopyranoside)	20
Cellobiose (4-*d*-glucopyranosyl-β-*d*-glucopyranoside)	<5
Melibiose (6-*d*-glucopyranosyl-α-*d*-galactopyranoside)	<5
Tri- and polysaccharides	
Raffinose	10
Glycogen (beef liver)	ca. 2
Glycogen (oyster)	ca. 2
Starch	ca. 2
Dextrin	10
Inulin	<10
Potassium hyaluronate (umbilical cord)	2
Potassium hyaluronate (vitreous humor)	<1.5
Chondroitin sulfate	<1.5
Hexahydric alcohols	
Inositol	<5
Dulcitol	<10
Mannitol	<5
Sorbitol	<5
Miscellaneous hexose derivatives	
Sodium thioglucose	<5
Glucosamine	30–50
N-Acetylglucosamine	<10
Glucose-1-phosphate	<5
Fructose-6-phosphate	<5
Fructose-1,6-diphosphate	<5
α-Methylglucoside	<5
Salicin	<5
Calcium maltobionate	<5

We are indebted to Dr. W. F. Goebel for supplying us with mammalian glycogen, to Dr. M. Levy for oyster glycogen, to Dr. M. P. Schubert for sodium thioglucose, *N*-acetylglucosamine, potassium hyaluronate, and chondroitin sulfate, and to Dr. F. H. Stodola for calcium maltobionate.

It is evident that none of the substances tested was as active as maltose. Approximately 10 times as much dextrin, and about 50 times as much glycogen, were required to produce the same effect as maltose. Of the sugars having configurations most closely resembling that of maltose, only trehalose was found to be active, and its activity was about one-fifth that of maltose. None of the pentoses was found to be active, and of the hexoses tested, only *d*-mannose possessed significant activity. It was about one-tenth as active as maltose. Other than maltose and trehalose, none of the α-glucosides, hexahydric alcohols, or phosphorylated hexoses tested was found to be active. In contrast to these findings, glucosamine proved to be highly active. In concentrations of M/10,000 to M/15,000, it produced as great an effect as M/30,000 maltose. *N*-Acetylglucosamine was found to have little or no activity.

DISCUSSION

Particular attention has been paid to the specificity of ribonucleic acid in inducing hemolysin formation, and the results show that no other substance tested, including desoxyribonucleic acid as well as acid and alkaline hydrolysis products of ribonucleic acid, produces the same effect as ribonucleic acid. It should be noted, however, that not all preparations of ribonucleic acid are active. The activity apparently depends upon the source. Preparations of ribonucleic acid derived from yeast, wheat germ, streptococci, and mammalian liver, were found to be active, while nucleic acid derived from tobacco mosaic virus was found to be inactive. It appears significant that after treatment with ribonuclease, the active preparations possessed, on a weight basis, approximately equal activity.

Attention may be called to the observation that ribonucleic acid prepared from the test strain of streptococcus, C203S, and subsequently treated with ribonuclease, was found to be approximately as active as ribonuclease-treated nucleic acid prepared from yeast. It is evident, therefore, that the cocci contain ribonucleic acid having potential streptolysin-forming activity, and it is evident, also, that this nucleic acid must have been synthesized by the streptococci because the cocci had been cultivated in a medium free of nucleic acid.

Since the quantity of streptococcal growth attained in a broth culture is less than 0.5 mg., dry weight, per cc., and since approximately 15 per cent (25) of the dry weight of streptococci is ribonucleic acid, it can be calculated that the concentration of streptococcal ribonucleic acid in fully grown broth cultures is not greater than 0.075 mg. per cc. Reference to Fig. 1 shows that this concentration of ribonucleic acid is only one-thirtieth of that necessary to induce the formation of 1000 hemolytic units. It is therefore not surprising to find that plain broth cultures of streptococci usually contain less than 100 hemolytic

units per cc., even though the cocci are able to synthesize nucleic acid which is potentially capable of inducing streptolysin formation.

The finding that nucleotides and their hydrolysis products fail to replace ribonucleic acid in forming streptolysin confirms statements to the same effect published by Itô (26). However, our finding that desoxyribonucleic acid is inactive, contrasts with observations made by Itô, in whose experience desoxyribonucleic acid prepared from cow spleen could replace yeast ribonucleic acid.

Fractionation of yeast nucleic acid shows that the activity is present in the ribonuclease-resistant portion, and with this knowledge, it has been possible to prepare a polynucleotide (AF) possessing activity many times greater than that of the starting material. The increased nitrogen:phosphorus ratio of AF, and the increased content of purine ribose, are in agreement with recent observations of Loring, Carpenter, and Roll (22) and with those of Schmidt, Cubiles, Swartz, and Thannhauser (27) on the nature of action of ribonuclease on yeast nucleic acid.

There remains the question of whether the activity studied in the present investigation is actually due to a fraction of ribonucleic acid or to a contaminating substance which tends to be coprecipitated with ribonucleic acid. In regard to yeast nucleic acid, it seems likely from the data reported in the foregoing section, that the streptolysin-inducing effect is caused by an integral component of yeast ribonucleic acid itself. In any event, it is clear that the active substance is either (1) a portion of the ribonuclease-resistant polynucleotide, or (2) a substance which is closely associated with that component. We are inclined to favor the former view, but it must be admitted that the evidence does not conclusively eliminate the second possibility.

The biological importance of desoxyribonucleic acids is clearly indicated by Avery, MacLeod, and McCarty's demonstration (28) of the capacity of desoxyribonucleic acid to direct specifically the synthesis of pneumococcal capsular polysaccharide. Although ribonucleic acid also is commonly believed to play an important rôle in cellular processes, there are but few instances in which its functions have been defined. For this reason, Okamoto's discovery of a specific biological effect of yeast nucleic acid seems to merit greater attention than has been accorded it hitherto. The results of the present study confirm and extend Okamoto's observation of the capacity of yeast nucleic acid to stimulate the formation of a potent streptolysin, and, in so doing, provide a technique which may prove useful in the study of nucleic acid structure and metabolism.

In regard to the specificity of maltose in promoting streptolysin formation, the results summarized in Table VIII show that of the disaccharides tested, only maltose and trehalose were active. It may be noted that both are α-glucosides. Although α-galactoside (melibiose) as well as disaccharides having the β-configuration were not active, the number of α-glucosides tested is so

small that no conclusion can be drawn concerning the activity of α-glucosides in general. It is of interest that cellobiose, a β-glucoside, but otherwise closely resembling maltose in structure, was not active. The streptolysin-promoting activity of trehalose suggests that a free aldehyde group is not essential for activity. The results are somewhat complicated by the finding that mannose possessed some activity, and still more by finding that glucosamine was almost as active as maltose.

There are on record several observations which show that certain carbohydrates favor the production of toxins by bacteria. In the production of diphtheria toxin, maltose has long been used in preference to glucose as the chief energy-supplying compound (29). In studying the production of α-toxin of *Clostridium welchii*, Logan, Tytell, Danielson, and Griner (30) have shown that the best yields of toxin are obtained when the medium contains dextrin as the sole or principal carbohydrate. This observation has been confirmed by Adams, Hendee, and Pappenheimer (31). In both investigations, it was observed that maltose was less effective than dextrin, although more effective than glucose, in promoting toxin production. It is not known whether a common mechanism underlies these effects on the production of toxin by different bacteria.

On the basis of the information presented, it is scarcely permissible to speculate on the rôle of AF and of carbohydrates in promoting streptolysin formation. There is one point, however, which is worthy of comment. The results show that very small amounts of either maltose or glucosamine can be detected in the presence of relatively large amounts of glucose. In the case of maltose, 6 γ per cc. produces a detectable effect in a culture containing, initially, 80 times as much glucose. Although the streptococci can utilize either maltose or glucosamine as a source of energy, it may be questioned whether they do so in the presence of a relatively high concentration of glucose. The possibility that maltose and glucosamine are not oxidized, but are used instead, as units from which larger molecules are synthesized, should be considered.

As a consequence of the present study, it is possible to produce the nucleic acid–induced streptolysin under comparatively well defined conditions. Using a peptone-free medium, supplemented with apporpriate amounts of AF, maltose, and glucose, streptolysin has been obtained in fair yield in cultures of 5 liters in volume. The hemolytic titers obtained in this medium have been about half as great as those which can be got in peptone-infusion broth containing AF. The conditions required for maximum streptolysin production, therefore, are still not completely defined. The peptone-free medium, however, affords advantages which offset the disadvantage of a reduced yield. For example, by employing this medium, the nucleic acid–induced streptolysin has been prepared in concentrated, partially purified form with relatively little difficulty.

SUMMARY

1. Ribonucleic acid of yeast causes the formation of a potent hemolysin in broth cultures of *Streptococcus pyogenes*.

2. The hemolysin whose formation is induced by yeast ribonucleic acid appears to be identical with streptolysin S.

3. Desoxyribonucleic acid, products of acid or alkaline hydrolysis of ribonucleic acid, or many other substances tested, fail to produce a similar effect.

4. Digestion by ribonuclease increases markedly the streptolysin-inducing activity of certain preparations of ribonucleic acid.

5. A fraction (AF) of yeast nucleic acid has been isolated which possesses approximately 100 times the streptolysin-inducing capacity of the starting material. Some of the properties which distinguish AF, a polynucleotide, from ordinary yeast nucleic acid are described. AF is associated with the ribonuclease-resistant fraction of yeast nucleic acid.

6. Ribonucleic acid prepared from streptococci, wheat germ, and mammalian liver, and subsequently treated with ribonuclease, is about as active in causing streptolysin formation as ribonuclease-treated yeast nucleic acid.

7. Ribonucleic acid of tobacco mosaic virus, tested under comparable conditions, was found to be inactive.

8. Ribonucleic acid prepared from streptococci, wheat germ, and tobacco mosaic virus resembles yeast nucleic acid in possessing a ribonuclease-resistant fraction.

9. In addition to AF, a factor (or factors), present in meat infusion and in peptone, was found to be required for the formation of streptolysin.

10. The factor can be partially replaced by any one of several carbohydrates, the most active being maltose, glucosamine, and trehalose, in that order.

11. When appropriate concentrations of AF, maltose, and glucose are used, the nucleic acid–induced streptolysin can be produced in a medium whose chemical composition is essentially defined.

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