# STUDIES ON STREPTOCOCCUS BACTERIOPHAGE.

## I. A Powerful Lytic Principle against Hemolytic Streptococci of Erysipelas Origin.

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### INTRODUCTION.

The influence of the bacteriophage upon streptococci was studied by several authors. Piorkowski (1) was the first to report a lytic principle against streptococci. He mixed 2 cc. of blood from a patient suffering from subacute bacterial endocarditis with glucose broth and filtered this mixture after 4 days incubation. The filtrate showed lysis of the streptococcus obtained from the blood of the same patient. The lytic principle was, according to Piorkowski, transmissible. The qualitative and quantitative potency of this principle was not studied, or were observations made as to whether the principle had any effect upon other pathogenic streptococci.

McKinley (2) claimed that he was able to obtain a lytic principle against a large group of Gram-negative and Gram-positive cocci, including streptococci. To demonstrate such a principle he filtered a given culture containing anti-b. Shiga phage, reinoculated the filtrate, incubated, and filtered again. This was repeated three or four times in succession. When 5 cc. of such a filtrate, diluted 1:10 with broth, was inoculated with the organism from which it was obtained, it failed to grow. This author did not do any experiments on the properties of this principle; namely, on the qualitative and quantitative potency, formation of resistants under its influence, and regeneration of this principle by the resistants. It would be impossible, therefore, to accept these inhibitory factors as evidence of bacteriophage.

Dutton (3) studied the appearance of colonies of various strains of

streptococcus. A large number of strains contained irregular and "moth eaten" colonies and, therefore, suggested symbiotic existence of bacteriophage. Only one strain had proven itself susceptible to a lytic principle obtained from another strain which failed to grow on transplant. The evidence brought forward suggested the existence of streptococcus bacteriophage of a low order which in the majority of instances is not able to produce the classical picture of the bacteriophage phenomenon.

Further work was reported by Hadley and Dabney (4). These authors were able to obtain a potent bacteriophage for one strain of *Streptococcus fæcalis*.

Clark and Clark (5, 6) obtained from activated sludge a bacteriophage which was highly potent against two strains of hemolytic streptococci isolated from rabbit and guinea pig lung infections. They failed to adapt this principle to five strains of human pathogenic streptococci.

It is evident that great difficulties accompany attempts to produce an active lytic principle against streptococci. The isolated positive results with a few strains, only two of which were of human origin (Piorkowski (1) and Dutton (3)), leaves the field open for further investigation. The question therefore arises as to whether streptococci as a genus are susceptible to the bacteriophage phenomenon or whether the few strains of streptococci in which this phenomenon was observed carried a factor, not common to other streptococci as a genus, which enabled them to be affected by the phage. With these considerations in mind the author attempted to obtain an active principle against pathogenic human streptococci. The following plan was adopted for this work:

1. To study the effect of Clark and Clark's "sludge" bacteriophage upon the susceptible strain of rabbit streptococcus in order to determine the properties of this principle, its specificity and relation to the classical bacteriophage phenomenon.

2. To make attempts to obtain a powerful bacteriophage for pathogenic streptococci by means of the Clark and Clark phage as well as by other lytic principles.

3. Should these attempts succeed, to study the properties and specificity of such a bacteriophage.

## EXPERIMENTAL.

### *Lytic Principles Tested against Pathogenic Streptococci.*

The following lytic principles tested on various occasions against streptococci will be mentioned in the course of this paper:

*B.H.*—A lytic principle kindly sent to me by Dr. A. Gratia, isolated by him from vaccinia pulp. This principle was potent against numerous strains of *Staphylococcus aureus* and *albus*.

*Anti-B. coli Phages.*—A large series of extremely potent bacteriophages was obtained by the author from stools of various animals. They will be mentioned under the name of the animal from which they were obtained.

*$L_{12}$ and C/2.*—$L_{12}$ represents anti-b. Shiga bacteriophage kindly sent to me by Dr. J. Bronfenbrenner. C/2 is the same phage but adapted to *B. coli*.

*"Sludge" phage.*—This principle was isolated by Drs. Clark and Clark from activated sludge and kindly sent to me a year ago. It proved itself extremely active against a *Streptococcus hæmolyticus* isolated by the same authors from a rabbit. The susceptible strain will be referred to as "Rb" streptococcus.

### *Strains of Streptococci Employed in This Work.*

Numerous strains of hemolytic and green-producing streptococci employed in this work were isolated from various sources in this laboratory. Others were kindly sent to me by Dr. A. W. Williams, Dr. Konrad Birkhaugh, and by the American Type Culture Collection. The nature of these strains will be described as the occasion arises.

### I.

Observations on general properties of "sludge" phage corroborate Clark and Clark's report (6). The following requirements which are necessary to identify this principle with the classical bacteriophage can be fully answered:

1. Lysis can be demonstrated on solid media (in the form of so called "eaten up" areas) and in fluid media. The principle diluted to $1 \times 10^{-8}$ cc. is still able to produce complete inhibition of growth. After lysis occurs such a culture shows again the full concentration of undiluted lytic principle.

2. Lysed culture eventually gives rise to growth. Such a growth is able to regenerate bacteriophage in turn when transplanted into a fluid medium.

3. Formation of resistants is accomplished by prolonged cultiva-

tion of susceptible streptococci with at first highly diluted and then undiluted lytic principle.*

4. Immunization of rabbits with lysed cultures brings about formation of antibacteriophage serum.

5. In addition it should be mentioned here that this principle is less stable than *coli* dysentery phages. When "sludge" phage is heated to 58° for ½ hour its lytic exponent ($E_L$) is reduced from −8 to −3. Control heating of dysentery phage to the same $t°$ does not affect it whatsoever. The lytic potency is also liable to become greatly reduced on standing in the refrigerator for 2 to 3 weeks.

6. The effect of "sludge" phage on other streptococci was studied in fluid media and observations were made 24 hours and 2 to 3 days later. In the course of 1 year 102 strains of pathogenic streptococci of human origin were tested, as follows: 12 strains of the group of scarlet fever hemolytic streptococci; 18 strains of *Streptococcus erysipelatis;* 3 strains isolated by Tunnicliff from cases of measles; 1 strain of septic sore throat, 1 strain from Rosenow's ulcerative colitis case; 1 strain from Rosenow's case of acute anterior poliomyelitis; 12 strains of green-producing streptococci from various human infections; 16 strains of anhemolytic streptococci of human origin, and 38 strains of hemolytic *pyogenes* strains. In not a single instance did "sludge" phage show the slightest suggestion of lysis. The specific lytic effect of this bacteriophage only upon the two strains of streptococcus mentioned above is very striking. It was of interest therefore to study the nature of this resistance as compared to the same phenomenon encountered in various strains of bacteria generally susceptible to the phage.

From the studies on the bacteriophage phenomenon it is evident that two types of resistance to phage can be differentiated.

1. *Apparent Resistance.*—Among the genera susceptible to phage, occasional strains can be found which do not undergo any lysis when brought into contact with a given principle. However, these strains are able to regenerate bacteriophage and carry it from generation to generation without being attacked themselves.

* The first three points were published by Clark and Clark when this work was almost completed.

2. *True Resistance.*—Certain genera of bacteria for which no bacteriophage was as yet found; or occasional strains of susceptible bacteria; or individual colonies of bacteriophage carrying strains show complete indifference to this principle. They are not attacked by it and are not able to regenerate it. In order to determine to which of these categories the resistance of streptococcus belongs, experiments were made in a quantitative manner and the fate of "sludge" phage in resistant cultures was followed up. Strains 55 (Williams—scarlet fever); E, (Birkhaugh—erysipelas); V-(*viridans*—endocarditis); H (*hæmolyticus*—meningitis) were used for these experiments. Flasks of broth containing $1 \times 10^{-5}$ cc. dilution of Clark's phage ($E_L - 8$) were inoculated with these strains. In 24 hours the cultures were filtered and the filtrates tested in various dilutions against "Rb" streptococcus. All these filtrates were able to produce lysis of "Rb" streptococcus in dilutions up to $1 \times 10^{-2}$ cc. only. It is evident that no regeneration of lytic principle was attained by any of the streptococci employed in these experiments. The conclusion was, therefore, that streptococci showed true resistance to lytic principle. In view of the fact that reproduction of the bacteriophage phenomenon is impossible without the ability of regeneration on the part of bacteria, it was thought advisable to direct the work towards creating favorable conditions which would enable streptococci to regenerate phage. Should these efforts fail it would then be of interest to attempt to train streptococci to perform this function.

II.

*Adaptation of Various Lytic Principles to Hemolytic Streptococci.*

In this experiment the usual method of "adaptation" of phage to a resistant microorganism was used. Various anti-*coli* phages, B.H. and "sludge" phage, were titrated against three strains of scarlet fever streptococci (Williams—55, 108, 130); one strain of erysipelas (Birkhaugh—$E_1$), and five strains of *pyogenes* hemolytic streptococci. In 24 hours each titration was then pooled in a flask, diluted with broth, and incubated for a few days. These cultures were filtered, the filtrates reinoculated with the respective strains, some lytic principle added, and the mixtures incubated again. This process of successive filtration,

reinoculation, and incubation was repeated four times. The final filtrates were tested against the above mentioned strains of streptococci. Occasionally, a filtrate used in dilution 1:10 would show a suggestion of lysis. However, it was never possible to duplicate such a result and all these trials remained frankly negative. Since in the majority of instances exact record was kept of the amount of lytic principle added, it was possible to determine by means of experiments described in Part I that there was never any regeneration of these principles.

### *The Influence of Age of Culture upon Susceptibility to the Bacteriophage.*

The original lytic principles as well as various filtrates of the preceding experiment were tested against the above mentioned scarlet fever streptococcus cultures of various ages from 6 hours to 1 month old. Only in one case, "pig" anti-*B. coli* phage showed complete lysis with 1 month old culture of Strain 55 without regeneration however. It was impossible to repeat this result. All the other results were distinctly negative.

### *The Influence of Various Degrees of Anaerobiosis Tested at Different Hydrogen Ion Concentration upon the Susceptibility of Streptococci to Bacteriophage.*

The author has previously demonstrated (7) that a quantitatively weak principle was able to produce complete lysis in culture of *B. coli* placed under partially anaerobic conditions in cases where it failed to do so under frankly aerobic conditions. Moreover (8) definite reciprocal relationship had to be observed between the hydrogen ion concentration and oxygen tension in order to enable *B. coli* to retain its susceptibility to phage. These observations were applied to the studies on streptococci with the hope that more favorable conditions would be found for obtaining lysis and regeneration of bacteriophage. For this purpose Strains 55 and 108 were cultured for three generations in broth of different pH under various oxygen tension conditions. The different cultures were then tested against several lytic principles under similar conditions. No lysis was observed in any of these cultures and no regeneration obtained.

*The Influence of Temperature on Lysis of Streptococci.*

Experiments with principle B. H. and staphylococcus showed the following: If B. H. was not regenerated for a period of 2 to 3 weeks it was apt to lose its potency almost completely. However, if a culture of staphylococcus containing such a principle was left at room temperature for a few days sudden complete clearing occurred. Such a lysed culture then contained a lytic principle which was able in turn to produce prompt lysis at 37°. It was decided to apply this observation to the studies upon streptococci and combine it with the method of the preceding experiment. The preceding experiment was repeated but cultures containing lytic principle were left at room temperature for over 2 weeks. No lysis appeared under these conditions and no regeneration of lytic principles was noticed.

*Addition of "Activating" Factors to Cultures of Resistant Streptococci.*

On the assumption that spontaneously resistant strains of streptococci may not produce regeneration on account of the absence of some activating factors, the following experiments were undertaken: These hypothetical factors were looked for in cultures of "Rb" streptococcus which always showed remarkable susceptibility. Cultures of 55,108, and 130 strains were grown in broth containing a suspension of live "Rb" streptococcus, or filtrate of live culture, or a suspension of this culture autolysed by freezing and thawing. (Autolysis was made aerobically and anaerobically.) In no instance did the strains prepared in such a manner show any change in susceptibility to "sludge" phage.

*"Training" of Streptococci to Regenerate Bacteriophage.*

The lack of power regeneration under various supposedly favorable conditions suggested the necessity of employing some method which would "train" the strains under question to perform this function. In the methods ordinarily employed the lytic principle was "adapted" to the microorganisms. In contrast to this method (page 501), the author attempted to "adapt" the microorganism to the lytic principle. Various strains of human pathogenic streptococci chosen for this experiment were subcultured every 24 hours in broth containing 1:10 dilution of lytic principle. After several such passages

the cultures were inoculated into flasks of broth, incubated for 24 hours, filtered, and 0.5 cc. of each of these filtrates tested against homologous normal cultures of streptococci. Table I represents the results obtained.

As is seen from this table, sixteen strains of hemolytic streptococci of erysipelas origin acquired the property of regenerating lytic principle active against normal cultures of their homologous strains. The

TABLE I.

| Strain of streptococcus | Lytic principle | Number of passages through phage | Degree of lysis of normal culture *vs.* filtrate of "adapted" culture of the same strain |
|---|---|---|---|
| Birkhaugh erysipelas $E_1$ | C/2 | 15 | 0 |
| 10 erysipelas strains (Birkhaugh) $E_1$–$E_{10}$ | "Sludge" | 12 | 4+ |
| Erysipelas—E | C/2 | 36 | 0 |
| *Viridans* subacute bacterial, endocarditis | "Sludge" | 30 | 0 |
| Scarlet fever 55, 108, 130, 4, 84, 42, "L", "M" | "Sludge" | 20 | 0 |
| Scarlet fever 55, 108, 130 | "Sludge" | 70 | 0 |
| "Rb" streptococcus | C/2 | 15 | 0 |
| "Rb" streptococcus | "Pig" | 20 | 0 |
| *Pyogenes* hemolytic 2 strains | "Sludge" | 37 | 0 |
| Scarlet fever 55, 108, 130 | C/2 | 19 | 0 |
| Scarlet fever 55, 130 | "Pig" | 18 | 0 |
| 2 strains *pyogenes* hemolytic streptococcus | C/2 | 31 | 0 |
| Erysipelas strains Birkhaugh 243, W 248, 214, 268. | "Sludge" | 12 | 4+ |
| American Type Culture Collection erysipelas 766, 777 | "Sludge" | 12 | 4+ |
| American Type Culture Collection erysipelas 768, 425 | "Sludge" | 12 | 0 |
| 769 Birkhaugh 239, 218 | "Sludge" | 12 | 0 |

same method failed to give any results with the same principle cultivated with other pathogenic streptococci, and another lytic principle failed to give results with the erysipelas strain. The following two explanations offered themselves to account for the failure of numerous other streptococci to acquire the property of regeneration.

It is quite possible that the strains which could be eventually trained to regenerate phage normally possessed this property in a *latent* condi-

tion before the experiment began, while the other strains which failed to acquire this property by this method—*entirely* lacked it. However, C/2 phage failed to evoke this property in a strain of erysipelas streptococcus, which was shown to undergo this change with "sludge" phage. There remained, therefore, the second possibility, namely that the phage employed must also possess a special ability to evoke such a change. The question as to whether the resistant streptococci would not eventually demonstrate the function of regeneration if only a proper phage is offered to them in the process of "training" is at present being followed up.

*Streptococcus Erysipelas Bacteriophage.*

The following observations were made on the erysipelas streptococcus bacteriophage:

Quantitative and qualitative estimation of potency of phage was made by titration in broth. The filtrate of a broth culture of a strain which was trained to regenerate a powerful lytic principle showed lytic exponent $-4$. The last tube showing lysis was filtered. The filtrate then showed $E_L - 8$. Such a lytic principle could be kept at this titer and transmitted indefinitely through contact with normal cultures of erysipelas strains. Care had to be taken to regenerate the phage frequently since the lytic principle had a tendency to partially lose its potency on standing. As is seen from these observations, the lytic principle regenerated by a "trained" strain was afterwards easily regenerated by normal cultures. Evidently, therefore, the process of obtaining phage for a resistant strain consists of the following stages:

(*a*) "Adaptation" of such a strain to the "sludge" lytic principle bringing about appearance of power of regeneration. (*b*) Change of the "sludge" lytic principle offered to the strain to a state which allows its regeneration by a normal and, therefore, unprepared culture of the same strain. To sum up the results so far obtained: (1) It is interesting to note that resistant strains of streptococcus may lack completely the ability of regeneration for one type of phage (C/2). (2) They may have a "latent" potentiality of regeneration for another type of phage ("sludge"). (3) They may have this property in an active state for the third type of phage (erysipelas phage).

The strain of erysipelas streptococcus which was "trained" to regenerate an antierysipelas streptococcus phage was studied.

1. When a subculture on solid medium was tested against streptococcus erysipelas phage it showed complete resistance as demonstrated by the absence of "eaten up" areas in those areas where a drop of phage was put on the surface of a bacterial film. Nor did any suggestion of lysis occur in fluid medium.

TABLE II.

| | "Sludge" phage *vs.* Rb streptococcus | "Sludge" phage *vs.* erysipelas streptococcus | "Erysipelas" phage *vs.* Rb streptococcus. | "Erysipelas" phage *vs.* erysipelas streptococcus |
|---|---|---|---|---|
| Degree of lysis | 4+ | 0 | 4+ | 4+ |
| Lytic exponent | −8 | 0 | −8 | −8 |

TABLE III.

*Degree of Lysis.*

| Dilutions of sera | −1 | −2 | −3 | −4 |
|---|---|---|---|---|
| Serum 87 *vs.* 0.5 cc. "sludge" phage + Rb streptococcus.. | 0 | 0 | 0 | 0 |
| Serum 87 *vs.* 0.5 cc. erysipelas phage + erysipelas streptococcus | 0 | 0 | 0 | 0 |
| Serum 212 *vs.* 0.5 cc. of "sludge" phage + Rb streptococcus | 0 | 0 | 4+ | 4+ |
| Serum 212 *vs.* 0.5 cc. erysipelas phage + erysipelas streptococcus | 0 | 0 | 0 | 0 |

4+ = complete lysis; 0 = normal growth.
Serum 87 prepared by immunization with "sludge" phage.
Serum 212 prepared by immunization with erysipelas phage.

2. The above mentioned strain was also streaked on the surface of a plain agar plate. Microscopic examination of colonies appearing in the course of 1 week did not show any striking morphological changes such as described for other microorganisms acted upon by phage.

The following colonies were observed: large smooth but slightly irregular with occasional erosions in the center or at the margin of the

colony; small smooth colonies with irregular margin; irregular fine masses of cocci without any definite colony formation. These colonies were grown in broth and tested against erysipelas bacteriophage. They all showed the same degree of resistance. The filtrates of fluid cultures contained active lytic principle. It was, therefore, impossible to obtain resistant but bacteriophage-free colonies, such as can be done with other microorganisms. A more painstaking search should, however, be made. The above observations leave no doubt that the lytic principle obtained is in no respect different from the classical bacteriophage.

TABLE IV.

| Strains tested *vs.* erysipelas phage | Lytic exponent | Degree of lysis |
|---|---|---|
| 10 strains of streptococcus erysipelas (Birkhaugh) $E_1$–$E_{10}$ | −8 | 4+ |
| Birkhaugh, erysipelas streptococcus 243, W 248, 214, 268 | −8 | 4+ |
| American Type Culture Collection 766, 767 streptococcus erysipelas | −8 | 4+ |
| Williams, scarlet fever streptococcus 55, 130, 108, 4, 84, 42 | 0 | 0 |
| Rb *Streptococcus hæmolyticus* | −8 | 4+ |
| Mt. Sinai, scarlet fever streptococcus "M", "L" | 0 | 0 |
| Streptococcus erysipelas, Birkhaugh 239, 218 | 0 | 0 |
| Mt. Sinai, hemolytic pyogenic streptococci, 36 strains | 0 | 0 |
| American Type Culture Collection streptococcus erysipelas, 768, 425, 769 | 0 | 0 |
| Mt. Sinai, green-producing streptococci, various conditions, 8 strains | 0 | 0 |
| Mt. Sinai, anhemolytic streptococci, 5 strains | 0 | 0 |
| American Type Culture Collection anhemolytic streptococci 421, 422, 423, 349, 345 hemolytic, sore throat 543 | 0 | 0 |

In order to determine the relations of "sludge" phage to the erysipelas lytic principle, antiphage sera were prepared with both phages. Care was taken to "purify" the erysipelas phage by several transmissions through normal cultures of erysipelas strains. Tables II and III represent the results obtained.

As is to be noted, both phages are able to lyse "Rb" streptococcus, while only erysipelas phage is able to dissolve the erysipelas strain. It can be assumed, therefore, that both phages contain a common component and that the erysipelas phage has also a specific portion which makes possible lysis of erysipelas streptococcus. Since the "sludge" phage serum is also able to neutralize erysipelas phage it is apparent

that the presence of the common component in addition to the specific portion is essential for the action of the erysipelas phage. A difference can be detected in the effect of antierysipelas phage serum upon both phages. This serum neutralizes the "sludge" phage in dilution 1:100 and the erysipelas phage in dilution 1:10,000. No explanation is at present offered for this result.

Studies on the specificity of the erysipelas streptococcus bacteriophage are recorded in Table IV.

Thus this bacteriophage is able to attack about 76 per cent (16 out of 21) of all the tested hemolytic streptococci of erysipelas origin besides the "Rb" streptococcus. The specificity of this phage is of extreme interest. As is seen, no other streptococci out of 64 strains tested is attacked to the slightest degree. It would be of interest to study the relation of the specific agglutinogen of this group to the susceptibility to this phage.

## SUMMARY AND CONCLUSIONS.

1. The "sludge" phage obtained by Clark and Clark answers all requirements for pronouncing it identical with the classical bacteriophage.

2. The "sludge" phage failed to produce lysis in any of the 102 human pathogenic streptococci tested.

3. Numerous attempts to induce regeneration of various lytic principles by human streptococci resulted in failure.

4. It was possible, however, to "train" erysipelas streptococci to regenerate a lytic principle active against 76 per cent of strains of this group.

5. The erysipelas phage showed remarkable specificity.

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