STUDIES ON BACTERIOPHAGES OF HEMOLYTIC STREPTOCOCCI

I. Factors Influencing the Interaction of Phage and Susceptible Host Cell

By RICHARD M. KRAUSE, M.D.

(From The Rockefeller Institute for Medical Research)

Plate 37

(Received for publication, May 1, 1957)

The bacteriophages of β-hemolytic streptococci studied by Evans (1–4) were classified into several races or types. It is clear from this early work that the phages demonstrated some degree of specificity for streptococcal groups. For instance, a bacteriophage which lysed almost all group C streptococci did not lyse group A streptococci. However, much of this work was done prior to the serologic classification of hemolytic streptococci into groups on the basis of their group-specific carbohydrate, and the later separation of group A streptococci into numerous types on the basis of the type specific M protein (5). It was of interest, therefore, to reinvestigate the bacteriophages of hemolytic streptococci in the light of current knowledge.

Since this work was begun, Maxted (6) and Kjems (7) have reported recent studies with these phages. Maxted investigated four group A phages, identical with those used in the present study, and his findings indicated that they are not specific for their propagating type strains. He demonstrated the role of the hyaluronic acid capsule by showing that mucoid encapsulated strains which were phage-resistant could be rendered sensitive by the use of hyaluronidase. Kjems was able to obtain bacteriophage from many strains isolated from the oropharynx of patients with streptococcal sore throat, thus indicating the existence of lysogenic streptococci.

The specificity of the streptococcal phages for their respective homologous serologic group and type of streptococci was investigated in the present study. The initial experiments on host range support the view that the specificity of the phages is determined in part by the serologic group of the hemolytic streptococci. The work of many investigators (8) has indicated that a specific constituent of the bacterial cell surface can be identified as a phage inhibitor and therefore presumably the attachment site for the infecting phage. Much information concerning the antigenic and chemical composition of the streptococcal cell wall is now available as a result of the studies of Lancefield (5) and McCarty (9, 10) and it has been found that the C carbohydrate which determines the serological group of streptococci comprises at least 50 per cent of the cell wall. In view of this fact, experiments were conducted to determine whether this constituent of the cell surface plays the role of a phage receptor.

The work reported demonstrates quite clearly that group C phages are inactivated by the chemically purified cell wall carbohydrate of group C streptococci, indicating it may serve as the locus of phage attachment. As yet it is not clear which component of the group A streptococcal cell wall plays this role in the case of group A bacteriophages.

Finally, evidence will be presented showing that following phage lysis of streptococci the group carbohydrate antigen can be identified both in the centrifuged sediment and the supernatent of a lysate, thus indicating some dissolution of the cell wall during lysis. Furthermore, it has been found that fresh phage lysates of group C streptococci are capable of causing rapid lysis of groups A and C streptococci and of their isolated cell walls. This process is presumably enzymatic in character and also results in the release of group carbohydrate into solution.

Materials and Methods

Bacteriophages.—Bacteriophages labelled A1, A6, and A25 were obtained from DR. W. F. Goebel, and phage A12 from Dr. C. H. Rammelkamp. Originally A1, A6, A25, and C1 phages had come from W. R. Maxted. The designation A1 refers to the fact that the indicator strain used in propagation of the phage is a group A, type 1 streptococcus. Similarly A6, A12, and A25 are propagated in group A, type 6, group A type 12 and group A type 25 respectively. C1 bacteriophage is active against most strains of group C streptococci. Stock phage lysates were prepared in Todd-Hewitt broth.

Strains of Streptococci.—Stock phage lysates were prepared with the following strains: strain 1RP80 (group A, type 1) for A1, strain T12 (group A, non-typable variant of type 12) for A12 and strain T25 (group A, type 25) for A25. These strains were from The Rockefeller Institute collection. A6 lysate was prepared with strain 8709 (group A, type 6) and C1 lysate with strain 4540 (group C). These two strains were received from W. R. Maxted.

Todd-Hewitt Broth.—This beef heart infusion was prepared as described (11).

Dialysate Media.—In order to obtain satisfactory plaque formation with hemolytic streptococci, it was necessary to use specially prepared agar media. The nutrient broths of these agar media contain two major components; a concentrated dialysate of beef heart infusion and a dialysate of peptone. The medium for group C phage differs from that used for group A phages. Essentially this difference is that the group A medium is made with neopeptone (Difco), while the group C medium contains a much larger quantity of a different peptone (Pfanstiehl). Finally, for the preparation of agar plates, 2.4 per cent melted agar is combined with an equal volume of the dialysate broth. The medium used in the preparation of agar plates for plating group A and group C phages are designated medium A and medium C respectively. The preparation of the dialysate broth is by a method published by Dole (12).

The dialysate beef heart infusion concentrate is prepared as follows. Ten pounds of ground lean fresh hearts is stripped of all fat, and infused overnight at 4°C in 2 liters of tap water. The following morning, the infusion is brought to 85°C., removed from the flame, and after cooling for 45 minutes, filtered through filter paper. The infusion is concentrated down to 300 cc. *in vacuo* and dialyzed three times for 12 hours against distilled water. The three dialysates are combined, concentrated down to about 400 cc. *in vacuo* and stored in the frozen state. One-fortieth of a lot makes 1 liter of medium A and one-twentieth of a lot makes 1 liter of medium C.

Two liters of medium A are prepared by the following procedure. Fifty gm. of neopeptone, 200 cc. of water and 2 gm. of charcoal are heated to 80°C. for 15 minutes, filtered through

filter cel, and cooled to room temperature. To this neopeptone are added $\frac{1}{20}$ of a lot of beef heart concentrate, 4 gm. of NaCl, and 2 gm. of dextrose. The pH is adjusted to 7.5 with NaOH and the volume brought up to 1 liter. This completed broth is filtered through a Coors P3 filter for sterilization and stored at 4°C. The final medium is prepared from this concentrated broth by the addition of 1 liter of melted agar at the time of pouring plates as described below.

Medium C used for preparation of agar plates for group C streptococci is made as follows.[1] To make 2 liters of medium C, 250 gm. of Pfanstiehl peptone and 10 gm. of charcoal are heated in 400 cc. of distilled water to 80°C. for 15 minutes, and while hot, filtered through filter cel. The solution is then dialyzed two times for 18 hours against 400 cc. of distilled water. The dialysate is readsorbed with 42 gm. of charcoal by heating to 80°C., filtered through filter cel, and cooled to room temperature. To this dialysate is added $\frac{1}{10}$ of a lot of beef heart infusion extract, 10 gm. of dextrose, and 9 gm. of NaCl. After the pH is adjusted to 7.5, the volume is brought to 1 liter with distilled water. The complete broth is filtered through a Coors P3 filter and stored at 4°C. This broth is then mixed with one liter of melted agar just before agar plates are prepared.

Preparations of Agar Plates.—Agar plates are prepared as follows. Agar is dissolved in distilled water at 2.4 per cent concentration, the pH adjusted to 7.5, and sterilized in the autoclave. For each liter of agar medium, 500 cc. of 2.4 per cent melted agar at 50°C. is added to 500 cc. of dialysate broth to which had been added 2 gm. of $NaHCO_3$. The $NaHCO_3$ is dissolved in a small volume of broth, candle-filtered, and added to the broth just before use. The buffered dialysate broth is brought to 46°C. before it is mixed with the equal volume of agar. About 35 cc. of agar medium is dispensed into Petri dishes.

Agar medium for the soft agar layer is prepared in a similar way except that 1.2 per cent agar in distilled water is used instead of 2.4 per cent agar. This agar medium for the soft layer with a final agar concentration of 0.6 per cent is prepared just before use and kept liquified at 46°C.

Plating and Counting Bacteriophage.—The soft agar layer method of plating phage and streptococci is used to demonstrate plaque-forming particles (13). The streptococci used for plating are grown on agar slants of dialysate medium for 18 hours, washed off with 5 cc. of broth and 0.1 cc. of this bacterial suspension added to the tubes of soft agar at the time of plating.

In some experiments, hyaluronidase[2] was incorporated into the agar plates. When this was done, the final concentration of hyaluronidase in both the nutrient and soft layers of the plate was 0.2 mg. per cc.

Phage Dilutions.—The broth used for phage dilutions was prepared by mixing equal volumes of dialysate broth with sterile distilled water. For this purpose the broth of medium A was used for group A phages, and the broth of medium C for group C phage.

Streptococcal Grouping and Typing.—Extraction of intact streptococci or cell walls was done by the *Streptomyces albus* enzyme procedure (9) or dilute HCl. Serologic identification of extracts was done with ring tests or capillary tube precipitin tests (14) using streptococcal group and type-specific rabbit antisera.

Bacteriophage Antibody.—Phage antibody was prepared by injecting rabbits with stock phage lysates made as outlined above. For each phage, two rabbits were injected intravenously with 5 cc. of lysate three times a week for 4 weeks. At the end of the 5th week, 50 cc. of blood was collected from each rabbit, and the antisera stored at 4°C. Normal sera were obtained on all rabbits prior to administration of phage lysates.

---

[1] The broth in this medium was made by a modification of a procedure devised by Dr. L. W. Wannamaker.

[2] Hyaluronidase, bovine testes, Worthington Biochemical Corporation, Freehold, New Jersey.

Preparation of Cell Walls.—The cell walls were prepared by the method of Salton (15) in which the streptococci, following several washings in distilled water, are disrupted in a Mickle disintegrator. The cell walls are separated from the remaining cellular material by centrifugation, washed four times with distilled water, and finally stored as an aqueous suspension at 4°C.

Preparation of Cell Wall Carbohydrates.—Following treatment of cell walls with the *Streptomyces albus* enzymes, the group-specific carbohydrates were prepared by methods previously described by McCarty (10).

Analytical Methods.—Quantitative hexosamine determinations were done by a modification of the Elson and Morgan procedure (16). Methylpentose was determined by the method of Dische and Shettles (17).

EXPERIMENTAL

Plaque Formation—The production of a luxuriant growth of hemolytic streptococci in surface agar layers suitable for the formation of distinct phage plaques proved to be a special problem. While Todd-Hewitt broth is usually excellent for the growth of streptococci, as the sole nutrient source in agar plates it does not support the production of a satisfactory bacterial lawn and visible phage plaques. For this reason, methods were devised for the use of dialysate beef infusion media which consistently supported good bacterial growth and distinct phage plaques. The A1 and A6 phage plaques were 1 to 2 mm. in diameter and the A12 and A25 phage plaques 2 to 4 mm. in diameter when the medium A was used, but no plaques formed on medium C. Plaques of C1 phage were 3 to 4 mm. in diameter when plated on medium C.

Specificity of the Phages for the Streptococcal Serologic Groups and Types.—The five phages studied here were propagated through strains in their respective streptococcal group and type; however, preliminary experiments indicated that the group A phages would form plaques on heterologous types of group A streptococci. An experiment was designed, therefore, to determine the specificity of these phages for their homologous streptococcus as well as the extent and degree of cross-reactions.

The strain of streptococcus which was being checked for phage lysis was plated by the soft agar layer technique. Tenfold serial dilutions were made of A1, A6, A12, A25, and C1 phage lysates so that the 10^{-6} dilution contained 500 plaque-forming particles per cc. when plated on the indicator strain. One-hundredth of a cc. of each of the dilutions from 10^{-1} to 10^{-6} of each lysate was spotted on the soft agar layer and the plates were then incubated overnight. The highest dilution of each lysate which caused unequivocal lysis of the test strain of streptococcus was noted.

This method permitted a rapid screening of many streptococcal strains for their ability to support plaque formation by homologous and heterologous phages. Table I indicates the number of strains of group A, type 1, 6, 12, and 25 and group C streptococci checked for phage lysis. Of the 48 group A streptococcal strains, 32 were lysed by homologous type phages, and 16 strains were not lysed. However, the group A phages were not uniformly specific,

since 24 strains were lysed by one or more heterologous phages. In general, a test strain would by lysed by the 10^{-5} or 10^{-6} dilution of homologous type phage but only by 10^{-2} to 10^{-3} dilutions of the heterologous type phages; however, some strains were lysed by the 10^{-5} or 10^{-6} dilutions of both the homologous and heterologous type phages. It was clear that group C phage had no activity on group A streptococci and *vice versa*.

In addition, 25 strains of various other types of group A streptococci were checked for lysis by the four group A phages, and several strains of other streptococcal groups were also checked by the group A phages and group C phage. No lysis was noted.

TABLE I

Lysis of β-Hemolytic Streptococci by Homologous and Heterologous Bacteriophages

Hemolytic streptococcus		Number of strains	Homologous phage		Lysis by one or more heterologous group A phages
Group	Type		Lysis	No lysis	
A	1	16	7	9	7
A	6	15	13	2	11
A	12	9	7	2	1
A	25	8	5	3	5
C	—	10	9	1	0

While this method of screening streptococci for phage activity permitted a survey of a large number of cultures, it did not provide an accurate quantitative estimate of the cross-reaction between streptococci and heterologous phages. The primary reason for this is that the technique of spotting drops of lysate on the surface of an agar plate is not an efficient method of plating. Therefore, dilutions of A12, A25 and C1 phages were plated by the standard soft agar layer procedure on hyaluronidase agar plates with the homologous and heterologous streptococci T12, T25/41 and 4540 (group C). The results are recorded in Table II. A considerable degree of cross-reactivity of A12 and A25 phages is apparent, and yet it is evident that there was a marked specificity of the phages for their homologous streptococcal group even though C1 phage did form plaques on T25/41 when plated in high concentration. However, these plaques were small and indistinct, and thus far attempts to propagate phage from these plaques in broth cultures of strain T25/41 have been unsuccessful.

Since the group A phages showed a limited type specificity, and since M substance accounts for the serological type specificity of streptococci, the group A streptococcal strains listed in Table I were checked for the presence or absence of M substance by the routine acid extraction procedure. Table III indicates quite clearly that there was no correlation between the content of

TABLE II

Variation in Plaque Count with Phages Plated on Homologous and Heterologous Hemolytic Streptococci

Phage	Streptococcus	Plaque count per cc. of dilutions						
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
A12	Type 12	++++	++++	++++	++++	+++	++	79
	Type 25	++++	++++	++++	++	134	12	0
	Group C	0	0	0	0	0	0	0
A25	Type 12	++++	+++	++	138	12	0	0
	Type 25	++++	++++	++++	++++	++	166	27
	Group C	0	0	0	0	0	0	0
C 1	Type 12	0	0	0	0	0	0	0
	Type 25	++	335	37	0	0	0	0
	Group C	++++	++++	++++	++++	++	270	37

++, plaques too numerous to count.
+++, Almost complete lysis of the plate.
++++, complete confluent lysis of the plate.

TABLE III

Bacteriophage Lysis of "M" and "Non-M" Variants of Group A β-Hemolytic Streptococci

Streptococcal types		Lysis	No lysis
Type 1	M	6	3
	Non-M	1	6
Type 6	M	11	2
	Non-M	2	—
Type 12	M	2	2
	Non-M	5	—
Type 25	M	3	—
	Non-M	2	3

All the strains were from The Rockefeller Institute collection. At the time of the initial isolation they were grouped and typed as indicated in the left column. Since isolation these strains have been stored in the dried state and during this time some of them lost M substance. Such strains are listed as non-M variants. Those strains which retained M substance are listed under M.

M substance of a strain and its susceptibility to phage lysis. Both the M and the non-M variants were lysed by the bacteriophage.

The Inhibition of Phage Infection by the Hyaluronic Acid Capsule.—It has been shown by Maxted (6) that the hyaluronic acid capsule of streptococci can

inhibit the interaction between phage and bacterium. This phenomenon was also encountered in the present study. For example, several strains included in Table I which had been phage-resistant became phage-sensitive when plated in a medium to which hyaluronidase had been added. When passed through mice, non-encapsulated strains of streptococci frequently become encapsulated, grow as large mucoid colonies on blood agar plates, and generally appear to be phage-resistant, even though the parent strains are lysed by phage. When the encapsulated forms are plated on agar with hyaluronidase, they are readily lysed by bacteriophage. Table IV indicates several examples of mouse-passed strains which became sensitive when grown on hyaluronidase agar plates.

TABLE IV

Bacteriophage Lysis of Hemolytic Streptococci before and after Mouse Passage

Strain*	Streptococcal type	Mucoid colonies	Lysis on plain agar plates	Lysis on hyaluronidase agar plates
S43	NT	0	+	+
S43/100	6	+	0	+
T12	NT	0	+	+
T12/36	12	+	0	+
T25	NT	0	+	+
T25/41	25	0	+	+
K43 original	1	+	0	+
K43 variant‡	1	+	0	+

* The number of mouse passages are indicated by the number after the diagonal line.

The designation NT indicates an extract of the strain did not react with the available typing antisera.

‡ This strain was obtained by passage of K43 through rabbits. It became group A variant rather than group A. See McCarty and Lancefield (18).

An additional indication of the role of the hyaluronic acid capsule comes from the fact that phage-resistant variants isolated from plates after exposure to large concentrations of phage are commonly heavily encapsulated. For example, a phage-sensitive strain of streptococci, T25/41, was plated on agar plates and seeded with homologous A25 lysate in such concentration to produce confluent lysis after 18 hours' incubation. Isolated large mucoid colonies were picked and restreaked on sheep blood plates where they developed into large mucoid colonies. These encapsulated variants were serologically identical with the parent glossy strain, but they were lysed by bacteriophage only when grown on agar plates with hyaluronidase.

Lysogeny.—Several strains of various types of group A streptococci were shown to be lysogenic.

Strains of streptococci were grown for 6 hours in 5 cc. of dialysate broth, centrifuged, and the supernate decanted. Dilutions of this material were plated in soft agar plates with T12 and T25/41 as indicator strains. These strains were used since they were frequently lysed by heterologous phages. Following incubation for 18 hours, the plates were observed for plaques.

The type distribution of 57 strains checked in this fashion is indicated in Table V. Twelve strains were shown to be lysogenic. Phage from the lysogenic types 18 and 19 streptococci formed plaques only on strain T25/41. Some effort was made to find phage-sensitive strains of types 18 and 19, but this was unsuccessful. Plaques formed by the phages from all the lysogenic streptococci were small with a diameter of about 1 mm. Plaques from each strain were picked,

TABLE V

Strains of Group A β-Hemolytic Streptococci Checked for Lysogeny*

Streptococcal type	Number of strains	Number of lysogenic strains
1	1	0
6	9	0
12‡	4	4
18	12	2
19	26	6

The bacteriophage from types 18 and 19 lysogenic streptococci formed plaques only on strain T25/41.

* Forty-four of these strains were supplied by Dr. Harold Houser, Sampson Air Force Base, New York. They had been isolated from Air Force recruits with streptococcal sore throats or acute respiratory disease.

‡ Three of these strains were isolated from patients with acute hemorrhagic glomerulonephritis.

suspended in broth, diluted in a tenfold serial fashion, and plated on the appropriate streptococcal strains. The 10^{-2} or 10^{-3} dilutions of these plaque suspensions contained plaque-forming particles, thus indicating that the initial small irregular plaques contained phage. As would be expected, the lysogenic type 12 strains were resistant to A12 phage.

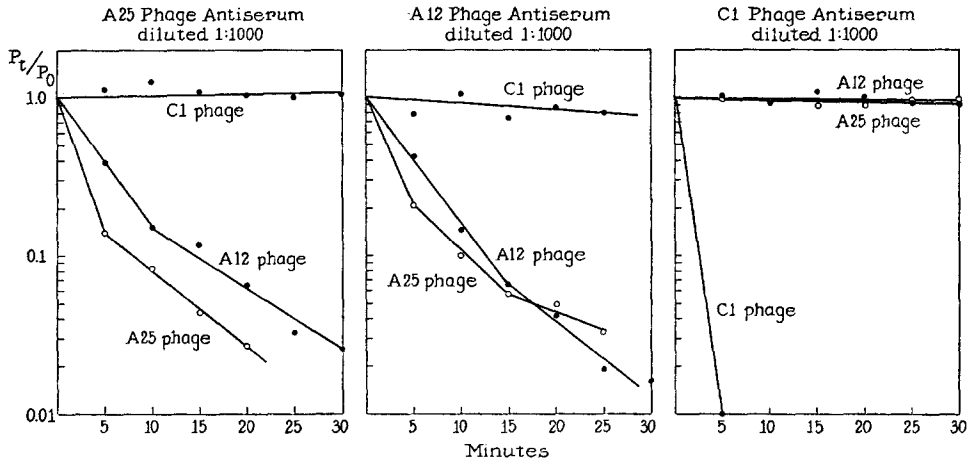
Bacteriophage Inactivation with Phage Antibody—Extensive work with other phage systems has shown that bacteriophages are antigenic. Furthermore, phages are neutralized by their homologous antiserum as well as by the antisera of related phages. Antisera, prepared by injecting rabbits with phage lysates, were used in experiments to demonstrate inactivation of phage by homologous and heterologous antisera.

The protocol for phage inactivation studies was essentially as outlined by Adams (19). Phage lysates were diluted in dialysate broth to approximately 1×10^7 plaque-forming particles per cc. One-tenth cc. of phage dilution was added to 0.9 cc. of the antiserum also diluted in broth so that the final antiserum dilution in the phage-antibody mixture was 1:100 or

1:1000. The mixture was incubated at 27°C. and 0.1 cc. samples were withdrawn at intervals and diluted into 9.9 cc. of dialysate broth. This sample or dilutions thereof were plated to determine the titer of bacteriophage in the phage-antibody mixture.

Most of the experiments to be reported were done with phage A12, A25, and C1 and their respective antisera. Preliminary tests indicated phage inactivation with a 1:1000 dilution of antisera. Rabbit sera obtained prior to lysate inoculation did not inactivate phage.

Text-fig. 1 shows the inactivation of A12, A25 and C1 phage by the 1:1000 dilutions of their homologous and the heterologous antisera. The plots are the ratios of the plaque counts at time t over the plaque counts at time zero



TEXT-FIG. 1. Neutralization of A12, A25, and C1 phage by homologous and heterologous phage antiserum.

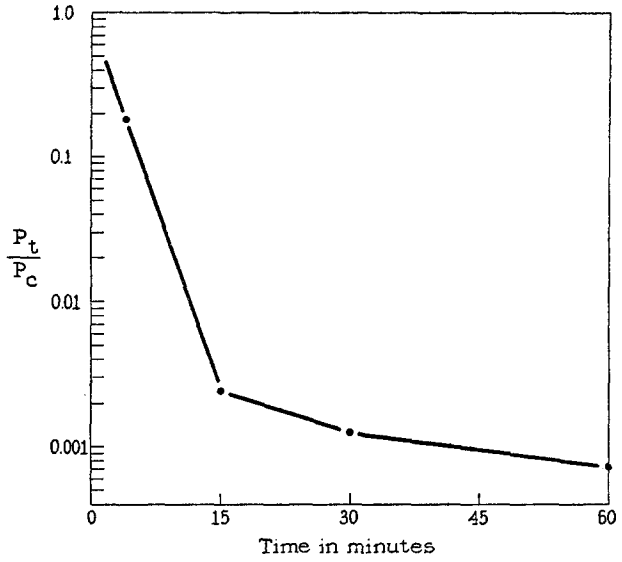
charted logarithmically. It is clear that A12 and A25 phages were inactivated by both A12 and A25 phage antisera, but not by C1 phage antiserum. The C1 phage is inactivated by C1 phage antiserum but not by the group A phage antisera. During the same 30 minute period, control tubes of phage in a 1:1000 dilution of normal rabbit serum revealed no decrease in phage count. Preliminary studies with A1 phage antiserum indicated it inactivated A1, A12, and A25 phages, but not C1 phage. Thus, the phage antisera appear to be group-specific but show no type specificity within group A.

Since the phage lysates used for rabbit injection contained group-specific streptococcal carbohydrate, the rabbit antisera were tested for carbohydrate antibody by the standard ring test procedure. However, there was no indication that the rabbit phage antisera contained carbohydrate antibody.

Phage Inactivation by Streptococcal Products.—Serologically, there are many types of group A streptococci with a specific surface protein for each type.

The groups A and C carbohydrates are the common antigenic constituents of all groups A and C streptococci respectively. In each case, the carbohydrate is a major constituent of the bacterial cell wall. The specificity of the bacteriophages for their homologous streptococcal group suggested that the carbohydrate may serve as the receptor site on the cell surface for phage attachment. Experiments were devised therefore, to test the effect of isolated cell walls and cell wall carbohydrate in phage inactivation experiments.

Streptococci were broken up by the Mickle disintegrator and the cell walls separated by differential centrifugation. This material after repeated washings appeared free from other particulate material when examined under the phase microscope. The cell walls were suspended

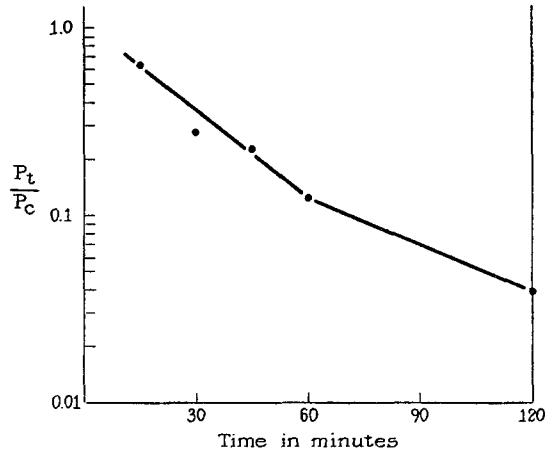


TEXT-FIG. 2. Inactivation of group C phage by group C streptococcal cell walls

in dialysate broth so that there were at least 10^8 cell walls per cc. One tenth of a cubic centimeter of phage lysate diluted to contain about 1×10^7 plaque-forming particles per cc. was mixed with 0.9 cc. of the cell wall suspension, incubated at 37°C. and sampled at intervals in the same fashion as described for the antibody studies. Control tubes contained heterologous cell walls and plain dialysate broth.

The first series of experiments were performed with C1 phage and group C streptococci. Text-fig. 2 shows the inactivation of C1 phage by group C cell walls. The results are recorded as the ratio of the plaque counts at time t over the plaque counts of the broth control. In 60 minutes, this ratio dropped from 1.0 to 0.001. Since the ratio of phage to cell walls was at least 1 to 10, it is unlikely that the fall in phage titer was due to adsorption of more than one phage onto one cell wall. The titers in the control tubes were constant throughout the 60 minute period.

Crude *S. albus* enzyme extracts of the group A cell walls were prepared which gave a strong precipitin reaction with group C antiserum. In experiments designed as described in the foregoing experiment, group C phage was inactivated by the enzyme extracts. The phage titer fell nearly 2 log units in 30 minutes. However, in the control tube of C1 phage and *S. albus* enzyme there was a fall in titer in 6 to 8 hours, suggesting that this enzyme slowly inactivated bacteriophage. These preliminary studies indicated that a complex mixture containing the group C carbohydrate inactivated C1 phage. Furthermore this reaction was group-specific, thus suggesting that the carbohydrate was



TEXT-FIG. 3. Inactivation of C1 phage by C carbohydrate of group C streptococci

the active agent in the crude extracts of the streptococcal cell wall. Therefore, the isolated carbohydrate was used in phage inactivation experiments.

The group C carbohydrate prepared from a *S. albus* enzyme extract of group C streptococcal cell walls gave a precipitin reaction with homologous group C antiserum, but not with heterologous antisera. For phage inactivation experiments, 2 mg. of the carbohydrate was dissolved in 0.9 cc. of C broth and mixed with 0.1 cc. of phage lysate diluted to 1×10^7 plaque-forming particles per cc. As in the previous studies, the mixture was incubated at 37°C., sampled at intervals and the surviving phages were counted. Control counts were determined with a mixture of phage and C broth.

Text-fig. 3 shows the results of incubating C1 phage with the group C streptococcal carbohydrate. The results are plotted as in Text-fig. 2. Over a 60 minute period, there was approximately a tenfold decrease in the number of plaque-forming particles, indicating that the group C carbohydrate inactivated the group C phage. Other control tubes included mixtures of C1 phage with group A carbohydrate, A12 and A25 phage with group C carbohydrate, and A12 and A25 phage with broth alone. There was no decrease of phage titer in these controls over a 2 hour period. Identical results were obtained

with a second batch of group C carbohydrate prepared from a different strain of group C streptococci. A comparison of the rhamnose and hexosamine content of groups A and C carbohydrate preparations are indicated in Table VI. The results on the carbohydrates prepared from both strains of group C streptococci indicate a rhamnose-hexosamine ratio of 1, while extensive experience with group A carbohydrate reveals a ratio which approaches two (18).

Extensive studies with group A cell walls, crude enzymatic extracts, and group A carbohydrate on the infectivity of group A phages have not been successful in demonstrating phage inactivation. Preliminary experiments indicated that group A phages were readily adsorbed onto the group A cell walls, but this proved to be a reversible reaction since the adsorbed phages were still plaque-forming particles. Furthermore, this reversible reaction was non-specific and occurred with mixtures of group A and group C cell walls, and group A cell walls and group C phage.

TABLE VI

Comparison of Chemical Composition of Group A and C Carbohydrate

	Hexosamine	Rhamnose
	per cent	*per cent*
Group C, strain 4540	27.8	31.7
Group C, strain H46A*	23.5	23.4
Group A average‡	23–28	42–49

* The carbohydrate of this strain was prepared by Dr. M. McCarty.

‡ These figures are from McCarty and Lancefield (18).

It would appear, therefore, that while the evidence suggests that the group-specific carbohydrate may play a role as the phage receptor for the group C phage, there is no indication for a similar function for group A carbohydrate.

The Breakdown of the Cell Wall during Phage Lysis of Streptococci.—Investigation of phage lysates has indicated that at least a part of the bacterial cell wall may remain intact following phage lysis (20). Since much is known concerning the antigenic and chemical composition of streptococcal cell walls prepared by mechanical means (10), experiments were conducted to determine the nature of the cellular debris resulting from phage lysis of streptococci.

In order to visualize the sediment of a lysate, 100 cc. of a fresh lysate of strain T25/41 was centrifuged at 3000 R.P.M. in a refrigerated angle head centrifuge, and the small quantity of sedimented material washed two times in 20 cc. of distilled water, and examined under the light microscope and the phage microscope. The same material was also mounted on grids, shadowed with chromium, and observed under the electron microscope.

A Gram stain of the washed sediment of a lysate showed Gram-negative amorphous debris; however, a wet preparation under the phase microscope

revealed a suspension of cell wall ghosts of streptococci. An electronmicrograph of this material is shown in Fig. 1. The structures are somewhat uneven and irregular with a beaded border which is felt to represent the heads of adsorbed phages. Free bacteriophages with the characteristic head and tail structure are also apparent and may have been liberated from the cell walls by suspension in distilled water. Fig. 2 shows cell walls of strain T25/41 prepared by the Mickle disintegrator. It is clear from a comparison of the two preparations that the structures from a phage lysate are more irregular and less uniform in density than the cell walls obtained by mechanical means. This suggests that during phage lysis there has been some disintegration of cell wall structure. The experiments to follow were designed to show that cell wall constituents are present in both a sedimentable and a non-sedimentable fraction of a lysate, thus indicating destruction of the cell wall when a culture undergoes lysis. This was demonstrated by antigenic and chemical analysis of a lysate, which revealed that the group-specific cell wall carbohydrate was present in sedimentable and soluble fractions.

A25 phage was added to a culture of T25/41 in the logarithmic phase of growth. Lysis occurred in 35 minutes. Bacterial counts using the pour plate technique indicated the colony count fell from 10^8 to 10^4 with phage lysis of the culture. Ten cc. of lysate was centrifuged at 10,000 R.P.M. for 1 hour, the sediment taken up in distilled water and divided into 2 portions. A *S. albus* enzyme extract was prepared with one portion and the presence of the group C carbohydrate demonstrated by group-specific antiserum. By comparing the precipitin reactions of dilutions of this extract with dilutions of a solution of known group carbohydrate concentration it was possible to estimate the amount of group carbohydrate in the sedimentable fraction. Rhamnose was determined in the second portion of the sedimentable fraction.

The non-sedimentable group C carbohydrate was recovered from the lysate as follows. Twenty cc. of the T25/41 lysate was centrifuged at 10,000 R.P.M. for 1 hour, and the carbohydrate in the supernate precipitated by the addition of antibody. This carbohydrate was recovered from the antigen-antibody precipitate by a published procedure (18). Carbohydrate obtained in this fashion was identified serologically by group antiserum and the amount estimated by the method described in the preceding paragraph. Rhamnose was also determined.

Table VII gives the analytical data from these experiments. Approximately equal quantities of group A carbohydrate are present in the sedimentable and the non-sedimentable fraction of strain T25/41 lysate. McCarty has shown that all of the rhamnose of the cell wall is in the group carbohydrate component (10). Therefore the identification of rhamnose in the sedimentable and non-sedimentable fractions confirms the presence of group carbohydrate in both fractions. These chemical data certainly suggest that antigenic components are broken off from the cell wall during phage lysis of a streptococcal culture.

Parallel observations with group C streptococcal lysates have not been extensive, but certain facts are of interest. There obviously was less cell wall debris obtained from centrifuged group C streptococcal lysates than from strain

T25/41 lysate. Analysis of the distribution of rhamnose in a group C lysate is only preliminary, but it indicates that the non-sedimentable fraction contains much more rhamnose than the sedimentable fraction. Both the soluble and the sedimentable fraction of the carbohydrate gave a specific group C precipitin reaction with streptococcal grouping rabbit antisera. It seems likely that this breakdown of the cell wall with release of antigens may be due to an enzyme either associated with or entirely separate from the phage particle.

Observations on a Labile Lytic Factor in Group C Phage Lysates.—In pursuing an explanation for the mechanism of this cell wall breakdown with phage lysis, it was observed that fresh phage lysates in certain cases lysed streptococcal cultures in a period of 5 minutes. This was particularly evident when a fresh group C phage lysate was added to a group C culture, but it was not consistently demonstrable with group A phage lysates and group A strepto-

TABLE VII

Distribution of Rhamnose and C Carbohydrate in the Sedimentable and Soluble Fractions of Cell Wall Carbohydrate from a Phage Lysate of T25 Hemolytic Streptococci

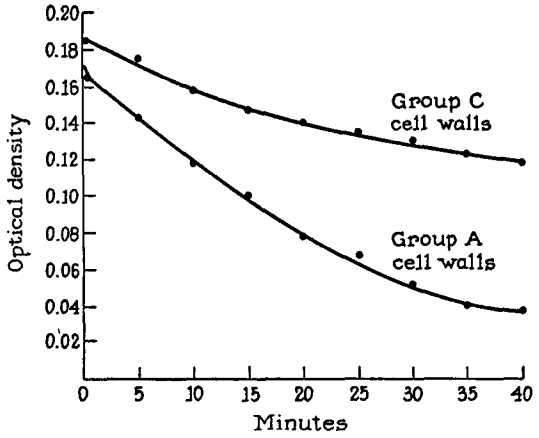
Sample	Rhamnose		C Carbohydrate determined serologically
	Experiment I	Experiment II	Experiment II
	μg.	μg.	μg.
Lysate 1 cc., sedimentable fraction	6.02	5.27	6.24
Lysate 1 cc., soluble fraction	6.93	3.24	6.24

cocci. Of special interest for the study of group A streptococci, however, was the lytic activity of fresh group C lysates for group A streptococci. Upon testing the effect of serial dilutions of a fresh group C lysate on rapidly growing cultures, the immediate lysis of the group A streptococci was more apparent than that of the group C streptococci. For example, group A cultures were lysed in 5 minutes by a 1:16 dilution of fresh group C lysate, whereas group C cultures were lysed only by the undiluted lysate in the same period of time. Of particular interest is the fact that the supernatent fluids of these lysed cultures contain their respective group-specific carbohydrates. Immediately after lysis, the cultures were centrifuged to remove bacterial debris and the group carbohydrates identified in the supernate with the group-specific antisera using ring tests. Thus during this immediate lysis of a culture following the addition of group C lysate the group-specific carbohydrate of the cell wall is released in a serologically active form. It seems unlikely that this phenomenon is "lysis from without" since the phage count was not appreciably higher than the bacterial count. Furthermore, the earlier studies in this paper clearly indicate that group C phage do not lyse group A streptococci to any significant extent. This immediate lysis only occurs when fresh group C lysate is used in

the experiments. A C1 lysate after storage at 4°C. for several days is no longer active, though it still contains infectious phage particles.

These considerations seemed to suggest that lysis of a culture was occurring following the addition of a lysate which did not require the complete cycle of viral attachment, penetration, and replication ending in cell lysis. Such a view was confirmed by using cell walls rather than living streptococci in experiments to demonstrate this immediate lytic effect.

Cell walls of streptococcal group C strain 26RP66 and group A strain T12 were prepared as described. Suspensions of the cell walls in distilled water were adjusted to the same optical density. To the cell wall from 1 cc. of each suspension was added 1 cc. of a fresh group C lysate. The mixtures were incubated at 37°C., agitated every 5 minutes, and the optical density determined at 630 mμ.



TEXT-FIG. 4. Lysis of groups A and C cell walls by a fresh group C phage lysate

The decrease in turbidity of groups A and C cell walls suspended in fresh group C lysate is illustrated in Text-fig. 4. This indicates there has been lysis of the cell wall, a view which is confirmed by the serological identification of the group-specific carbohydrate in the supernatant fluids obtained from the suspensions centrifuged at the end of the incubation period.

Thus fresh group C lysates contain a labile lytic factor which lyses both groups A and C streptococci and their isolated cell walls with release of cell wall carbohydrate into solution. Communication with W. R. Maxted indicates he has observed a similar phenomenon. It is interesting that this process as well as regular phage lysis of a culture results in the release of group carbohydrate from the cell wall, suggesting in each case an enzymatic process similar to the *S. albus* enzyme action on streptococcal cell walls. The nature of this labile lytic factor in group C lysates and its possible relationship to the usual process of phage lysis is under investigation.

DISCUSSION

The experiments described in this paper deal with the relationships between hemolytic streptococci and their bacteriophages. In particular, an attempt has been made to determine whether the bacteriophage receptor on the cell surface can be identified with any of the known antigenic or chemical components of the streptococcal cell wall. A possible clue to the nature of the receptor is provided by the fact that the limited collection of bacteriophages studied possess group specificity. Thus, the group A phages do not lyse group C streptococci and *vice versa*, and it is suggested that the receptors may be related to structures concerned with group specificity. At the same time, the host range studies indicate that type-specific components of the group A cell surface are probably not intimately concerned with phage attachment, since several different types of group A streptococci can be lysed by the same bacteriophage. The serologic experiments with phage rabbit antisera indicate that the several group A phages are related immunologically and quite distinct from the group C phage. Such results parallel the experience with some other phage bacterial systems; namely that phages which share host range are serologically homologous while phages of antigenically distinct bacteria are heterologous.

In further study of the phage receptors, it was necessary to take into consideration the various factors which interfere with phage lysis. The occurrence of these factors is well illustrated by the finding that not all strains of group A streptococci within a single type are lysed by the homologous phage. The presence of a hyaluronic acid capsule was shown by Maxted (6) to be one factor which can lead to phage resistance. This observation and the fact that the effect can be eliminated by hyaluronidase were confirmed in the present study. A second factor which can be responsible for phage resistance is lysogeny, since an organism carrying a temperate phage is usually found to be immune to infection. Lysogeny of group A streptococci has been reported previously (7), and in the present studies it was shown to be of significance by the finding that one of the type 12 resistant strains was lysogenic. A possible third factor which influences the efficiency of infection of group A streptococci by phage is suggested by the work of Maxted (6) indicating that the presence of M protein, or other surface protein digestible by trypsin, can reduce the susceptibility to lysis. However in the present study both M and non-M variants of a given type were lysed by the homologous phage.

In addition to these factors there must be others, as yet undefined, which are responsible for the limited type specificity of group A phages. However, after these exceptions are noted it is clear that sensitivity of streptococci to the phages studied is most closely related to serological group. This view is supported by the finding that the group C cell wall carbohydrate specifically inactivates group C phage. This carbohydrate has no effect on group A phage

nor does the group A cell wall carbohydrate inactivate group C phage. As in the case of group A carbohydrate, rhamnose and hexosamine are the major constituents of the group C carbohydrate, but an additional amino sugar, galactosamine, is also present (21). There is no significant serological cross reaction between the two carbohydrates.

The observations relating the cell wall carbohydrate to the inactivation of group C phage are not parallelled in the case of group A phages. It has not been possible to demonstrate inactivation of group A phages either with group A carbohydrate or with intact group A cell walls. While it is possible that some alteration or blocking of the carbohydrate may be responsible for these results, the simplest interpretation is that some entirely different substance may serve as the receptor site. This latter view receives some support from the fact that A1 phage lyses both K43 and K43 variant streptococci. The serological specificity of group A carbohydrate appears to depend largely on N-acetylglucosamine side-chains which are entirely lacking in the carbohydrate of variant strains such as K43 variant (22). Thus, it is clear that the presence of the classical group A carbohydrate is not necessary for phage sensitivity.

In the extensive studies with other bacterial virus systems, it has frequently been possible to demonstrate specific inhibition of phage by antigens extracted from the bacterial cell (8). However, it should be mentioned that in certain instances purification of the somatic antigen results in loss of antiviral activity. Thus the lipocarbohydrate of Phase II *Sh. sonnei* obtained by degradation of the lipomucoprotein does not inactivate phages T2 and T6, even though the original somatic antigen inactivates all of the phages to which Phase II *Sh. sonnei* is susceptible (23).

The observation that fresh group C lysates can bring about immediate lysis of group A streptococci and their isolated cell walls is of interest from many points of view. The findings indicate that lysis is dependent on the presence of a relatively labile accessory lytic factor. This effect of group C lysates has been independently observed by Maxted, and in all probability it is responsible for the phenomenon of "nascent bacteriophage" described by Evans many years ago (4). Evans found that group A streptococci were lysed by group C phage if living group C streptococci were added to the mixture. Under these conditions, the lytic factor released by bacteriophage lysis of the group C streptococci would in turn lyse the group A organisms.

Evidence has been presented which indicates that phage lysis of streptococci results in considerable disintegration of the cell wall. There are interesting similarities between this process and the immediate lysis of groups A and C cell walls by the labile lytic factor of group C phage lysates. Both processes result in the release of group carbohydrate into solution. This action on the cell wall suggests the possibility of an enzymatic effect comparable to that of the non-proteolytic component of the *Streptomyces albus* enzyme.

SUMMARY

The host ranges of bacteriophages for group A, types 1, 6, 12, and 25 and group C streptococci have been determined. The findings indicate that the susceptibility to these phages is primarily a group-specific phenomenon, although it is modified by several factors such as the hyaluronic acid capsule, lysogeny, and possibly the presence of surface proteins. Phage antibody studies indicate that while the group A phages are antigenically related, they are distinct from the group C phage. This is in agreement with the observation that group A phages are not specific for their homologous streptococcal types.

The purified group C carbohydrate inactivates group C phage but not the group A phages, thus suggesting that the carbohydrate, a component of the cell wall, may serve as the phage receptor site. It has not been possible to inactivate the group A phages with group A carbohydrate.

Phage lysis of groups A and C streptococci is accompanied by fragmentation of the cell wall since the C carbohydrate has been identified serologically and chemically in the supernate of centrifuged lysates. The immediate lysis of groups A and C hemolytic streptococci and their isolated cell walls by an accesory heat-labile lytic factor in fresh group C lysates is also described.

BIBLIOGRAPHY

1. Evans, A. C., Streptococcus bacteriophage: A study of four serological types, *Pub. Health Rep., U. S. P. H. S.*, 1934, **49**, 1386.
2. Evans, A. C., Studies on hemolytic streptococci. I. Methods of classification, *J. Bact.*, 1936, **31**, 423.
3. Evans, A. C., The potency of nascent streptococcus bacteriophage B, *J. Bact.*, 1940, **39**, 597.
4. Evans, A. C., Technique for the determination of the sensitivity of a strain of streptococcus to bacteriophage of type A, B, C, or D, *J. Bact.*, 1942, **44**, 207.
5. Lancefield, R. C., Specific relationship of cell composition to biological activity of hemolytic streptococci, *Harvey Lectures*, 1940–41, **36**, 251.
6. Maxted, W. R., The influence of bacteriophage on *Streptococcus pyrogenes*, *J. Gen. Microbiol.*, 1955, **12**, 484.
7. Kjems, E., Studies on streptococcal bacteriophages. I. Technique of isolating phage-producing strains, *Acta Path. et Microbiol. Scand.*, 1955, **36**, 433.
8. Tolmach, L. J., Attachment and penetration of cells by viruses *in* Advances in Virus Research, (M. A. Lauffer and K. M. Smith, editors), New York, Academic Press, Inc., 1957, **4**, 63.
9. McCarty, M., The lysis of group A hemolytic streptococci by extracellular enzymes of *Streptomyces albus*. I. Production and fractionation of the lytic enzymes, *J. Exp. Med.*, 1952, **96**, 555.
10. McCarty, M., The lysis of group A hemolytic streptococci by extracellular enzymes of *Streptomyces albus*. II. Nature of the cellular substrate attacked by the lytic enzymes, *J. Exp. Med.*, 1952, **96**, 569.

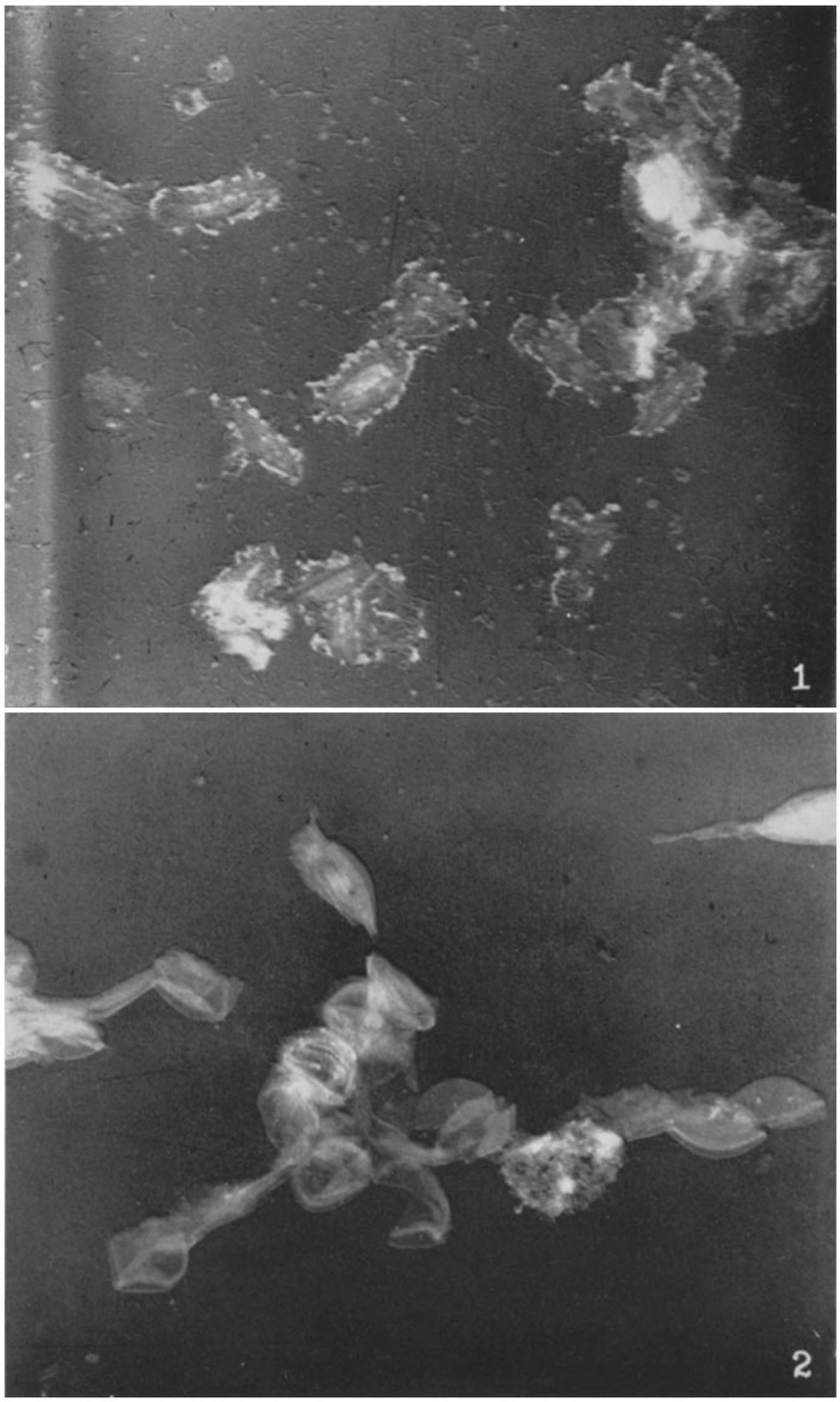
11. Todd, E. W., and Hewitt, L. F., A new culture medium for the production of antigenic streptococcal haemolysin, *J. Path. and Bact.*, 1932, **35,** 973.
12. Dole, V. P., A dialyzable medium for cultivation of group A hemolytic streptococci, *Proc. Soc. Exp. Biol. and Med.*, 1946, **63,** 122.
13. Gratia, A., Numerical relations between lysogenic bacteria and particles of bacteriophage, *Ann. Inst. Pasteur*, 1936, **57,** 657.
14. Swift, H. F., Wilson, A. T., and Lancefield, R. C., Typing group A hemolytic streptococci by M precipitin reactions in capillary pipettes, *J. Exp. Med.*, 1943, **78,** 127.
15. Salton, M. R. J., and Horne, R. W., Studies of the bacterial cell wall. I. Electron microscopical observations on heated bacteria, *Biochim. et Biophysic. Acta.* 1951, **7,** 19; II. Methods of preparation and some properties of cell walls, *Biochim. et. Biophysic. Acta,* 1951, **7,** 177.
16. Elson, L. A., and Morgan, W. T. J., A colorimetric method for the determination of glucosamine and chondrosamine, *Biochem. J.*, 1933, **27,** 1824.
17. Dische, Z. and Shettles, L. B., A specific color reaction of methylpentoses and a spectrophotometric micromethod for their determination, *J. Biol. Chem.*, 1948, **175,** 595.
18. McCarty, M., and Lancefield, R. C., Variation in the group-specific carbohydrate of group A streptococci. I. Immunochemical studies on the carbohydrates of variant strains, *J. Exp. Med.*, 1955, **102,** 11.
19. Adams, M. H., Methods of study of bacterial viruses *in* Methods in Medical Research (J. H. Comroe, Jr., editor-in-chief), Chicago, The Year Book Publishers, Inc., 1950, **2,** 1.
20. Luria, S. E., Delbrück, M., and Anderson, T. F., Electron microscope studies of bacterial viruses, *J. Bact.*, 1943, **46,** 57.
21. Cummins, C. S., and Harris, H., The chemical composition of the cell wall in some Gram-positive bacteria and its possible value as a taxonomic character, *J. Gen. Microbiol.*, 1956, **14,** 583.
22. McCarty, M. Variation in the group-specific carbohydrate of group A streptococci. II. Studies on the chemical basis for serological specificity of the carbohydrates, *J. Exp. Med.*, 1956, **104,** 629.
23. Jesaitis, M. A., and Goebel, W. F., The chemical and antiviral properties of the somatic antigen of Phase II *Shigella sonnei, J. Exp. Med.*, 1952, **96,** 409.

EXPLANATION OF PLATE 37

The micrographs were made through the courtesy of Dr. Keith R. Porter.

FIG. 1. Electronmicrograph of the centrifuged sediment of a phage lysate of strain T25/41. Preparation shadowed with chromium at an angle of 12°. Magnification approximately 12,000.

FIG. 2. Electronmicrograph of cell walls of streptococcal strain T25/41 disrupted in the Mickle disintegrator. Preparation shadowed with chromium at an angle of 12°. Magnification approximately 12,000.



(Krause: Bacteriophages of hemolytic streptococci. I)