THE INHIBITION OF BACTERIOPHAGY BY BACTERIAL AND NONBACTERIAL POLYSACCHARIDES

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Studies of susceptibility to bacteriophage have shown that polysaccharides associated with somatic bacterial antigens may inhibit the lytic action of bacteriophages upon susceptible homologous organisms. Levine and Frisch (1933 a,b) demonstrated this inhibitory effect with extracts from certain Salmonella and Shigella cultures. Burnet (1934) confirmed these findings; and Gough and Burnet (1934) reported that the agent inactivating the bacteriophage was a complex polysaccharide. The phenomenon was observed among staphylococci by White (1936), Pandit et al. (1936), Rakieten et al. (1936), and Freeman (1937).

The relationship between polysaccharide and bacteriophage has been considered to be analogous to that between antigen and antibody, the carbohydrate representing a heat-stable surface antigen. It follows that susceptibility to bacteriophage may be associated with immunological specificity. Although the most potent extracts were usually obtained from the most susceptible bacteria, some preparations from resistant cultures were inhibitory to bacteriophage activity (Levine and Frisch, 1933a,b; Burnet, 1934; Rakieten et al. 1936). Furthermore, Slanetz and Jawetz (1941) reported that extracts from highly susceptible strains of staphylococci did not adsorb bacteriophage as completely as one would expect, assuming that adsorption preliminary to lysis is a function of a surface antigen. Recently Miller and Goebel (1949) observed that lysis of phase I Shigella sonnei by bacteriophages T3, T4, and T7 was specifically inhibited by the purified homologous lipocarbohydrate-protein somatic antigen. This finding supports the notion that some relationship exists between the bacteriophage sensitivity of smooth strains of gram-negative bacilli and their somatic antigens. On the other hand, lysis of phase I and phase II Shigella sonnei by bacteriophages T2 and T8 was not affected by the homologous type-specific antigens.

A preliminary report of the nonspecific inhibition of bacteriophage by starch, glycogen, and gum arabic was presented by Ashenburg et al. (1940). Ellis and Spizizen (1941) confirmed this observation employing starch, gum arabic, inulin, and acetylated gum arabic. Recently Maurer and Woolley (1948) found that

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citrus pectin would prevent the lysis of *Escherichia coli* by a suitable bacteriophage. Gum acacia and starch were ineffective in this regard.

The present study deals with the effect of certain nonbacterial polysaccharides and of capsular polysaccharides derived from *Aerobacter* and *Klebsiella* upon the activity of a bacteriophage that lyses bacteria from these two genera.

**MATERIALS AND METHODS**

*Culture media.* The host organisms and those employed as the source of polysaccharide were grown in peptone water. All lytic activity was tested in semisolid nutrient agar.

*Bacteria.* Four *Aerobacter* strains and one *Klebsiella* strain, all of proved cultural characteristics, were used as the source of the bacterial polysaccharides. One organism, *Aerobacter aerogenes*, strain 1, was resistant to the test bacteriophage.

The following strains were employed as host organisms: *Aerobacter aerogenes* 3, *Aerobacter aerogenes* 18, *Aerobacter oxytoca* 20, *Klebsiella pneumoniae* type B (Julianelle), *Aerobacter aerogenes* 2, *Aerobacter aerogenes* 6, and *Aerobacter oxytoca* 30. The first four strains served in addition as sources of polysaccharide. The latter three strains did not yield suitable polysaccharide preparations.

*Bacteriophage.* A Friedländer B bacteriophage (Rakieten), active against the test organisms, was used throughout the study. A fresh preparation was made in peptone water employing as the host the homologous organism, *Klebsiella pneumoniae* type B (Julianelle).

*Nonbacterial polysaccharides.* Soluble starch (Baker, cp analyzed), glycogen (Pfanstiehl), and gum arabic (McKesson and Robbins) were weighed directly into Erlenmeyer flasks, and sufficient physiological saline was added to produce a polysaccharide concentration of 2 mg per ml. The solutions were sterilized by autoclaving.

*Bacterial polysaccharides.* The specific capsular polysaccharides were prepared according to the method of Heidelberger, Kendall, and Scherp (1936) with slight modifications. A 10-liter batch of peptone water in a 5-gallon carboy was seeded with 100 ml of an 18-hour-old culture of a source organism and incubated for 10 days at 37 C. Fifty g of crystalline sodium acetate and 1.5 volumes of 95 per cent ethanol were added to the peptone water culture. The mixture was shaken thoroughly and allowed to stand at 5 C for 48 hours. Following decantation of the supernatant, the precipitate was collected by centrifugation and then was dissolved in 400 ml of 0.1 N sodium acetate, acetic acid buffer

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4 Peptone water: peptone (Difco), 20 g; NaCl, 5 g; distilled water, 1,000 ml; pH, 7.4 to 7.6.

5 Semisolid nutrient agar: peptone (Difco), 5 g; beef extract (Difco), 3 g; agar (Difco), 5 g; distilled water, 1,000 ml; pH, 7.4 to 7.6.

6 In the case of *Klebsiella pneumoniae* type B, the culture was made 0.25 N in NaOH and incubated at 37 C for an additional 24 hours before the first alcoholic precipitation. This procedure seemed necessary to set free the polysaccharide; without it only insignificant yields were obtained.
solution (pH 4.8). The solution was distributed into centrifuge bottles, and 20 ml of chloroform and 10 ml of n-butanol were added to each portion. The mixtures were stirred at high speed for 30 minutes and then centrifuged. A 3-layer separation was obtained, with the polysaccharide in the top aqueous layer, an emulsion of denatured protein in the center, and the excess chloroform and n-butanol on the bottom. The top layer was decanted and subjected to repeated treatments with chloroform and n-butanol until an emulsion layer was no longer obtained. All emulsion layers were pooled and washed with 50 ml of buffer solution. The washings were treated with chloroform and n-butanol until the protein was eliminated. Polysaccharide obtained from the washings was added to the main solution of polysaccharide, producing a total volume of about 450 ml. One and one-half volumes of 95 per cent ethanol were added to the solution and the polysaccharide was precipitated out as a white stringy mass. Following decantation of the supernatant the precipitate was collected by centrifugation, washed twice with redistilled 95 per cent ethanol, and placed in 50 ml of acetone for 48 hours. The polysaccharide was collected by centrifugation, the acetone was decanted, and the preparation was dried in vacuo to a constant weight. Physiological saline was added to the dried preparation to produce a polysaccharide concentration of 2 mg per ml. The solution was sterilized by filtration through a Berkefeld "V" candle. The filters retained, on an average, 50 per cent of the polysaccharide present in the original solution as shown by colorimetric analyses by the orcinol method (Tillmans and Philippi, 1929).

Except for one preparation, the precipitin titers of the bacterial polysaccharides ranged from 1:1,000,000 to 1:4,000,000 when tested with undiluted homologous immune rabbit sera. The organism (Aerobacter aerogenes 3) that yielded the polysaccharide with weak serologic activity (precipitin titer of 1:250,000) produced a colonial type not very mucoid in consistency.

The polysaccharides produced highly viscous solutions and gave strongly positive Molisch reactions. Five of the seven preparations were analyzed for nitrogen by a micro-Kjeldahl technique with the following results: Aerobacter aerogenes 1, preparation 1, 3.46 per cent; preparation 2, 1.43 per cent; preparation 3, 6.02 per cent; Aerobacter oxytocum 20, 1.80 per cent; Klebsiella pneumoniae type B, 1.07 per cent. This nitrogen appeared to be nonprotein because samples of the polysaccharide solutions containing an amount of nitrogen equivalent to 1 mg of protein gave negative biuret and trichloracetic acid tests. The polysaccharides could not be precipitated as barium salts, but did form insoluble cupric compounds. The character of the present investigation did not warrant more extensive chemical analysis or purification of these materials.

Experimental technique. Preliminary titrations were performed with each test organism for the purpose of determining the dilutions of bacteriophage that would produce numbers of plaques suitable for accurate counting. The effect of the polysaccharides upon the activity of the bacteriophage was then tested. Two ml of a polysaccharide solution were thoroughly mixed with 2 ml of a suitable dilution of bacteriophage in peptone water. This produced a final concentration
of 0.5 mg per ml for the bacterial polysaccharides and 1.0 mg per ml for the non-
bacterial polysaccharides. After incubating the bacteriophage-polysaccharide
mixture at 37 C for 24 hours, 0.1-ml aliquots were transferred to 20 petri dishes
respectively. One-tenth ml of an 18-hour-old culture of the host organism in
peptone water was added to each dish, followed by from 10 to 15 ml of melted
semisolid agar. The dishes were gently rotated to obtain thorough mixing and
then were incubated at 37 C for 24 hours. The number of plaques on each plate
was recorded and subjected to statistical analysis. The control in each case
was prepared by adding 2 ml of sterile peptone water to 2 ml of the suitable
dilution of bacteriophage, incubating at 37 C for 24 hours and testing 20 0.1-ml
aliquots against the host organism, as described previously. The difference be-
tween control and experimental mean plaque counts was tested for statistical
significance. Table 1 is presented to illustrate the type of data collected for each
experiment.

TABLE 1
Inhibition by bacterial and nonbacterial polysaccharides of the lysis of Aerobacter aerogenes,
strain 3, by a Friedländer B bacteriophage

<table>
<thead>
<tr>
<th>DILUTION OF BACTERIOPHAGE</th>
<th>10^6</th>
<th>10^7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Polysaccharide from A. aerogenes</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Polysaccharide from A. aerogenes</td>
</tr>
<tr>
<td>Per cent reduction in mean plaque count ..........</td>
<td>—</td>
<td>69.7</td>
</tr>
<tr>
<td>Mean plaque count of 20 plates ..................</td>
<td>65.1</td>
<td>19.7</td>
</tr>
<tr>
<td>Standard deviation ..........</td>
<td>28.7</td>
<td>7.1</td>
</tr>
<tr>
<td>Critical ratio* ..........</td>
<td>—</td>
<td>6.9</td>
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</table>

* Difference of the means/standard error of the difference of the means.

RESULTS

The influence of the various polysaccharides upon the activity of the Fried-
länder B bacteriophage is summarized in table 2 as the percentage of reduction
in mean plaque counts. All of the polysaccharides possessed some ability to in-
hit the lytic action of the bacteriophage. Only one instance was observed in
which the difference between the control mean plaque count and the experimen-
tal mean plaque count was not statistically significant.

Starch and the polysaccharide from the bacteriophage-resistant organism
(Aerobacter aerogenes 1) inhibited lytic activity as much as did polysaccharides
derived from homologous susceptible strains. In one experiment comparison
was made of the effect of starch, glycogen, and gum arabic upon the lytic activity
of the bacteriophage. One can see from the data that the inhibitory influence
of these nonbacterial polysaccharides was marked and of relatively identical
magnitude in each case.
Aerobacter oxytocum 20 and Klebsiella pneumoniae type B, both very mucoid strains, were highly susceptible to lysis by the test bacteriophage. The polysaccharides obtained from these strains exhibited strong serological activity with homologous immune rabbit sera. Such observations might lead one to expect marked inhibitory activity of these polysaccharides upon the lysis of the homologous susceptible strains by the test bacteriophage. However, the inactivation percentages in this case were among the lowest encountered in the entire study.

Inhibitory activity varied with the test organism. The capacity of starch and the polysaccharide from Aerobacter aerogenes 1 to inhibit bacteriophagy was relatively high when Aerobacter oxytocum 30 and Aerobacter aerogenes 3 were used as the host organism, but was much lower with Aerobacter aerogenes 2 and Aerobacter aerogenes 18 as host organisms.

In 1934 Burnet showed that the inhibition of bacteriophagy by bacterial extracts followed the “percentage law” of Andrewes and Elford (1933). That is, the percentage reduction in the number of plaques would be approximately the same regardless of the number of bacteriophage particles. The data in the present study, in general, agree with this principle.

**DISCUSSION**

Nonspecific inhibition of bacteriophagy by bacterial and nonbacterial polysaccharides is not necessarily contrary to the hypothesis that susceptibility to lysis is related to antigenic structure. One is tempted to imagine that by
virtue of similar chemical structure the nonspecific polysaccharide competes with receptor units on the bacterial surface for chemical groupings on the bacteriophage and thus partially blocks lytic activity. However, Ginsberg et al. (1948) have shown that one can destroy the serological activity of the polysaccharide of Klebsiella pneumoniae type B by chemical means and at the same time one does not diminish its inhibitory activity against mumps virus. Conversely, one can destroy inhibitory activity chemically without altering serological activity greatly. Maurer and Woolley (1948) could not demonstrate a competitive inhibition between pectin and receptor substance in the bacterial cell for bacteriophage. No substance was isolated from the bacterial cell that would antagonize the protective action of pectin. The latter investigators suggested that pectin may have formed a protective layer around the bacterial cell in some manner preventing lysis even though adsorption and multiplication of bacteriophage occurred. They pointed out the close analogy existing between this situation and that seen with lysogenic bacteria.

Recently Ginsberg and Horsfall (1949), from the results of studies of the inhibition of mumps virus by the polysaccharide of Klebsiella pneumoniae type B, suggested various possibilities by which a polysaccharide might exert its inhibitory action. The carbohydrate might inhibit (1) virus-cell combination (2) invasion of the cell by virus, (3) release of virus from the cell after multiplication, or (4) an intracellular process essential for multiplication of virus. Of these proposals they believe the last to be most consistent with their experimental results.

One might also consider the question of chemical combination of polysaccharide with bacteriophage interfering with the metabolism of the latter. Another possible mechanism is the inhibition by the polysaccharide of bacterial surface enzymes essential for bacteriophage metabolism.

SUMMARY

A study has been made of the effect of homologous and heterologous capsular bacterial polysaccharides and of starch, glycogen, and gum arabic upon the ability of a Friedländer B bacteriophage to lyse seven host organisms (Klebsiella pneumoniae type B and six strains of Aerobacter).

All of the polysaccharides inhibited the lytic action of the bacteriophage. In no instance was the inhibition complete. In many cases it followed the "percentage law" of Andrewes and Elford. The degree of inhibition varied with the polysaccharide and with the host organism.

REFERENCES


INHIBITION OF BACTERIOPHAGY


