Inhibition of Coli Bacteriophage T₂ by Apple Pectin

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SUMMARY: Apple pectin in a complex organic medium partly protected *Escherichia coli* strain B from lysis by T₂ phage. It was not bactericidal or virucidal. The rate of adsorption of the phage was unaltered, but part of the initially adsorbed phage could be eluted with distilled water at 0°, as the second irreversible step of adsorption was inhibited by pectin. It was shown in one-step growth and single cell burst experiments that phage multiplication was reduced. The release of any formed phage from the host was not affected. The protective effect of the pectin resulted from the failure of some of the phage particles to penetrate into the host cell and from its action in decreasing phage synthesis in those cells where penetration did take place. It is suggested that this non-specific polysaccharide may exert its protective action because of its polymeric electrolyte nature.

D'Herelle (1926) early on cited the inhibition of phage lysis by gelatin, tragacanth, etc. Bacterial and non-bacterial polysaccharides have been reported to inhibit bacteriophage. Levine & Frisch (1933) demonstrated that extracts from certain salmonella and shigella cultures inactivated phage. Burnet (1934) and Gough & Burnet (1934) confirmed this and found that the phage-inactivating agent was a complex polysaccharide, probably identical with the bacterial surface component on which the phage was adsorbed and which determines the specificity of the phage-host relationship.

Ashenburg, Sandholzer, Sherp & Berry (1940) reported that non-specific polysaccharides like starch, glycogen and gum arabic had an effect similar to specific polysaccharides. They suggested later (Ashenburg, Sandholzer, Sherp & Berry, 1950) that this non-specific inhibition by polysaccharides was not necessarily in conflict with the hypothesis that susceptibility to phage is related to the antigenic structure of the organism. Non-specific polysaccharides may, by virtue of similar chemical structures, compete with the receptor units on the bacterial surface for chemical groupings on the phage and thus partially block lytic activity, a view which was first put forward in relation to bacterial polysaccharides by Gough & Burnet (1934).

The work cited thus far does not describe inhibition of phage multiplication but an inactivation of free phage. Maurer & Woolley (1948), working with *Escherichia coli* and T₂ phage, reported that citrus and apple pectin were non-bactericidal and non-virucidal and did not prevent the adsorption of phage, but prevented the lysis of the cells, perhaps by forming a protective layer around them. Multiplication of phage took place and they compared the effect with a lysogenic system (liberation of phage without lysis as some workers interpreted the phenomenon of lysogenicity at that time).

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Kleczkowski & Kleczkowski (1952) reported that a specific polysaccharide derived from the host bacteria (rhizobium) interfered with the multiplication of a homologous phage. He showed that in the first 3 hr. of incubation in the presence of 0.25 % (w/v) polysaccharides phage synthesis was only about half that in the control. This inhibitory effect disappeared after prolonged incubation.

This paper is concerned with the inhibition of the T₂ coli phage by a non-specific polysaccharide, apple pectin, in an organic medium.

METHODS

Escherichia coli B and phage T₂ were employed, using the routine methods in phage research as described by Adams (1950).

The medium consisted of 1 % peptone, 1 % Lemco meat extract, 0.3 % yeast extract (all Oxoid), 0.5 % sodium chloride in distilled water, pH 7.2. The pectin was dissolved as described by Maurer & Woolley (1948), except that it was found of advantage not to adjust the pH before sterilization. 4 % apple pectin (B.D.H. grade 240) was dissolved in water at 95° by adding it slowly with constant stirring. The resultant solution was then sterilized and the pH adjusted with 2M-caustic soda while still hot. This solution, half diluted with double-strength broth, constituted the pectin broth medium.

The media are referred to in the text as YG broth for yeast-glucose broth, and P broth for YG broth containing the supplement of 2 % (w/v) pectin.

The number of organisms was determined either by direct plating in nutrient agar, or with the aid of a nephelometer (Evans Electroselenium Ltd.).

Intracellular phage. The intracellular phage was determined in the manner described for the T₅ phage by Kay (1952). The organisms were artificially lysed by suspension in a saturated solution of glycine containing 0.015M-potassium cyanide at 37° for 2 hr. It was found that T₂ phage was rather more affected by glycine and potassium cyanide than T₅ phage, and the incubation time was reduced to 2 hr. instead of 3 hr. as used for T₅ phage.

Elution experiments. To determine whether phage was adsorbed reversibly or irreversibly elution experiments were carried out according to Garen & Puck (1951). T₂ phage was mixed with an excess of organisms at 37° and centrifuged after an adsorption period of 5 min. The separated organisms were then resuspended in double-distilled water at 0°. After 5 min. the organisms were again separated by centrifugation and then the eluted phage determined in the supernatant.

This process was repeated once, and the total of the eluted phage was then expressed as a percentage of the adsorbed phage.

RESULTS

Preliminary experiments confirmed the report of Maurer & Woolley (1948) that apple pectin did not prevent the adsorption of T₂ phage and was non-toxic to *Escherichia coli* B. It was also confirmed that the presence of pectin totally or partially inhibited clearing of cultures of *E. coli* B. by phage T₂; but the inhibition was less complete when the cultures were continuously aerated.

Exposure of T_2 phage to apple pectin did not inactivate it, for it gave the same plaque count after being suspended in P broth at 37° as before this treatment.

Rate of adsorption. While it was confirmed that adsorption did take place in the presence of pectin no data were available regarding the rate of adsorption. If adsorption were substantially retarded it could account for at least some of the protective action of pectin.

8×10^7 cells/ml. were infected with 8×10^7 phage/ml. in YG broth and P broth at 37° and kept for 5 min. after mixing (the rate of infection was the same as used for the single cell burst experiments). The unadsorbed free phage was then determined in the usual way.

The velocity constant K was practically identical for both media, namely, 3.9×10^{-9} ml./min. for YG broth, and 4.2×10^{-9} ml./min. for P broth.

Experiments with high concentrations of organisms (10^9 /ml.) and a low rate of phage infection (10^5 /ml.) as used in the elution experiments, confirmed the identical rate of adsorption in the two media.

One-step growth experiments and intracellular phage. Since the latent period of the T_2 coli phage at 37° was too short to permit the taking of sufficient samples for the determination of intracellular phage, the incubation temperature was lowered from the optimum of 37 to 30° .

A culture of *Escherichia coli* B, grown in YG broth to a concentration of 10^7 organisms/ml., was centrifuged and washed twice with distilled water. The packed cells were resuspended in YG broth and P broth respectively and incubated at 37° for 15 min. and then infected with 10^7 phage/ml. After an adsorption period of 5 min. the free, unadsorbed, phage was eliminated by centrifuging a 1:100 dilution of the infected culture and decanting the clear supernatant. The sediments containing the infected bacteria were resuspended in fresh media. The appropriate dilutions were then assayed for total and intracellular phage.

The glycine KCN method for artificial lysis (Kay, 1952), which was originally used for the T_5 phage, proved to be applicable for the estimation of intracellular T_2 phage as shown in Fig. 1. Very little intracellular phage was detected up to 15 min. after infection in YG broth which indicates, according to current conception of phage multiplication, that the adsorbed phage had been broken down inside the cell. Apparent burst sizes (i.e. the ratio of phage count at the end of the rise period to the initial phage count) from 120 to 150 phage particles were regularly obtained in this medium.

Fig. 2 shows a one-step growth curve in the presence of pectin. Such experiments gave apparent burst sizes of 10–30 phage particles. The level of the demonstrable intracellular phage during the early latent period was very much higher than in the absence of pectin. Much of the adsorbed phage was not broken down and presumably did not penetrate into the cell. The first noticeable increase in both total and intracellular phage was delayed, giving a longer latent period. The phage count at the end of the burst period was never increased by dissolution of the cells in glycine. The low phage yields obtained were, therefore, not due to any action of pectin which prevented the cells from liberating phage.

While the low phage yield in the presence of pectin was at least partly due to the failure of the phage to penetrate the host cell, it was found in one-step growth and in single cell burst experiments that the phage yield was also reduced when the adsorption took place in YG broth with subsequent dilution and growth in P broth.

Fig. 1

Fig. 2

Fig. 1. Phage multiplication in YG broth; one-step growth curve. Total phage/ml., ●; intracellular phage/ml., ○.

Fig. 2. Phage multiplication in P broth, one-step growth curve. Total phage/ml., ●; intracellular phage/ml., ○.

The data show that the yield of phage was appreciably reduced in the presence of pectin but they do not reveal how many of the infected cells go on to burst and what the range is in individual burst sizes.

Single cell burst experiments. The input ratio of phage to bacterium was 10:1 with an adsorption period of 5 min. at 37°. The free, unadsorbed, phage was again eliminated by sedimenting the infected cells and decanting the supernatant. The percentage of adsorption was 80 % under these conditions.

In YG (Table 1) the average burst size and broad range of distribution of burst sizes were as expected. In the presence of pectin throughout the whole period of adsorption and growth the individual burst sizes were not only much smaller but a good proportion of the positive plates always contained single plaques. It was, of course, ascertained that the single plaques did not originate from any free phage particles which may have been distributed in the individual 0.5 ml. samples with the infected cells during the patent period. The average burst size in this particular experiment was either 17 or 47/cell according to whether the positive plates showing single plaques are included or excluded in the calculation.

As the phage yield was influenced by the adsorption process in the presence of pectin it was necessary to determine the average burst size after adsorption

in YG broth and transfer of the infected cells to P broth. The average burst size was lower than in YG broth and practically identical with that found in the experiments in P broth if one disregards the single plaque plates in the calculation of the latter. It is evident that the single plaque plates did not occur unless pectin was present during the adsorption period.

It can be inferred from these results that the single plaques originated from infected cells which had failed to produce a burst.

Table 1. *Single cell burst experiments*

Adsorption medium	Growth medium	Individual burst sizes	Average burst size
YG broth	YG broth	1, 80, 81, 88, 104, 107, 122, 123, 161, 168, 177, 215, 223, 284, 340, 343, 400	150
P broth	P broth	1, 1, 1, 1, 1, 1, 1, 7, 14, 28, 94, 95	17 (47)*
YG broth	P broth	10, 10, 45, 45, 50, 62, 64, 65, 66, 101, 132	54

* Disregarding the single plaques.

Elution experiments

Both the determination of intracellular phage during the early latent period in the one-step growth experiments and the occurrence of positive plates with single plaques in the single cell burst experiments suggested that the pectin prevents the penetration of the phage into the host. Garen & Puck (1951) have shown that distilled water at 0° will elute phage which has been adsorbed under certain environmental conditions, as at a low temperature and in medium of suboptimal salt concentration. If the T₂ phage does not penetrate the host in P broth it is to be expected that it can be eluted.

Table 2. *Elution experiments*

Adsorption medium	Percentage of phage adsorbed	Percentage of adsorbed phage eluted
YG broth*	96	3
P broth	96	62
P broth + 0.1 M-NaCl	94	58
P broth + 0.5 M-NaCl	94	5

* Contains 0.1 M-NaCl.

10⁹ cells/ml. in YG broth and P broth were infected with 10⁵ phage/ml. at 37°, kept for 5 min., centrifuged and eluted with distilled water according to Garen & Puck. YG broth itself contains 0.1 M-sodium chloride and is, therefore, an optimum adsorption medium for T₂ phage with the given excess of cells (over 90 % adsorption). As expected almost no phage was eluted after adsorption in this medium (Table 2) because the adsorption was irreversible.

In the presence of pectin, however, a large proportion of phage was eluted, proving that a great part of the phage was reversibly bound to the host.

Decreasing concentrations of ions in the adsorption medium (e.g. below 0.1 M-NaCl) make the adsorption process increasingly more reversible. It is, therefore, possible that pectin acts as a chelating agent, reducing the ion concentration and permitting the elution of the phage. The addition of sodium chloride to the pectin medium might, therefore, be expected to prevent elution, and Table 2 shows that it has this effect. But no inference on the role of pectin in such a modified medium can be made, since pectin itself becomes altered and unstable after the addition of more than 0.1 M-NaCl. It is, therefore, not clear from these experiments whether NaCl acts by supplying the need of ions for the irreversible adsorption process or whether it destroys the inhibiting action of pectin itself.

DISCUSSION

Various explanations have been put forward to interpret the effect of non-specific polysaccharides on bacteriophage action, but so far as pectin is concerned, little experimental evidence is available. The results presented show that apple pectin does not itself inactivate phage, does not prevent the adsorption of phage to its host or reduce the rate of phage adsorption, and that it does not interfere with any liberation of synthesized phage from the cell.

Its action can be explained in the light of the present results in two ways. First, inhibition of the second step of adsorption, which is known to kill the host. This failure to complete the adsorption process in the presence of pectin was demonstrated by the elution of the phage, its recovery by artificial lysis of the infected cells during the early latent period and the appearance of single plaques in the single burst experiments. All these experiments showed that a large part of the phage remained extracellular in the presence of pectin. Secondly, the low rate of multiplication in P broth, even when adsorption took place in Y-D broth in the absence of pectin. This showed that the phage synthesis itself must be affected by the pectin.

The elution of phage adsorbed in the presence of pectin and its prevention by high concentrations of NaCl in the absorption medium could be explained by the sequestering effect of pectin which could act as a polymeric electrolyte. Such action of the pectin would, however, only be possible at a limited range of salt concentration, for it is known that neutral salts greatly influence the electrolytic nature of charged polymers themselves.

Another possible explanation may be considered, namely, that the pectin as a polyelectrolyte might be absorbed directly to the bacterial surface, interfering with both the adsorption and the multiplication of the phage. Bichovsky-Slomnitzky (1953) demonstrated that the positively charged polyelectrolyte polylysine attached itself to *Escherichia coli*. It may well be that the same could happen with the negatively charged polyelectrolyte given suitable ionic concentration in the medium.

Pectin, of course, is not a definite chemical entity and pectins from different sources vary considerably in their chemical composition. They have been found to vary also in their inhibition of coli T₂ phage (e.g. elutions of up to 95 % have been found with one batch of apple pectin).

It is to be expected, of course, that the chemical constitution of the pectin will influence its action in phage inhibition, and further explanations of this inhibition are likely to be found in the nature and extent of the active radicle groups present in the pectin.

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REFERENCES

- ADAMS, M. H. (1950). Methods of study of bacterial viruses. *Meth. med. Res.* **2**, 3.
- ASHENBURG, N. J., SANDHOLZER, L. A., SHERP, H. W. & BERRY, G. P. (1940). The influence of bacterial and non-bacterial polysaccharides upon bacteriophage. *J. Bact.* **39**, 71.
- ASHENBURG, N. J., SANDHOLZER, L. A., SHERP, H. W. & BERRY, G. P. (1950). The inhibition of bacteriophage and non-bacterial polysaccharides. *J. Bact.* **59**, 681.
- BICHOVSKY-SLOMNITZKY, L. (1953). Data cited by Katchalsky, A. Polyelectrolytes. *Endeavour*, **12**, 90.
- BURNET, F. M. (1934). The bacteriophage inactivating agent of bacterial extracts. *J. Path. Bact.* **38**, 285.
- GAREN, A. & PUCK, T. T. (1951). The first two steps of the invasion of host cells by bacterial viruses. II. *J. exp. Med.* **94**, 177.
- GOUGH, G. A. C. & BURNET, F. M. (1934). The chemical nature of the bacteriophage inactivating agent in bacterial extracts. *J. Path. Bact.* **38**, 301.
- D'HERELLE, F. (1926). *The Bacteriophage and its Behaviour*, p. 63. (English translation.) London: Baillière, Tindall and Cox.
- KAY, D. (1952). The intracellular multiplication of coli bacteriophage T₅st. *Brit. J. exp. Path.* **33**, 236.
- KLECZKOWSKI, J. & KLECZKOWSKI, A. (1952). Effect of specific polysaccharides from the host bacteria and of ribonuclease on the multiplication of rhizobium phages. *J. gen. Microbiol.* **7**, 340.
- LEVINE, P. & FRISCH, A. W. (1933). Specific inhibition of phage by bacterial extracts. *Proc. Soc. exp. Biol., N.Y.*, **30**, 993.
- MAURER, F. D. & WOOLLEY, D. W. (1948). Protection of *Escherichia coli* against bacteriophage with citrus pectin. *Proc. Soc. exp. Biol., N.Y.*, **67**, 379.

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