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Protection of *Escherichia coli* against Bacteriophage with Citrus Pectin.

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Since the discovery in this laboratory[1,2] that apple pectin and certain other polysaccharides inhibit the hemagglutinating activity and the multiplication of influenza A virus it became desirable to determine whether other viruses as well could be combatted with such polysaccharides. A suitable bacteriophage acting on *Escherichia coli* suggested itself as a useful system for this purpose, not only because of the facility with which various aspects of the virus-host relationship might be studied, but also because circumstantial evidence has pointed to the conclusion that the point of viral attack on the bacterium is carbohydrate in nature.[3] This latter point was attractive because the discovery of the effect of apple pectin in inhibiting influenza virus was based on belief in the polysaccharide nature of the cell receptor of this virus.[1,2] Just as in the case of influenza A virus, so with bacteriophage the host cells might be protected by supplying an antagonistic polysaccharide to compete with the receptor substance. For these reasons pectins were examined for ability to protect the bacterium from the phage, and it was found that citrus pectin was capable of doing this.

To demonstrate this phenomenon, the T_2 phage and its susceptible host strain of *Escherichia coli* were best suited.† Inocula for the tests were prepared by incubating the strain of *E. coli* in a synthetic medium[4] for 2.5 hours at 37°C. At this time growth was just plainly visible. 0.5 cc of this fresh young culture was used to seed each tube of the synthetic basal medium of Hook *et al.*[5] which was used in all of the tests, unless otherwise noted. The inoculum of T_2 bacteriophage was prepared by growing the virus in the susceptible strain of bacteria cultivated in Todd-Hewitt broth[6] for 20 hours at 37°C. The lysate was filtered through a Seitz pad to provide cell-free material and the filtrate was titered by the plaque count method.[7] Suitable dilutions were then used in the tests. The same phage filtrate was used throughout this investigation, and was maintained potent by storage at 4°C. Solutions of citrus pectin were prepared exactly as described previously,[2] and no other manner of obtaining solutions was found satisfactory.

When the synthetic basal medium of Hook *et al.* fortified with 100 mg of citrus pectin

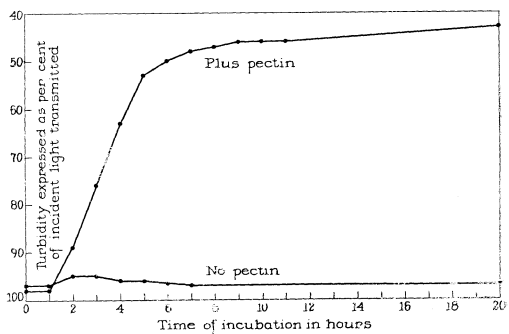

FIG. 1.

Effect of pectin on the growth curves of *E. coli* in the presence of T_2 bacteriophage.

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1 Woolley, D. W., and Green, R. H., *J. Bact.*, 1947, **54**, 63.

2 Green, R. H., and Woolley, D. W., *J. Exp. Med.*, 1947, **86**, 55.

3 Burnet, F. M., Koegh, E. V., and Lush, D., *Australian J. Exp. Biol. and Med. Sci.*, 1937, **15**, 227.

† We wish to thank Dr. S. E. Luria for transplants of these organisms.

4 Woolley, D. W., and White, A. G. C., *J. Exp. Med.*, 1943, **78**, 489.

5 Hook, A. E., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Biol. Chem.*, 1946, **165**, 241.

6 Todd, E. W., and Hewitt, L. F., *J. Path. and Bact.*, 1932, **35**, 973.

7 Hershey, A. D., Kalmanson, G., and Bronfenbrenner, J., *J. Immunol.*, 1943, **46**, 267.

TABLE I.
Influence of Citrus Pectin on Multiplication of *E. coli* and T_2 Bacteriophage. Counts Made After 20 Hours Incubation at 37°C.

Additions to basal medium	*E. coli* cells per cc	Phage particles per cc
Bacteria	1×10^9	
" + pectin	1×10^9	
" + phage	0	11×10^9
" + pectin + phage	2×10^9	5×10^9

per 6 cc was inoculated with 10^8 cells and then with the 10^9 phage particles, and the mixture was incubated at 37° overnight, heavy growth of the bacteria occurred. In contrast, when no pectin was added, no bacterial growth was visible. In the absence of pectin the virus had acted in its usual fashion and had lysed the host. Furthermore, the citrus pectin was not injurious to the growth of the bacteria in the absence of phage, because multiplication of coli in tubes of medium plus pectin was equal to that in the basal medium without pectin. Growth curves of *E. coli* in the presence of the T_2 phage, with and without citrus pectin are shown in Fig. 1. Quantitative data on the numbers of viable cells in experiments such as these will be found in Table I.

The concentration of citrus pectin needed to effect this protection of the bacterial cells was determined by adding graded amounts of the substance to the basal medium which was subsequently inoculated with *E. coli* and with T_2 phage, and incubated for 20 hours. Sixty mg of citrus pectin per 6 cc of culture was the smallest quantity which would allow good growth of the bacteria.

A number of polysaccharides were tested for ability to protect against the phage, and citrus pectin was found to be the most effective. Apple pectin at 125 mg per 6 cc would not allow growth of the bacteria in the presence of the virus when the incubation was for 20 hours, but in a test of 40 hours' duration this substance was almost as effective as citrus pectin. Gum acacia (125 mg per 6 cc‡) or starch (125 mg per 6 cc) were inactive. This order of potency was not the same as that for these substances acting against influenza virus. In that instance Green and Woolley[2] found that apple pectin was more active than citrus pectin in causing inhibition of multiplication of the virus, and that gum acacia had some potency. All 3 substances, but not starch, were inhibitors of hemagglutination caused by the virus.

The power of citrus pectin to enable *E. coli* to grow luxuriantly in the presence of its bacteriophage is the basic observation in this work. The experiments which are now to be outlined were performed in an effort to learn how this action was accomplished.

It was first necessary to ascertain whether the action of citrus pectin was attributable to the increased viscosity produced by it in the medium. Work of earlier investigators has led to the conclusion[8] that substances which made the medium highly viscous protected bacterial cells from destruction by phage. This old opinion proved not to be entirely correct, because in the present work it was found that viscosity and protective action against the virus were not always correlated. Potent protective substances with very low viscosity, as well as inactive materials of high viscosity were both found. As D'Herelle has stated many viscous substances exert protective action against lysis, but this correlation proved to be illusory because non-viscid material which was still quite active was found. Thus, a viscous material such as gelatin at 60 mg per cc of the culture fluid allowed good growth of *E. coli* in the presence of phage, and in this respect resembled citrus pectin. Both media were viscid (time of flow of the gelatin fortified basal medium 524 seconds in an Ostwold viscosimeter at 37°C and of the pectin containing medium 259 seconds). However, when the gelatin was digested with crystalline trypsin overnight,

‡ Subsequent experiments showed that 240 mg of acacia per 6 cc did exert some effect.

8 D'Herelle, F., *The Bacteriophage and Its Behavior*, Williams and Wilkins Co., 1926.

TABLE II.
Influence of Citrus Pectin on Survival of Cells in Presence of Bacteriophage as Shown by Plate Counts Made 15 Minutes After Mixing the Participants.

Additions to basal medium	Numbers of organisms after 15 min.	
	$10^2 \times$ *E. coli* cells per cc	$10^8 \times$ phage particles per cc
Phage		3
Bacteria	4,000,000	
" + pectin	5,000,000	
" + phage	3	1
" + pectin + phage	3,000,000	3

and the digest was dialyzed against running water, the nondialyzable portion retained full (and even slightly enhanced) powers of protection against lysis by the phage. Digestion so reduced the viscosity that basal medium containing the preparation at a level equivalent to 60 mg of gelatin per cc was scarcely more viscous than the basal medium without any additions (time of flow 99 seconds compared to 84 seconds). Possibly the polysaccharide portion of gelatin which remained after the proteolysis, was responsible for the protective effect against the phage. The lack of correlation of viscosity and protection from lysis may be demonstrated in the reverse manner. Thus, the basal medium fortified with Tween 80 (125 mg per cc) at pH 5.7, was more viscous than that containing an effective concentration of citrus pectin. Nevertheless, the Tween did not prevent lysis of the bacteria by the virus. Therefore, the protection afforded by citrus pectin was not due solely to the fact that it increased the viscosity of the medium.

The effect of the pectin was not to destroy the phage, because no virucidal power could be demonstrated. Thus, when 10^{10} phage particles were added to citrus pectin solution (20 mg per cc), and the mixture incubated for 30 minutes, diluted and assayed by the plaque method, 10^{10} particles were found.

The data of Table I show that while citrus pectin allowed the bacteria to grow in the presence of the phage, it did not prevent multiplication of the virus. Thus, nearly as many virus particles were found in 20-hour cultures containing abundant viable bacterial cells supported by pectin, as in the control cultures without pectin where few cells survived. This is exactly the situation which obtains with lysogenic strains[9] in which one finds growth of the host strain, and no lysis, concomitant with multiplication of the phage. Therefore, the addition of citrus pectin to the system of *E. coli* and T_2 phage has reproduced the phenomenon of lysogenic strains. This change was dependent on the continued presence of the pectin.

Citrus pectin did not prevent the attachment of the virus to the host cell, but it did protect the cell from lysis, *i.e.*, destruction. This protection from lysis may be seen from the results of the following experiments. Two tubes of basal medium, one containing pectin (100 mg per 6 cc), and the other none, were inoculated with approximately 10^8 young cells and 10^8 phage particles and the mixtures were incubated at 37°C for 15 minutes. During this time no multiplication of the bacteria would be expected. Dilutions were made, and in these the numbers of viable cells and of phage particles were determined by the usual plate counts. The results of a typical run are shown in Table II, where it can be seen that in the tube without pectin most of the viable cells had become infected and subsequently disappeared through lysis while in the tube with pectin no significant decrease had occurred.

The fact that pectin did not prevent attachment of the phage to the cells was shown in the following way. The experiment immediately preceding was repeated, except that after the 15-minute incubation period the cells were collected by centrifugation and washed 4 times with a solution of citrus pectin (20 mg per cc). Dilutions were then made in water, and the numbers of viable cells and of phage

[9] Northrop, J. H., *J. Gen. Physiol.*, 1939, **23**, 59.

particles were determined. The object of the pectin washing was to remove any virus particles which were not attached to cells. The results showed that in the tube which contained pectin when the host and parasite were mixed, 2 x 10^8 phage particles, the number actually introduced as inoculum, still remained. These must therefore have been fixed to the cells in a manner firm enough not to be dislodged by washing. The results were similar when water or physiological saline were used as wash. However, the pectin solution was used as wash so that excess phage could be removed without danger of subsequent infection of the cells. If water had been used, the pectin in the culture would have been diluted and thus rendered impotent before the excess of virus had been eliminated. Infection of the cells subsequent to the main experiment might thus have occurred.

From the above experiments it seemed probable that the pectin formed a protective layer around the bacterial cells. This layer did not prevent the attachment of the virus to the host; but it did preclude destruction of the cells. Furthermore, it did not prevent the multiplication of the virus. Moreover, this protective influence of the pectin remained with the cell during dilution of the culture and subsequent plating on agar. This fact is well illustrated by the data in line 5 of Table II.

An apparently therapeutic effect of citrus pectin on cells previously infected with the phage was observed, but this was shown to be spurious, and to have resulted from a very few uninfected cells among the infected ones. Special means were required to detect these uninfected cells, because by the ordinary plating methods none could be found. Thus, a suspension of 10^8 fresh young cells was mixed with 10^9 phage particles and the whole was incubated for 15 minutes. The cells were then collected by centrifugation and washed to remove excess virus. They were then used as inoculum for two tubes, one containing basal medium and the other basal medium plus citrus pectin. The tubes were incubated at 37° for 20 hours, and it was then found that the one with pectin contained luxuriant growth, while the one without had none. Plate counts had been made on dilutions of the inoculum of infected cells, and no living bacteria had been detected. However, when the counting was done on plates made from pectin-containing basal medium (solidified with agar) a few (43) viable, and therefore presumably uninfected cells were found. One may conclude that the presence of the pectin in the plating medium protected those uninfected cells from attack by the large amounts of virus liberated from their infected neighbors, and thus allowed detection. The probable explanation of the phenomenon observed in the tubes now seemed clear. The few cells which escaped infection during the period of exposure to phage were thereafter protected from the phage by the addition of pectin and proceeded to multiply.

The protection of bacterial cells from destruction by bacteriophage was not an isolated observation confined to one strain of the organisms employed. The same relationships were found with *E. coli* strain No. 8677 of the American Type Culture Collection in combination with either the T_2 phage, or with phage strain 8677 of the A.T.C.C. This bacterium, however, was less well adapted for the experiment than was the Luria strain, because when incubation of it with the phage was continued for 20 hours or longer, overgrowth of the previously lysed culture occurred. The effect of pectin, therefore, could be observed for a much shorter time.

One additional fact was, however, established with the A.T.C.C. organisms, namely, that the amount of pectin needed for protection was practically independent of the number of phage particles introduced. The amount of phage was varied over a 100-fold range without altering the influence of a minimal effective amount of pectin.

The discovery of the inhibiting action of apple pectin and certain other polysaccharides on influenza virus[1,2] arose from a working hypothesis which postulated a specific polysaccharide in the host cell which the virus attacked. This receptor substance was postulated to compete with pectin for the attention of the virus because of structural analogy o the two. Woolley (in press) has pursued this working hypothesis further, and has purifie

from erythrocytes of species susceptible to the virus, a polysaccharidelike substance which does compete with apple pectin for the virus acting as a hemagglutinin. Therefore, a search for a substance in *E. coli* which might antagonize the protective action of citrus pectin on this species seemed advisable. A number of attempts to demonstrate such a substance have been made, but none has succeeded. The testing procedure employed for this was to add the various samples to the basal medium plus citrus pectin (100 mg per 6 cc) and then to inoculate with 10^8 young cells and with 10^9 phage particles. Incubation of the mixtures for 20 hours should reveal an active preparation by failure of the bacteria to grow. This should follow logically because if the pectin were competing with a cell receptor, increasing the total concentration of the latter in the system should nullify the action of the pectin. The concentration of this receptor in the system is fixed by the number of bacteria, but it might be increased by adding extracts of the substance from the cells. Extracts were prepared from 20-hour cultures of *E. coli* by treating the cells with weak alkali, by grinding them with sand, by heating to 70° or 100°, by ultraviolet light irradiation, and by plasmolysis with ether or with chloroform. None was found able to overcome the antiviral action of citrus pectin. Needless to say, the influenza virus receptor substance extracted from human erythrocytes likewise was ineffective.

Summary. Citrus pectin was found to allow growth of *E. coli* in the presence of an excess of bacteriophage. The pectin was nontoxic to the bacteria, and was not virucidal. The action of the pectin was found not to be the prevention of attachment of the virus to the cell, and indeed, it did not inhibit the multiplication of the phage appreciably. It did, however, protect the cells from lysis, and hence in its presence a phenomenon similar to that seen with lysogenic strains was observed. Gum acacia and starch, representatives of other classes of polysaccharides, were ineffective. No substance could be demonstrated in the cells which was antagonistic to citrus pectin in the way that an influenza virus substrate in erythrocytes was antagonistic to apple pectin.

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Effect of Thyro-Parathyroidectomy upon the Blood and Plasma Volumes of the Rat.

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There have been few studies of blood and plasma volumes in myxedema or experimental hypothyroidism, none on the rat. Thompson[1] in an early study found the plasma volume in myxedematous patients to be 30% below normal, and also found that the values returned to normal upon treatment with thyroid extract. Holböll[2] likewise found the blood and plasma volume reduced in myxedema. In the most recent study, Gibson and Harris[3] studied 7 myxedematous patients. They found that

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1 Thompson, W. O., *J. Clin. Invest.*, 1926, **2**, 477.

2 Holböll, S. A., *Acta med. Scandinav.*, 1930, **73**, 538.

3 Gibson, J. G., 2nd, and Harris, A. W., *J. Clin. Invest.*, 1939, **18**, 59.