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Effect of Specific Polysaccharides from the Host Bacteria and of Ribonuclease on the Multiplication of Rhizobium Phages

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SUMMARY: Two serologically unrelated strains of nodule bacteria produced two different polysaccharides, only one of which precipitated with antiserum to its parent bacterium. Both polysaccharides interfered with the multiplication of two bacteriophages in liquid cultures of the two bacterial strains, each of which was susceptible to only one of the two bacteriophages. One polysaccharide was slightly more effective than the other in interfering with multiplication of both bacteriophages; one phage was much more susceptible than the other to the interfering action of both polysaccharides. Crystallized pancreatic ribonuclease interfered with multiplication of bacteriophages much more strongly than did the polysaccharides. Neither the polysaccharides nor ribonuclease destroyed the phage particles.

The chance that a bacteriophage particle will multiply in a liquid culture of Rhizobium bacteria decreases with increasing age of the culture (Kleczkowski & Kleczkowski, 1951). This can have various causes. Bacteria may become increasingly resistant to infection as their cultures age, or some metabolic products accumulating in the medium might interfere with phage activity. The literature contains many references suggesting that polysaccharides, or materials containing polysaccharides, produced by individual bacterial species specifically interfere with the activity of bacteriophages that attack those species (Levine & Frisch, 1933; Burnet, 1934; Gough & Burnet, 1934; White, 1936; Miller & Goebel, 1949). Some authors have also related polysaccharides capable of interfering specifically with bacteriophages to those that confer serological specificity to their host bacteria (see Burnet, Keogh & Lush, 1937). On the other hand, Ashenburg, Sandholzer, Scherp & Berry (1940) found no evidence for specific inhibition of activity of bacteriophage by a polysaccharide produced by its host bacteria, for polysaccharides of widely different origins inhibited similarly. Some polysaccharides have been found to interfere with the multiplication of certain animal viruses in the allantoic sac of the chick embryo (Green & Wooley, 1947; Ginsberg, Goebel & Horsfall, 1948) and with infectivity of plant viruses (Takahashi, 1942, 1946; Bawden & Freeman, 1952). There is no evidence of any specificity in the action of polysaccharides against individual viruses, but different plants differ in the degree to which their susceptibility is influenced (Bawden & Freeman, 1952).

The work described in the present paper was done to see whether strains of Rhizobium bacteria that differ serologically and in their susceptibility to different bacteriophages, produce different specific polysaccharides and, if so, whether these are active serologically and act specifically against a bacteriophage to which the species is susceptible.

For comparison, tests were made to see whether pancreatic ribonuclease, which is known to be a powerful inhibitor of activity of plant viruses (Loring, 1942; Kassanis & Kleczkowski, 1948), has any effect on activity of Rhizobium bacteriophages. It reduces the numbers of lesions formed by plant viruses on leaves of susceptible plants, but has no effect on numbers of plaques formed by one of the phages (Kassanis & Kleczkowski, 1948).

MATERIAL AND METHODS

Two strains of nodule bacteria, 317 derived from pea (obtained from the Agricultural Research Station, Wisconsin) and Cl_5 from clover and two homologous bacteriophages were used. Each bacterial strain was susceptible only to its homologous phage and was agglutinated only by its homologous antiserum. The phages were originally obtained from soil and isolated from single plaques. The media, the poured plate method of obtaining plaques, and other methods of cultivation were the same as used previously (Kleczkowski & Kleczkowski, 1951).

The polysaccharides were isolated from 3-week-old, liquid cultures of the bacteria. The cultures were centrifuged, 10 ml. 20 % NaCl were added to each litre of the clear supernatant fluid followed by 2 l. 96 % (v/v) ethanol. The precipitate was filtered off and suspended in 100 ml. of a 20 % solution of sodium acetate neutralized with glacial acetic acid. Material that did not dissolve within 24 hr. was removed by centrifugation. Further procedure was the same as that described by Heidelberger, Kendall & Scherp (1936) for isolating pneumococcal polysaccharides, except that, before removing protein by Sevag's procedure, the material was precipitated with ethanol, dissolved in 0·06 M-phosphate buffer at pH 7·0 and incubated for 24 hr. at 37° with the addition of $\frac{1}{30}$ vol. of filtered saliva. (This removed a material which gave the same colour as glycogen on treatment with iodine.) The polysaccharide was then precipitated with ethanol, dissolved in acidified 20 % solution of sodium acetate, and Sevag's treatment was applied. (This removed a material that gave a positive biuret test). The polysaccharide was precipitated from the resulting solution by ethanol, washed in 96 % ethanol, in absolute ethanol, in ether, dried and ground to a white powder. The yield was about 100 mg. from 1 l. bacterial culture.

Antisera were produced by injecting rabbits intraveneously with bacterial suspensions prepared by washing with physiological saline 3- to 5-day old cultures from agar slopes. Agglutinin tests with bacterial suspensions were made as previously described (Kleczkowski & Thornton, 1944). Precipitin tests with the bacterial polysaccharides were made similarly to the agglutinin tests except that the antiserum was used at a constant dilution and the polysaccharide concentration varied.

Crystallized beef pancreatic ribonuclease was prepared by the method described by Kunitz (1940).

For paper chromatography the polysaccharides were hydrolysed from 20 min. to $2\frac{1}{2}$ hr. at 120° in the presence of 5 % H_2SO_4, which was then removed

by adding an excess of $BaCO_3$, and centrifuged after a few hr. incubation at room temperature.

One-dimensional chromatograms were prepared as described by Partridge (1948), using two different solvents; water-saturated phenol (with ammonium-saturated atmosphere) and a water-saturated *n*-butanol-ethanol mixture with the volume ratio butanol : ethanol : water = 4·5 : 0·5 : 5·0.

Benzidine reagent (Horrocks, 1949) was used as a developer. For the quantitative estimation of sugars from the different spots after development, water extracts were made from areas corresponding with the spots but cut from part of the filter-paper that had not been sprayed with the developer. The reducing power of the extract was then determined by the Somogyi (1945) titrimetric procedure.

RESULTS

Some properties of the polysaccharides

Both polysaccharides were obtained as white powders, which formed clear colourless solutions in water and were highly viscous at 1 %. Solutions of polysaccharide 317 were obviously more viscous than those of Cl_5. They contained no nitrogen, did not stain with iodine and did not reduce Fehling's solution unless previously hydrolysed. After hydrolysis with 5 % H_2SO_4 for 1 hr. at 120° and subsequent neutralization with NaOH, their reducing power was about 85 % that of glucose. The intensity of colour developed when solutions of polysaccharides were heated with ten times their volume of 0·2 % orcinol in 66 % H_2SO_4, did not differ appreciably from that formed under the same conditions by an equally concentrated solution of glucose.

The polysaccharides had different compositions. The 317 produced only two spots on the paper chromatograms. Their positions corresponded with those of glucose and of a uronic acid. The Cl_5 produced three spots; the positions of two of them were identical with those of the components of the 317, that of the third corresponded with the position of fructose. This spot was weak, and it was absent if the time of hydrolysis was $2\frac{1}{2}$ hr., and the temperature was raised to 125°, under which conditions fructose is destroyed and gives no spot on the chromatogram. Over 90 % of the total reducing power of each hydrolysed polysaccharide was due to the component identified as glucose.

If hydrolysed for only 20 min., the hydrolysate of each polysaccharide contained a component that gave an additional sport on chromatograms. This occurred only when phenol was used as a solvent, and then the component moved very slowly (R_F about 0·015). The component was presumably stationary with the butanol-ethanol mixture so that its position coincided with that of the uronic acid. The component may be an aldobionic acid or an oligosaccharide that was hydrolysed when hydrolysis was prolonged.

Our results agree with those obtained by previous workers who examined polysaccharides produced by strains of nodule bacteria and found they contained glucose and glucuronic acid, the latter probably forming part of the

aldobionic acid (Hopkins, Peterson & Fred, 1931; Cooper, Daker & Stacey, 1938; Schlüchterer & Stacey, 1945).

The two polysaccharides differed serologically. The 317 polysaccharide precipitated with an antiserum to 317 bacteria (Table 1) but not with an antiserum to Cl_5 bacteria. The polysaccharide Cl_5 did not precipitate with either antiserum. Each antiserum agglutinated suspensions of its homologous bacterium up to a titre of 1/6000, but neither of the bacterial strains was agglutinated by antiserum to the other.

Although polysaccharides produced by different strains of clover *Rhizobium* may differ serologically, some of them may be closely related to polysaccharides produced by bacteria that do not belong to the Rhizobium group. The polysaccharide isolated by Schlüchterer & Stacey (1945) from 'Bartel A' strain of clover *Rhizobium* was found by Dr M. Heidelberger to give a positive precipitin test at a high dilution with pneumococcus III antiserum and also with mixed antisera to other types of pneumococcus.

The possibility that one or both of the polysaccharides 317 and Cl_5 was a mixture cannot be excluded. However, the high dilution end point at which the polysaccharide 317 precipitated with the antiserum to 317 bacteria makes it unlikely that the polysaccharide was a mixture of which the serologically active component was only a minor part.

Effect of the polysaccharides and of ribonuclease on the bacteriophages

Three types of experiment were made to see whether the polysaccharides and ribonuclease interfere with bacteriophage activity. Experiments of the first type were designed to show whether contact with these materials permanently inactivates phage particles. Bacteriophage 317 was incubated with the polysaccharide 317 and with ribonuclease, then diluted to obtain a conveniently countable number of plaques, mixed with host bacteria and plated. The details and results are given in Tables 2 and 3 which show that the incubation did not inactivate any phage particles.

Experiments of the second type were designed to test whether the presence of the polysaccharide or ribonuclease in the bacterial culture, to which a suitably diluted phage preparation was added and which was then plated directly, could render some phage particles inactive. Table 4 gives the details of one such experiment and shows that the polysaccharides had no such effect, for the number of plaques formed was not affected.

Experiments of the third type were designed to show whether the presence of the polysaccharide or of ribonuclease in a liquid culture of host bacteria interferes with phage multiplication in the culture. Table 5 shows that the polysaccharide 317, added to a concentration of 0·25 % to a culture of 317 bacteria, which was then inoculated with 317 bacteriophage, did interfere with multiplication of the phage, which, within the first 3 hr., increased only about 50 times compared to about 100 times in the control. The polysaccharide Cl_5 interfered with multiplication of 317 phage only slightly, though significantly, less than did the polysaccharide 317. Potato starch also interfered

Table 1. *Precipitin reaction of the polysaccharide* 317 *with an antiserum to* 317 *bacteria*

Time of reading (hr.)	Reciprocal of dilution of a 0·05 % solution of the polysaccharide										
	1	2	4	8	16	32	64	128	256	512	1024
1	−	−	±	+ +	+	+	±	−	−	−	−
2	−	−	+	+ + +	+ + +	+ +	+ +	+	±	−	−
4	−	+	+ + +	+ + +	+ + +	+ + +	+ +	+ +	+	±	−

1 ml. of the antiserum diluted 1/10 was added to 1 ml. of variously diluted solution of the polysaccharide, and incubated at 50°. + + +, + +, + and ± signs show the presence and the extent of precipitation; − sign shows the absence of precipitation.

Table 2. *Incubation of bacteriophage* 317 *with the polysaccharide* 317 *before mixing with host bacteria and plating*

Mixture no.	Composition of the mixtures: 0·5 % solution polysaccharide (ml.)	H_2O (ml.)	Phage culture diluted 1/50 (ml.)	Mean no. plaques/plate
1	1·0	—	1·0	41
2	—	1·0	1·0	38

The mixtures were incubated for 1 hr. at room temperature, then diluted 10^{-5} in 24 hr. bacterial cultures and plated. Four platings were made with each mixture.

Table 3. *Incubation of bacteriophage* 317 *with ribonuclease before mixing with host bacteria and plating*

Mixture no.	Composition of the mixtures: 0·4 % ribonuclease (ml.)	H_2O (ml.)	Phage culture dil. 10^{-4} (ml.)	Mean no. plaques/plate formed by mixture incubated for: 0 hr.	24 hr.
1	0·25	0·75	0·1	103	95
2	—	1·00	0·1	106	104

The mixtures were incubated for 24 hr. at 25°. Samples were taken at the beginning and at the end of incubation, diluted 1/100 in 24 hr. bacterial cultures and plated. Each plating was made in four replications.

although considerably less. Rabbit serum albumin used at the same concentration had no effect.

Ribonuclease when present at a concentration of 0·01 %, i.e. at $\frac{1}{25}$th of the concentration of the polysaccharides, inhibited phage multiplication much more strongly than did the polysaccharides, and at a concentration of 0·1 % it stopped its multiplication completely during the first 3 hr.

After 24 hr. incubation the ratios of the concentrations reached by bacteriophage in the presence of the interfering materials to that reached in their absence were the same as, or greater than, after 3 hr. incubation. It is obvious, therefore, that the inhibiting effect of the materials tested lasted less than 24 hr.

Preparations of crystallized ribonuclease obtained from beef pancreas by Kunitz's (1940) method contain a proteolytic enzyme (Kleczkowski, 1948) and possibly some other enzymes and proteins. Proteolytic activity of the preparations can be destroyed or greatly decreased by heating for 5 min. to 90–100° at pH 7·5, preserving about 30 % of the original ribonuclease activity (Kleczkowski, 1948). As almost all known enzymes are also destroyed by heating in these conditions, heated preparations of crystallized ribonuclease can be assumed to be free from active enzymes other than ribonuclease. After heating in 0·06 M-phosphate buffer at pH 7·5 for 5 min. in a water-bath at 100°, the ribonuclease preparation still interfered with phage multiplication to an extent proportional to its remaining ribonuclease activity.

The results given in Table 6 show that the extent of inhibition depends on the host bacterium. The growth of phage Cl_5 in Cl_5 bacterial culture was much more strongly inhibited by polysaccharides 317 and Cl_5 than was the growth of phage 317 in a culture of 317 bacteria. Again the polysaccharide 317 inhibited somewhat more strongly than did the polysaccharide Cl_5.

To see whether inhibition of phage multiplication by the tested materials could be a direct result of inhibition of growth of host bacteria, 24 hr. bacterial cultures were incubated at 25° with the materials at concentrations at which they inhibited phage growth. Samples were taken at intervals for haemocytometer counts. The presence of 0·25 % of the polysaccharides or of 0·01 % ribonuclease had no effect on multiplication of the bacteria, but the presence of 0·1 % ribonuclease decreased it considerably.

Experiments were also made to test whether the polysaccharide 317 interferes with infectivity of tobacco mosaic virus. Details and results of one of such experiments are given in Table 7, which shows that the presence of the polysaccharide in the inoculum reduced numbers of lesions formed on leaves of *Nicotiana glutinosa* more than did the presence of potato starch or rabbit serum albumin. The reduction was very slight by comparison with that caused by the polysaccharide obtained from yeast by Takahashi (1942), or by such substances as beef pancreatic ribonuclease or a protein isolated from *Phytolacca esculenta* (Kassanis & Kleczkowski, 1948).

Table 4. *Effect of adding polysaccharide* 317 *or ribonuclease to bacterial cultures with which bacteriophage* 317 *was plated*

Mixture no.	Composition of the mixtures: 1 % solution polysaccharide (ml.)	0·4 % solution ribonuclease (ml.)	H_2O (ml.)	24 hr. bacterial culture (ml.)	Phage culture diluted 10^{-6} (ml.)	Mean no. plaques/plate
1	0·25	—	—	0·75	0·1	38
2	—	0·25	—	0·75	0·1	31
3	—	—	0·25	0·75	0·1	33

The mixtures were mixed with 10 ml. molten agar medium, which had been cooled to 42°, and poured into Petri dishes. Four plates were made from each mixture.

Table 5. *Effect of the polysaccharides and of ribonuclease on the multiplication of* 317 *bacteriophage*

Composition of the mixtures	Concentration of bacteriophage in terms of no. of plaques/ml. of the mixtures: Immediately	After 3 hr.	After 24 hr.
3 ml. 24 hr. culture of 317 bacteria + 1 ml. of:			
1 % 317 polysaccharide	21×10^2	10×10^4	65×10^8
1 % Cl_5 polysaccharide	18×10^2	13×10^4	85×10^8
1 % potato starch	20×10^2	17×10^4	106×10^8
1 % rabbit serum albumin	19×10^2	24×10^4	120×10^8
0·4 % ribonuclease	17×10^2	15×10^2	$4{\cdot}5 \times 10^8$
0·04 % ribonuclease	17×10^2	26×10^2	46×10^8
0·004 % ribonuclease	19×10^2	23×10^4	125×10^8
H_2O	19×10^2	25×10^4	129×10^8

0·4 ml. of a culture of phage 317 diluted 10^{-5} was added to each mixture. The mixtures were then incubated at 25° and samples were taken at intervals for phage estimation.

Table 6. *Effect of polysaccharides (PSH) on multiplication of* Cl_5 *bacteriophage*

Mixture no.	Composition of mixtures: 24 hr. culture Cl_5 bacteria (ml.)	1 % 317 PSH (ml.)	1 % Cl_5 PSH (ml.)	H_2O (ml.)	Phage Cl_5 diluted 10^{-3} (ml.)	Concentration of bacteriophage in terms of no. plaques/ml. of the mixtures: Immediately	After 24 hr.
1	0·75	0·25	—	—	0·1	71×10^2	29×10^5
2	0·75	—	0·25	—	0·1	76×10^2	67×10^5
3	0·75	—	—	0·25	0·1	67×10^2	1050×10^5

The mixtures were incubated at 25° and samples were taken at stated intervals for phage estimation.

Table 7. *Effect of polysaccharide* 317 *on activity of tobacco mosaic virus*

Addition to the inoculum	Mean no. lesions/half-leaf with virus at 5 mg./l.	Mean no. lesions/half-leaf with virus at 0·5 mg./l.
0	93	8·5
0·1 % 317 polysaccharide	23	2
0·05 % 317 polysaccharide	49	5·5
0·025 % 317 polysaccharide	73	7·5
0·1 % potato starch	72	7
0·05 % potato starch	76	—
0·1 % rabbit serum albumin	—	4

Each inoculum was applied to twelve half-leaves distributed among six *Nicotiana glutinosa* plants according to the principle of the Latin square. Each column of numbers was obtained in a separate experiment.

DISCUSSION

The fact that specific bacterial polysaccharides can interfere with phage multiplication shows clearly that their accumulation in a liquid bacterial culture may decrease the chance of a phage particle to multiply. However, the ability to interfere with phage multiplication is shared by various other materials such as potato starch and beef pancreatic ribonuclease, the last one being by far the most effective. This suggests that, apart from specific polysaccharides, various other products of bacterial metabolism that accumulate in the medium are also likely to decrease the chance of a phage particle to multiply.

There is obviously no specific affinity between the bacterial polysaccharides and phage particles, because interference by the polysaccharides with phage multiplication is unspecific, i.e. not restricted to phages whose hosts produced the polysaccharide. Also it is not connected with the ability of the polysaccharides to precipitate specifically with antisera to host bacteria. The unspecific nature of the interference is further emphasized by the fact that the polysaccharides can also interfere with infectivity of tobacco mosaic virus.

The mechanism of interference by the polysaccharides or ribonuclease remains unknown, but some possibilities can be excluded. First, phage particles are not destroyed by these interfering materials. Secondly, the materials do not interfere with phage multiplication by affecting the rate of bacterial multiplication. It is true that ribonuclease, when present at a concentration of 0·1 % did interfere with bacterial multiplication, but it did not do so at 0·01 %, although it interfered strongly with phage multiplication at this concentration. It is possible, therefore, that ribonuclease interferes with phage multiplication by affecting bacterial metabolism. The latter is obviously affected by 0·1 % ribonuclease. It may therefore also be affected by 0·01 % ribonuclease though not sufficiently to influence bacterial multiplication and it may be similarly affected by the polysaccharides. It is also possible, however, that 0·01 % ribonuclease affects only the bacterial surface, making it less penetrable for bacteriophage, or preventing its adsorption; and the poly-

saccharides may interfere with phage activity by forming a protective coating on the bacterial surface.

A feature of the inhibitory effect of the polysaccharides and of ribonuclease on phage multiplication is that with young bacterial cultures it is only temporary; with 24 hr. cultures of 317 bacteria inoculated with 317 phage it lasts less than 24 hr. Various explanations can be suggested. For example, if the inhibitory mechanism is based on a protective coating of the bacterial surface, in a young fast-growing culture the surface may outgrow its protective coating and thus increase its vulnerability; if it is based on interference with bacterial metabolism, the metabolism may recover its original course either by adaptation or through some process directed against the inhibitor.

Although the interference by the polysaccharides is unspecific, different host-phage systems are affected to different extents. This is shown by the fact that the phage Cl_5 was much more affected by each of the two polysaccharides, 317 and Cl_5, than was the phage 317.

It may seem that there is a contradiction between a polysaccharide or ribonuclease interfering with phage multiplication in a liquid bacterial culture and having no effect on the numbers of plaques formed on a solid medium. The contradiction, however, is apparent only. To be plated, a phage-bacteria mixture is diluted 1/10 in molten agar medium, and this may suffice to make the inhibitor ineffective. However, should it remain effective, plaque numbers are still unlikely to be affected, because the inhibitor does not destroy phage particles or have more than a temporary effect on phage multiplication in a young bacterial culture.

Bawden & Freeman (1952) have discussed the interference by a polysaccharide and by ribonuclease with the activity of some plant viruses, and have concluded that it is most likely to occur because these substances affect the metabolism of the host cells. Thus there may be no basic difference between the effect of polysaccharides and of ribonuclease on the activity of plant viruses, on the one hand, and on that of bacteriophage, on the other. If so, the fact that these materials decrease the numbers of local lesions formed by plant viruses and have no effect on the numbers of plaques formed by a bacteriophage would mean that the lesions are not analogous to the plaques. There would be a closer analogy between the formation of lesions and lysis of liquid bacterial cultures. This analogy is also apparent when a comparison is made between the dilution curves obtained with numbers of lesions caused by plant viruses and the dilution curves obtained with proportions of liquid bacterial cultures lysed by a bacteriophage (Kleczkowski & Kleczkowski, 1951).

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