The Isolation and some Properties of a Virus-Inhibiting Protein from *Phytolacca esculenta*

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SUMMARY: An inhibitor of plant viruses can be isolated from the sap of *Phytolacca esculenta* by differential precipitation with ethanol followed by adsorption on celite and elution with 10% NaCl. Purified preparations contain 14–15% nitrogen and 8–12% carbohydrate and the inhibitor is probably a glycoprotein. Denaturation leads to loss of inhibiting power. The protein, unless denatured, is unaffected by pepsin and trypsin.

The glycoprotein is isoelectric at about pH 7. It can combine with tobacco mosaic virus, and when salt-free solutions of the two are mixed in certain proportions at pH values between their isoelectric points it precipitates the virus in the form of paracrystalline threads. The glycoprotein also precipitates tomato bushy stunt virus.

When added to several plant viruses, the glycoprotein causes an immediate reduction in infectivity, but has no effect on a bacteriophage. Non-infective mixtures regain infectivity when diluted. No evidence was found for a combining ratio of virus to inhibitor necessary to cause loss of infectivity. The mechanism of virus neutralization is discussed.

Substances that are known to inhibit the infectivity of tobacco mosaic virus include a polysaccharide isolated from yeast (Takahashi, 1942, 1946) and a number of proteins such as trypsin, ribonuclease, globin and clupein (Stanley, 1934; Loring, 1942; Kleczkowski, 1946). They vary in their efficiency as inhibitors and ribonuclease is the most active, but all show certain common features. Reduction of infectivity occurs immediately the virus and inhibiting substances are mixed, and infectivity can be restored by diluting the mixtures. Also all the proteins are isoelectric at pH values above 7.

Sap from a number of different plant species is also known to inhibit infectivity of tobacco mosaic virus and of some other mechanically transmissible plant viruses (Grant, 1934; Fulton, 1943; Kuntz & Walker, 1947) in much the same manner as the inhibitors named above, and of those so far studied saps from species of *Phytolacca* seem to be the strongest inhibitors. No attempts have been made to isolate the materials from plant saps responsible for inhibition, but from their studies on crude sap of spinach Kuntz & Walker (1947) conclude that there are two inhibiting principles present and that the properties of one of them suggest that it may be a protein.

This paper describes a method for concentrating and purifying the inhibitor from sap of *P. esculenta* and its preliminary identification as glycoprotein.

MATERIALS AND METHODS

The sap of different plants was obtained by passing their leaves through a domestic meat-mincer and expressing the juice through cheese-cloth. The sap was usually left for a few hours at room temperature and then clarified by centrifugation for ½ hr. at 3000 r.p.m.

Most of the inhibition tests were made with tobacco mosaic virus (T.M.V.), which was used as liquid crystalline preparations made from the sap of infected tobacco plants by the method described by Bawden & Pirie (1943). Purified preparations of tomato bushy stunt virus and potato virus *X* were also used and these were obtained from infected tomato plants by precipitation methods (Bawden, 1943). Cucumber mosaic virus and the Rothamsted culture of tobacco necrosis virus were used in the form of sap from infected tobacco plants clarified by centrifugation at 8000 r.p.m.

To assay the inhibitory effect of tested fluids, they were mixed with equal volumes of solutions of purified tobacco mosaic virus, and the infectivity of the mixtures was estimated by the local lesion method using *Nicotiana glutinosa* as a test plant. Each mixture was inoculated to at least twelve half-leaves, and the half-leaves allotted to each treatment were distributed among several plants so as to form a randomized block. Inoculations were made by rubbing the leaf surface with the forefinger wet with inoculum, and after inoculation the leaves were rinsed with water. Infection tests with tomato bushy stunt virus were also made by the local lesion method in *N. glutinosa*, with potato virus *X* in *N. tabacum* and with tobacco necrosis virus in bean (*Phaseolus vulgaris*, var. Prince). With cucumber virus tobacco was used as a test plant and inhibition shown by failure to cause systemic symptoms. Each mixture was inoculated to at least ten tobacco plants.

The inhibitory power of any one material on the same virus preparation can vary when tested on different sets of test plants, so that any critical comparison between two inhibiting materials has to be made in the same experiment.

Proteolytic and ribonuclease activity were tested by methods previously described (Kleczkowski, 1944, 1946), and phosphatase (nucleotidase) activity by a method based on estimation of inorganic phosphate released from a solution of nucleic acid previously depolymerized by incubation at pH 9. Nitrogen was determined by the micro-Kjeldahl method and carbohydrate and phosphorus colorimetrically, the carbohydrate by the orcinol-sulphuric acid method using glucose as the standard of comparison, and the phosphorus by a method based on reduction of phosphomolybdic acid by stannous chloride in the presence of sulphuric acid (Kleczkowski, 1946). When the total phosphorus content of different materials was estimated, they were first incinerated with sulphuric acid and cleared by adding a few drops of perhydrol.

Properties of the inhibitor in clarified sap

Table 1 shows the results of two experiments in which the inhibiting effect on 0·02 % tobacco mosaic virus (T.M.V.) of sap from *Phytolacca esculenta* (*P.E.*) was compared with that of several other plants. The inhibitory power of *P.E.* sap was much greater than that of sap from any of the other plants tested, and that of tobacco and tomato sap was negligible.

Sap from *P.E.* was also an effective inhibitor of tomato bushy stunt virus, potato virus *X*, tobacco necrosis virus and cucumber virus. This was also found

to be true with the purified inhibitor, but no attempts were made to compare in detail the relative susceptibility of the different viruses. However, neither purified *P.E.*-inhibitor nor ribonuclease affected the activity of a bacteriophage. Tests with a bacteriophage ($S2P_{11}$) of *Rhizobium* sp. were kindly made by Dr J. Kleczkowska. Bacteriophage activity was estimated by plaque counts

Table 1. *The effect of sap from different plants on the infectivity of tobacco mosaic virus*

Sap from	Dilution of sap	Average no. of lesions/leaf
	Experiment 1	
Phytolacca esculenta	1/10	0
	1/100	5
	1/1,000	33
	1/10,000	87
Datura stramonium	1/1	2
	1/50	58
Tobacco	1/1	29
	1/50	92
Tomato	1/1	24
	1/50	84
Water control		89
	Experiment 2	
Phytolacca esculenta	1/50	0
	1/500	4
Spinach	1/1	0
	1/50	10
Sugar beet	1/1	1
	1/50	10
Water control		70

obtained by the poured plate method (Kleczkowska, 1945). A crude bacteriophage culture was diluted 10^{-5} in the sterile liquid medium containing the isolated *P.E.*-inhibitor or ribonuclease at various concentrations between 0·05 and 0·001 %. The fluid was then mixed with 24 hr. liquid cultures of the host bacteria, and the mixture was added to melted agar, cooled to 42°, and plated. Equal numbers of plaques (about 400 per plate) were produced on the control plates and on those containing *P.E.*-inhibitor or crystallized ribonuclease.

Table 2. *The recovery of infectivity by diluting a mixture of tobacco mosaic virus and sap of* Phytolacca esculenta

Mixture	Average no. of lesions/leaf obtained with mixtures diluted in water to			
	1/1	1/10	1/100	1/1000
1 vol. 0·005 % T.M.V. + 1 vol. sap. dil. 1/10	0	3	6	4
1 vol. 0·005 % T.M.V. + 1 vol. H_2O	110	64	20	4

The inhibition of plant viruses occurred immediately the sap of *P.E.* and virus solutions were mixed and there was no further fall in infectivity when the mixtures were incubated at room temperature or at 37°. Diluting non-infective mixtures in water restored infectivity of the virus (Table 2).

The sap not only inhibited infectivity when it was mixed with a virus solution and the mixture was inoculated to test plants, but also when it was first rubbed over the leaves, which were then rinsed with water, dried with blotting paper and the virus solution inoculated immediately after. The reduction in infectivity produced by the two methods with a purified preparation of the inhibitor is shown in Table 3. At first sight the results suggest that inoculation with the inhibitor first is as effective as inoculation of a mixture of the virus and inhibitor. However, it will be seen that a previous rubbing of the leaves with water alone considerably reduces the number of lesions produced, and this needs to be taken into consideration when comparing the results of tests in which leaves were rubbed once or twice.

Table 3. *The effect of inoculating the inhibitor and tobacco mosaic virus separately and together*

First inoculation	Second inoculation	Average no. of lesions/leaf
1 vol. 0·0002 % inhibitor +1 vol. 0·015 % T.M.V.	None	4
0·0001 % inhibitor	0·0075 % T.M.V.	3
0·0075 % T.M.V.	0·0001 % inhibitor	10
Water	0·0075 % T.M.V.	17
0·0075 % T.M.V.	Water	34
0·0075 % T.M.V.	None	47

The inhibitory power of the sap was unaffected by heating for 10 min. at 70°, but was diminished by 10 min. at 80° and completely destroyed by 10 min. at 100°. Drying the sap in a desiccator at 37° and suspending the dry residue in the original volume of water did not affect the inhibitor; nor did freezing and thawing, changes of pH between 2·5 and 8·0, or dialysing the sap in a cellophan bag against distilled water, or centrifugation for 1 hr. at 40,000 r.p.m. Filtration through a porcelain filter L_1 did not affect its inhibiting power, but subsequent filtration through an L_3 or L_5 filter considerably diminished it.

Sap expressed from minced leaves of *P.E.* is viscous and rather frothy. After keeping for 24 hr. at room temperature and centrifuging for ½ hr. at 3000 r.p.m., the clarified sap contains about 7 % of dry matter. Much inactive material can be coagulated and removed by heating the sap to 60–70°, freezing and thawing, or precipitation with half a volume of 96 % ethanol.

The inhibitor is precipitated quantitatively by adding two volumes of ethanol to one volume of the sap and can be redissolved completely in a 2 % NaCl solution. The precipitate produced by ethanol, however, must be taken up in 2 % NaCl within 1 hr., for if the time is prolonged the inhibiting power of the resulting solution is progressively diminished. If water is used instead of 2 % NaCl, only part of the inhibitor is recovered.

The inhibitor begins to be precipitable by ammonium sulphate when the concentration reaches half-saturation, and progressively more is precipitated as the concentration of the salt increases. Precipitation is almost complete when one volume of the sap is mixed with twenty volumes of saturated ammonium sulphate solution. The precipitated inhibitor dissolves readily in water.

Isolation of the inhibitor based on fractional precipitation with ethanol and ammonium sulphate, combined with dialysis against distilled water and adjusting the pH to various values, gave concentrated preparations, but usually a number of fractions, all with some inhibitory power, were obtained. The fractions differed from each other in various respects and were not homogeneous, as they could usually be fractionated still further. They contained 20–60 % of carbohydrate and had no detectable proteolytic or ribonuclease activity, but they were invariably rich in a phosphatase (nucleotidase) having a maximal activity between pH 5 and 6.

In preparations concentrated by precipitation, phosphatase and inhibitor were so closely associated that it seemed probable that the enzyme was the inhibitor. Eventually, however, it was found that the inhibitor but not the phosphatase was readily adsorbed from salt-free solutions by a number of adsorbents. Of the various adsorbents tested charcoal and celite (diatomaceous silica Filter-Aid, Johns-Manville) were found to be the most effective. It was not possible to elute the inhibitor from the two adsorbents with distilled water, but from celite it could be eluted with 10 % NaCl solution, though not from charcoal.

The method of isolation of the inhibitor

Of many methods of isolation tested the following was finally adopted as the most reliable in producing apparently homogeneous preparations. Five hundred ml. of 96 % ethanol are added to 1 l. of sap, and the resulting bulky precipitate is removed by centrifugation. To the clear, brown supernatant liquid 1500 ml. of ethanol are added, and the precipitate, which contains the inhibitor, is centrifuged down. The precipitate is suspended in 250 ml. of 2 % NaCl, and the undissolved material is removed by centrifugation. The clear greyish supernatant liquid is dialysed in a cellophan bag for 2 days against distilled water. The liquid, which is usually slightly acid, is then neutralized, and some insoluble material produced during dialysis is removed by centrifugation. This involves some loss of the inhibitor, usually less than 25 %, probably because it is adsorbed on the insoluble material. Adjusting the pH to 7 decreases the amount lost. Separation of insoluble material, and consequently loss of inhibitor, reaches its maximum when the pH is around 4·5.

At this stage the liquid contains about 0·5 % of dry matter, of which 50 % is carbohydrate and 8 % nitrogen. The liquid gives a strongly positive biuret test and has strong phosphatase activity. Celite is next added to the liquid, 2 g. to each 100 ml. The mixture is kept for 1 hr. at room temperature with occasional shaking, and the celite removed by filtration through a filter-paper on a Buchner funnel. In this way almost all the inhibitor is removed from the liquid, although the solid content of the liquid is reduced by less than 10 % and its phosphatase activity remains almost unchanged. The celite is washed in the funnel with 200 ml. of distilled water and suspended in 30 ml. of 10 % NaCl solution. The suspension is kept for 4 hr. at room temperature with occasional stirring, and the celite is again filtered off. The water-clear filtrate is dialysed in a cellophan bag for 24 hr. against frequently changed distilled

water. A slight turbidity sometimes appears during dialysis, and is removed by filtration. About 20 % more inhibitor can be obtained from the celite by repeating the elution.

The yield of purified material from 1 l. of sap varied from 20 to 60 mg. Inhibition by a 0·0002 % solution of the purified preparation was approximately the same as that of sap diluted 1/100. Thus, assuming that the inhibitory power of the material is unaffected during purification, its initial content in crude sap is about 200 mg./l. and 60–90 % is lost during purification. However, as exposure to ethanol has a deleterious effect on the inhibitor, it may be that the recovery is greater than this but that the purified product is, weight for weight, less active than the inhibitor in crude sap.

When the purification procedure was carried out on tobacco sap, which does not inhibit infectivity of T.M.V. to any appreciable extent (see Table 1), nothing was recovered in the elute from the celite, showing that the method is selective.

Properties of the purified inhibitor

The nitrogen content of purified products varied from 14 to 15 % and the carbohydrate content from 8 to 12 %. No phosphorus was detected in any preparation. The material gives a positive biuret test, is precipitated by 95 % saturated ammonium sulphate solution and by 5 % trichloroacetic acid. When salt-free solutions are boiled, they become slightly opalescent, while in the presence of 1 % NaCl a coagulum separates. These properties all indicate that the material is a protein. Since most of the carbohydrate is not separated from the protein by precipitation with ammonium sulphate or trichloroacetic acid, it appears that the two are combined as a glycoprotein, and the inhibitor will be referred to as such.

Table 4. *Relative inhibitory powers of ribonuclease and the glycoprotein*

	Concentration (%)	Average no. of lesions/leaf
Ribonuclease	0·05	1
	0·001	4
	0·0002	15
Glycoprotein	0·0002	16
Water control		90

The fluids were mixed with equal volumes of 0·015 % T.M.V.

No critical tests for homogeneity were made, and it may be that the purified preparation contains more than one protein. However, there is a good deal of evidence connecting the inhibitor with the glycoprotein that forms at any rate the major constituent of the preparation. Of the various inhibiting substances previously tested, ribonuclease is by far the most efficient (Loring, 1942). Table 4 shows the results of an experiment comparing the inhibitory power of the purified glycoprotein from *P.E.* and that of crystallized ribonuclease prepared by Kunitz's (1940) method. It will be seen that, weight for weight, the two have similar inhibitory powers, so that if the inhibition produced by

the preparation from *P.E.* is brought about by a minor component, then this must possess a much higher order of activity than ribonuclease. Again, processes that denature the glycoprotein—heating, trichloroacetic acid, or exposure to ethanol—also cause a proportional loss of inhibitory power. The glycoprotein is not hydrolysed by pepsin or trypsin, unless it has been previously denatured, and the inhibiting power similarly is not diminished by these enzymes. Drying neither denatures the glycoprotein nor affects its inhibiting power.

Denaturation of the glycoprotein by ethanol is slow, and may be demonstrated by the loss of solubility in water. The addition of two volumes of 96 % ethanol to one volume of salt-free solutions of the glycoprotein, produces an opalescence, but flocculation occurs only in the presence of salt. After precipitation with ethanol a proportion of the glycoprotein usually becomes insoluble in water, the proportion increasing with increasing time of contact with ethanol, so that after a few days all the glycoprotein becomes insoluble. The stability of suspensions of ethanol-denatured glycoprotein depends on the pH and this property was used to find its isoelectric point.

When suspensions containing 0·05 % of the glycoprotein were adjusted to different pH values (measured with a glass electrode) with HCl or NaOH, it was found that they flocculated in a few minutes at pH values between 6·7 and 7·3, but they remained stable for several hours at pH values outside this range. From this it was concluded that the isoelectric point of denatured glycoprotein is near pH 7. It is known that denaturation can alter isoelectric points of some proteins, but the alteration is usually slight and does not exceed 0·5 of the pH unit (Neurath, Greenstein, Putnam & Erickson, 1944). Thus the isoelectric point of the undenatured glycoprotein, which is soluble over a wide pH range, is probably also about pH 7, a feature which it shares with other proteins, such as ribonuclease and trypsin, which inhibit infectivity of T.M.V.

The isoelectric point of salt-free solutions of T.M.V. is at pH 4·2 (Bawden & Pirie, 1937), so that between pH 4·2 and pH 7 the glycoprotein and the virus are oppositely charged. Some other pairs of proteins that are oppositely charged precipitate one another when mixed (Kleczkowski, 1946) and this also occurs with the glycoprotein and T.M.V. When the two are mixed in salt-free solutions at pH values between their isoelectric points, they combine, mutually discharge one another and produce a visible precipitate.

No attempts have been made to determine the minimum concentrations at which T.M.V. and the glycoprotein must be mixed to give a visible precipitate, but precipitation is pronounced with 0·01 % solutions of the two. Precipitation only occurs when the ratio of the weight of virus to that of glycoprotein is between 1 : 1 and 1 : 2. Outside this range, the fluids merely become opalescent and develop a satin-like sheen, the intensity of which decreases the further the ratio of the two components departs from that causing precipitation. The addition of NaCl up to 1 %, or changing the pH to below 3 or above 7, causes immediate solution of precipitate or disappearance of opalescence. Under the microscope the precipitates are seen to consist of long, curvilinear, birefringent

threads, similar to those formed in mixtures of T.M.V. with ribonuclease, clupein or serum globulin (Bawden & Pirie, 1937; Loring, 1942; Kleczkowski, 1946). Precipitation also occurs in mixtures of salt-free solutions of glycoprotein and tomato bushy stunt virus at pH values between 5 and 6·5. Using the glycoprotein at 0·005 %, precipitation was optimal when the concentration of tomato bushy stunt virus was 0·01 %. Microscopically the precipitate appeared to be granular, but no definite crystals could be identified.

Mixtures of equal volumes of 0·05 % glycoprotein and 0·05 % clupein sulphate became slightly opalescent at pH 9 but not at pH 6. The isoelectric point of clupein is at pH 12 (Miyake, 1927), so that pH 9 is in the inter-isoelectric zone, whereas pH 6 is not.

No opalescence or flocculation was observed when sap from *P.E.*, clarified by heating at 70° and centrifugation, was mixed with equal volumes of solutions of T.M.V. at different concentrations. Tests were also made using dialysed sap to avoid the possibility that precipitation was being prevented by inorganic salts, but again there was no precipitation. The negative result cannot be explained by assuming that the concentration of the glycoprotein in the sap is too low to cause precipitation, as it is higher than 0·005 %, at which the isolated glycoprotein precipitates the virus. Dialysed sap contains 5–7 % of dry matter and only 0·01–0·02 % of the glycoprotein, so that some of the non-dialysable constituents of sap seem to act as stabilizers and prevent the virus from being precipitated.

By means of ultracentrifugation it was possible to show that T.M.V. and the glycoprotein combine when they are mixed at ratios which cause no opalescence or precipitate. These tests also produced further evidence identifying the glycoprotein with the inhibiting entity. When 0·1 % solutions of the glycoprotein were centrifuged for 1 hr. at 40,000 r.p.m., the glycoprotein was not sedimented nor was the inhibiting power of the fluid reduced. When similar solutions containing 3 mg./ml. of T.M.V. were centrifuged, however, almost all the glycoprotein and the inhibitor were removed from the supernatant fluid. Some of the glycoprotein with its inhibiting power could be recovered from the virus by dissolving the pellet in water, and precipitating the virus with one-third saturated ammonium sulphate solution. After removing the virus by centrifugation at low speed, the glycoprotein was present in the supernatant fluid with its original inhibiting power.

By analogy with toxin-antitoxin reactions, it seemed possible that inactivation of the virus by the glycoprotein might be based on combination between equivalent quantities of the two. To test this possibility, attempts were made to find the highest concentrations of T.M.V. solutions whose infectivity could be completely inhibited by solutions of the glycoprotein at different concentrations. Table 5 gives the results of an experiment in which the neutralizing power of four different concentrations of glycoprotein was tested against different concentrations of the virus. Inactivation was considered as complete in mixtures that produced an average of less than one lesion per inoculated half-leaf, and the results are given as the highest concentrations of the virus inhibited to this degree by the glycoprotein at four different concentrations.

It can be seen that as the concentrations of the virus increased, the ratio of glycoprotein to virus needed to give complete inactivation decreased. Within the range tested, the virus concentration was proportional to the third power of that of the glycoprotein. This result differs strikingly from that obtained

Table 5. *The ratio of inhibitor to virus required to give complete inhibition at different virus concentrations*

Concentration of the inhibitor (mg./ml.) x	Concentration of T.M.V. (mg./ml.) y	x/y
0·024	0·72	0·03
0·012	0·09	0·13
0·006	0·01	0·60
0·003	0·0015	2·00

The relationship between y and x can be expressed by the equation $y = 51290x^3$.

with toxins and antitoxins, and shows that the higher the virus concentration, the smaller was the amount of the glycoprotein that was needed to neutralize each unit weight of the virus. There are clearly no fixed equivalent quantities of the virus and glycoprotein which combine before the virus is rendered non-infective.

DISCUSSION

From our results it is clear that sap from *Phytolacca esculenta* contains a protein that is not present in the sap from tobacco plants. The evidence suggests that it is a glycoprotein and that it is the substance in sap of *P. esculenta* responsible for the inhibition of infectivity of plant viruses. Weight for weight it is as active as an inhibitor as ribonuclease, and its identification adds a new type of substance to those known to inhibit plant viruses. How these varied substances produce their effect is uncertain, but superficially at least they appear to act similarly.

Since the inhibitory effect of trypsin was discovered (Caldwell, 1933), there has been much speculation on the mechanism of its action, some workers maintaining that it acts on the virus and others that it acts on the host plant. The fact that an inhibitory material used at constant concentration caused the same relative reduction of infectivity irrespective of virus concentration, was often considered as evidence that the inhibiting material affected susceptibility of the host plant (Chester, 1934; Stanley, 1934; Caldwell, 1938).

The results with the glycoprotein, in addition to those with some other of the inhibiting substances (Kleczkowski, 1944, 1946), show that the inhibitors do combine with tobacco mosaic virus, so that the simplest explanation of the phenomenon would be that in combining with the virus the inhibitors block some groups on it that are essential for the establishment of infection. However, there are various other facts that do not fit in with this view of a simple quantitative neutralization of the virus. First, of the substances that can combine with and precipitate T.M.V., not all are equally active as inhibitors, and some, such as clupein and globin, have only little neutralizing power.

Hence mere combination of a protein with the virus is not enough to explain inhibition, unless different substances combine with and block different groups. Secondly, if inhibition were dependent on, and a consequence of, combination with the virus, it is to be expected that for complete neutralization of infectivity a given weight of virus would need to combine with a certain minimum quantity of inhibitor, but there is no evidence for such a neutralizing ratio. On the contrary, there is good evidence that no such ratio exists. This is partly implied by the development of infectivity when non-infective mixtures of virus and inhibitor are greatly diluted, for dilution leaves the ratio of the two unchanged. However, this is not conclusive, for if the combination is reversible, dilution could disrupt the combination and leave free virus particles. More positive evidence for the lack of a neutralizing ratio is supplied by the fact that the higher the virus concentration, the smaller is the ratio of combined inhibitor to virus that coincides with complete neutralization. Hence, there is no reason to assume that the combination between the two is at all essential for inhibition to occur. This suggests an effect on the host rather than on the virus, a suggestion supported by the fact that the number of lesions produced is reduced if the leaves are inoculated first with the inhibitor alone. In our present state of knowledge of the processes involved in the establishing of infection, any attempt to distinguish between effects on the virus and on the host is probably premature. All that can be said with certainty is that the virus and inhibitors can combine, and that the presence of the inhibitors prevents infection. It is also possible that the inhibitors can combine with some constituents of the host cells, but this has not been demonstrated.

Many examples are known of substances that are structurally similar being biologically antagonistic. The inhibitors could be further examples of such biological antagonism, acting either because they resemble some cell constituents with which the viruses must combine to establish infection or because they themselves combine with these constituents and so prevent the virus from doing so. Although this is a possibility that needs further study, the wide range of substances that can act as inhibitors ranging from carbohydrates, through various enzymes, to the glycoproteins, does not suggest that any great structural specificity is involved. A lack of specificity is also apparent in that substances such as the glycoprotein from *P. esculenta* and ribonuclease are effective inhibitors of all the plant viruses against which they have been tested. However, neither substance affected the activity of a bacteriophage, suggesting that the inhibitors may act specifically against some phase of the infection process essential for the establishment of mechanically transmitted plant viruses but absent from the process of infection of bacteria by bacteriophage.

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(*Received* 31 *October* 1947)