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THE LIBERATION OF VIRUS, TOGETHER WITH MATERIALS THAT INHIBIT ITS PRECIPITATION WITH ANTISERUM, FROM THE SOLID LEAF RESIDUES OF TOMATO PLANTS SUFFERING FROM BUSHY STUNT.

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IN all previous work on the properties of plant viruses the starting material has been the sap that is expressed from macerated tissues by squeezing through cloth. Leaves or whole plants have usually been macerated by passage through a domestic meat mincer or by pounding them with a pestle in a mortar. Most workers have frozen the leaves, the mince or the sap, as this facilitates clarification and purification but it does not increase the amount of virus extracted. Washing the leaf residues yields little more virus, usually only the amount to be expected from the volume of sap contained by the residues. It has therefore been assumed that the leaf tissues contain no significant amount of virus, and the published figures for the virus content of infected plants refer solely to the amounts extracted in the sap.

This assumption is unjustified, for we have found that the leaf tissues hold virus in considerable quantities. This virus, however, is bound and is liberated only if the tissues are subjected to special treatment. Brief reference has already been made to the extraction of tomato bushy stunt virus by the fine milling of infected tomato leaves (Bawden and Pirie, 1943a). The present paper describes some of the conditions affecting the liberation of this normally insoluble virus by milling and other treatments.

The broad principles are fairly clear, but it is difficult to be definite about details for different lots of plants behave very differently. The leaf residues contain approximately as much virus as is extracted in the sap, and it is most completely liberated by incubating the residues with a commercial trypsin preparation and then grinding finely. Either incubation with commercial trypsin or grinding will liberate some of the virus, but the relative proportions liberated by the two treatments vary from one lot of plants to another. The component of commercial trypsin preparations responsible for the liberation of virus is soluble and does not diffuse through cellophane, but we have no evidence that it is actually trypsin. For convenience this phenomenon will be referred to as liberation by " trypsin."

We have made no attempt to standardize the cultural and environmental conditions of the host plants, and the variations in results with different batches of leaves presumably have their origin in differences in these conditions. Tomato plants growing at different times of the year vary in their susceptibility to infection with bushy stunt virus, in the type and severity of symptoms they show and in their virus content ; they also, of course, vary in their vigour, colour and habit of growth.

We have found no differences between purified virus obtained from the sap and from the leaf residues, but extracts from infected residues have serological properties different from any previously described. Some contain virus in the form of a non-precipitating antigen, while others contain materials that prevent the normal precipitation of the virus by its antiserum.

Preparations of leaf residues.

All the work described has been done with young tomato plants of the variety Kondine Red, which were grown in glasshouses with heat but no extra illumination. In the summer months seedlings rapidly pass through their most susceptible period, and to get good systemic infection, with a high virus content, they must be inoculated when not more than 6 in. high. In the winter, plants retain their susceptibility for longer, and the symptoms are usually more necrotic and less chlorotic than in the summer. Leaves are picked from 8–14 days after infection, when the symptoms are well developed. The main stem and bases of the petioles are not included, as these contain only about one-eighth of the virus present in the laminae and their presence greatly complicates all later operations.

The leaves are minced with a domestic meat mincer and the sap expressed by hand through madapollam ; from 100 g. of fresh leaves there is, on an average, 80 g. of sap and 20 g. of residue. If the leaves or the mince have been frozen, or if the residue is minced again after one squeezing, there is a slightly greater yield of sap. Provided the pH is unaltered, the amount of sap that can be expressed by hand is not significantly altered by further or finer grinding. This suggests that most of the cells are sufficiently damaged by freezing and mincing, or by two successive mincings, to liberate their sap, and that the effect of fine milling is not simply to damage further cells.

Sap remaining in the leaf residues can be removed by suspending them in a volume of water equal to that of the sap and squeezing through madapollam. Even with the most infective sap, four such washes have been sufficient to reduce the virus content of extracts below that necessary to give a visible precipitate with antiserum. For the first two washes the water is expressed after a few minutes in contact with the residues, and for the last two it is left in contact for some hours. Material prepared in this way, pressed as dry as possible by hand, will be referred to as washed fibre ; it forms the starting material for the experiments described below.

Milling the washed fibre.

For fine grinding we have used a triple-roller ointment mill (made by the Pascall Engineering Company, London) that grinds 1–2 g. wet weight of fibre per minute. This has three porcelain rollers, cylinders 10 cm. long and 5 cm. diameter, driven by a gear train at 160, 350 and 520 r.p.m. respectively. The middle roller revolves in the opposite direction to the other two, so that material fed between the slow and the middle roller is carried on the undersurface of the middle roller and ground again between this and the fast roller ; it is finally removed by a scraper. The fineness of grinding is controlled by the force holding the rollers together ; this is normally adjusted so that the ¼ h.p. motor driving the mill begins to " labour " when a charge of proper consistency is passing through.

Grinding is only effective over a narrow range of water content ; if the charge is too wet it does not cling to the rollers, and if it is too dry there is insufficient lubrication where the rollers rub together. The moisture content of washed fibre in the usual pH range (5·7 to 7·2) is about 70 per cent. This is too low for satisfactory grinding, and one part of water should be added to three of fibre to get the best consistency.

Separation of fluid from milled fibre.

Fluid can be separated from the finely ground fibre by squeezing the paste in a madapollam bag, but by assiduous kneading much of the solid also passes through the bag. This leads to great variations in results, for the cloth becomes clogged and

less porous as the separation proceeds. This phenomenon becomes more pronounced with increasing pH, which increases the water-holding capacity of the ground fibre. Above pH 8 separation of fluid and solid is no longer practicable by squeezing through a cloth. It can be done by centrifugation, but within the pH range used the water content of sediments is so high that this is an unsatisfactory method of separation, for, unless such large volumes of extracting fluid are used that there is excessive dilution of virus, a large proportion of the virus will be in the free fluid remaining in the sediment. Therefore, for quantities of ground fibre between 2 and 15 g., we have used the following method, which gives reasonably reproducible results. A piece of cloth is fastened over the mouth of a 50 or 250 ml. centrifuge tube with a rubber band, and adjusted so that there is enough slack to form a bag in the mouth of the tube. With properly adjusted tension on the band, the system can be centrifuged at 1000 r.p.m. All the fluid that can be separated passes through the cloth in 10 minutes at this speed. The area of cloth over which the material is distributed is always small, so that the recovery of both extract and residue is more nearly quantitative than when separation is done by hand.

Neutralization of fibre.

For testing the effect of enzymes in liberating virus from leaf residue it is necessary to control the pH of the suspension. This needs careful checking, as the pH value of neutralized fibre drifts considerably. The pH of washed fibre is between 5·8 and 6, and very little alkali is needed to take it to 8. However, the pH soon drifts back, and frequent addition of small amounts of alkali is needed during 24 hours at room temperature to stabilize the pH at 7·6 to 8. A total of 3 ml. of $N/5$ NaOH is needed for 10 g. of washed fibre. Bacteria grow readily on the neutralized suspension, and to prevent this we have used chloroform as an antiseptic. During the neutralization 1 ml. of chloroform is added for each 10 g. of washed fibre ; for ease of stirring during the addition of NaOH, the fibre is suspended in a volume of water equal to twice that of the sap previously expressed from it, i.e. 60–80 ml. per 10 g. Washed fibre neutralized in this way was used in all the incubation experiments described, and all subsequent operations were made with fluids saturated with chloroform.

Methods of testing.

The virus content of the extracts from infected plants has been estimated by titration against antiserum prepared by injecting rabbits with purified bushy stunt virus. Occasional infectivity tests showed that this antigen was active virus ; these were done by the local lesion method, using *Nicotiana glutinosa* as a host plant. In the precipitin tests 1 ml. of antiserum at a constant dilution was added to a series of tubes each containing 1 ml. of extract at varying dilutions. The tubes were then placed in a water-bath at 50° C., with their fluid column half immersed to ensure mixing by convection, and the greatest dilution at which the extract gave a visible precipitate was taken as the end-point. Usually the extracts were diluted serially with a factor of two, and no attempt was made to detect smaller differences.

The sap of plants clarified by centrifugation at 3000 r.p.m. may give unspecific precipitates under the conditions of test, but this is easily recognized by controls with saline or normal rabbit serum. This tendency is reduced if the leaves or sap have been frozen and thawed before centrifuging, or if a little Na_2HPO_4 has been added or the sap has been left for a few hours at room temperature. After such treatments, clarified sap is clear brown and suitable for immediate use in precipitin tests.

Extracts made from milled fibre or from fibre incubated with " trypsin " need further treatments before precipitin tests are a reliable index of their virus content.

The extracts made from milled fibre are deep green, and the chromoprotein is not removed by centrifugation at 3000 r.p.m. even after they have been stored for some days at room temperature. The yield of chromoprotein is higher in the summer than in the winter and its extraction is favoured by low salt content, high pH and fine grinding. These extracts sometimes give bulky unspecific precipitates in the water bath, but they are more often stable, and the chief complication in serological tests is the failure to obtain a specific precipitate with antiserum when the virus content is adequate. Hence the chromoprotein must be removed before precipitin tests are made. This is most conveniently done by freezing for 15 hours at $-7°$ C., which coagulates the chromoprotein but, at the virus concentration, salt content and pH usual in these extracts, does not inactivate the virus (Bawden and Pirie, 1943*b*).

The conditions used for enzymic digestion usually give extracts that are too dilute for satisfactory serological tests ; after incubation with " trypsin " such extracts may also contain material that prevents the precipitation of virus by antiserum. Before testing, the extracts are concentrated and the inhibiting material removed. The fibre and fluid are first separated by centrifuging through cloth as already described. After removing the cloth from the tube, any insoluble material in the fluid is packed by centrifuging for 15 minutes at 3000 r.p.m., when the supernatant fluid is poured off and half saturated with ammonium sulphate. If digestion has been prolonged there is immediate precipitation, but if it has been brief or with little enzyme, precipi-tation is delayed. The mixture is therefore left for 3 hours at 25° C. to ensure complete precipitation. After centrifuging for 30 minutes at 3000 r.p.m. the pellet is suspended in 1·5 ml. of water for each gramme of fibre extracted, and 1 drop of 10 per cent. acetic acid is added to the thoroughly mixed suspension to bring the pH to 4–4·5. The insoluble material, which contains the inhibitor, is removed by a few minutes' centri-fuging at 3000 r.p.m. After neutralization, the supernatant fluid is suitable for testing serologically.

<center>RESULTS.</center>

Liberation of virus by milling and enzymic digestion.

After freezing and centrifuging, extracts made by suspending milled fibre in three times its weight of water are clear and only pale straw colour. Their virus content varies with different lots of plants, as does the virus content of sap. The precipitin titre of such extracts may be the same as that of sap, but more usually it is one-half or one-quarter. Variations in pH between 5 and 7·5 or in the nature and concentration of the buffer solution have had no significant effect on the liberation of virus. A second extraction of the milled fibre yields additional virus, but there is usually a greater liberation if the milling is repeated before the fibre is extracted. With some batches of leaves the amount of virus released by successive millings falls slowly, whereas with others nearly all the virus is released by one milling. Table I illustrates these variations ; in general, more of the virus is liberated by one milling from plants grown in the winter than from those grown in the summer. In making these extracts the milled fibre was soaked in the fluid for 30 minutes before separation ; longer soaking does not bring out significantly more virus.

The amount of virus liberated by incubation with commercial trypsin preparations (British Drug Houses) depends on the pH, amount of " trypsin " and duration and temperature of incubation. We have standardized the last three factors by allowing 0·1 per cent. solutions of " trypsin " to act for 15 hours at 42° C. More intense digestion does not give sufficient extra virus to be detected by the methods of testing used. Column 1 of Table II gives the results of incubating washed fibre at different pH values with " trypsin," and shows a clear optimum between 6 and 7. No attempt has been

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TABLE I.—*Virus Content of Sap and Successive Mill Extracts.*

Month of harvesting plants.	Serological titres.			
	Sap.	First mill extract.	Second mill extract.	Third mill extract.
January	256	256	8	0
February	256	180	8	0
March	660	350	60	10
May	64	32	16	8
July	450	64	24	6
September	96	40	20	10
December	80	16	1	0

Washed fibre was milled and extracted twice with 2–3 times its weight of M/50 pH 7 phosphate buffer ; the two extracts were mixed and prepared for testing as described in the text. The fibre residue was then milled again and extracted in the same way. The titres given are the observed precipitation end-points of sap or mill extract multiplied by a factor to bring them to the standard ratio of 1 ml. of fluid from 1 g. wet weight of washed fibre.

TABLE II.—*The Effect of pH on the Liberation of Virus from Fibre by Incubation with "Trypsin."*

pH.	Serological titres.		
	1. Extract from fibre incubated with " trypsin."	2. Residues from 1 re-extracted at pH 7.	3. Residues from 2 milled and extracted.
4	2	2	32
5	16	4	32
6	64	0	32
7	48	0	32
8	16	0	24
9	0	0	16
Control fibre milled without previous treatment			8

Titres are reduced to a standard basis of 1 ml. of extract from 1 g. of washed fibre.

made to determine the optimum more closely ; the pH is a matter of uncertainty when over 6·5, as there is a considerable liberation of acid during incubation. 1 ml. of buffer solution, made by adjusting a 4 per cent. solution of Na_2HPO_4 to the required pH with acetic acid, is added for each gramme of fibre, but this is insufficient to hold the pH at the required value, and the pH must be adjusted three or four times during the first five hours if it is to be kept constant to within 0·5 of a unit.

Provided adequate amounts of antiseptic have been present throughout the operations on the washed fibre, incubation without the addition of " trypsin " usually fails to liberate virus, although occasionally it has given an eighth as much as has incubation with " trypsin." Incubation with pepsin or papain, with or without cyanide, has liberated no more virus than incubation alone. These two enzymes are not ineffective because they destroy the virus, for provided that the incubation has not been carried out at a pH below 3, virus can still be liberated by subsequent milling or by incubation with " trypsin."

When no more virus can be released by further milling, some can still be obtained by incubation with " trypsin." The amount varies with different lots of leaves and is never large ; it seems to be greater with plants grown in the winter. By contrast, from washed fibre that has been incubated with " trypsin " there is a copious liberation of further virus on milling, although no more is liberated by increased incubation. Indeed, extracts with the highest precipitin titres are often obtained by this method,

in spite of the fact that virus has previously been extracted as a result of the incubation with " trypsin." Table III shows that there is greater liberation of virus when incubation with " trypsin " precedes milling than when the sequence is reversed ; it also shows how the discrepancy between the yield of virus by the two methods increases the more often the fibre has been milled before it is incubated with " trypsin."

Even at pH values at which incubation with " trypsin " gives little direct liberation, there is some loosening of the virus from its connection with the fibre. This is shown in Table II. Fibre that has been incubated with " trypsin " on the acid side of the optimum releases some virus when subsequently extracted at pH 7 (Table II, column

TABLE III.—*The Effect of the Sequence of Treatments on the Serological Titres of Successive Extracts.*

Trypsin	64	Trypsin	64	Mill	16	Mill	16	Mill	16	Mill	16
Trypsin	16	Mill	64	Mill	1	Mill	1	Mill	1	Trypsin	64
Trypsin	2	Trypsin	16	Mill	0	Mill	0	Trypsin	32	Mill	4
Trypsin	0			Mill	0	Trypsin	16	Mill	2	Trypsin	0
Mill	128			Trypsin	4	Mill	2	Trypsin	0		
						Trypsin	0				

Each column deals with a separate 2 g. sample from the same lot of washed fibre, which was subjected successively to the treatments listed. After each treatment the fibre was extracted twice with a total of 12 ml. of water ; the two extracts were combined and precipitated with acid and ammonium sulphate before testing serologically ; the residue was then subjected to the next treatment. Titres are reduced to a standard basis of 1 ml. of extract per g. of washed fibre.

2). Column 3 gives the virus contents of extracts made by milling, and here also the effect is clearly shown, for extracts from fibres incubated with " trypsin " at all pH values between 4 and 9 contain more virus than the mill extract of unincubated washed fibre. Incubation without the addition of " trypsin " has never led to a similar increase in the amount of virus liberated by milling, provided that antiseptic precautions were taken ; occasionally incubation alone has reduced the amount of virus liberated by milling.

Properties of virus from sap and residues.

Purified preparations of bushy stunt virus have been made by the method recently described (Bawden and Pirie, 1943a) from infective sap, and from the extracts prepared by subjecting the residues to fine grinding or incubation with " trypsin." Preparations from the two sources have not been found to differ in any significant manner.

TABLE IV.—*Activity of Purified Virus Preparations made from Sap and Fibre.*

Source of virus.	Serological titre.	Infectivity. Average number of lesions per leaf at—		
		10^{-5}.	10^{-6}.	10^{-7}.
Sap	1/300,000	66	12	1
Milled fibre	1/300,000	60	15	2
Sap	1/300,000	90	17	2
Milled fibre	1/300,000	78	15	1
Sap	1/300,000	115	36	—
Milled fibre	1/300,000	103	38	—
Trypsined fibre	1/300,000	99	23	—
Sap	1/400,000	41	19	4
Milled fibre	1/400,000	49	26	6

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They have similar serological activity and infectivity (Table IV), and similar phosphorus and carbohydrate contents ; they crystallize in the same form and behave in the same way during crystallization.

Measurements of sedimentation constants have not been made, but comparisons of the weight of virus compacted into a pellet when preparations from sap and mill extracts are ultracentrifuged in the same conditions show that there is no gross difference. In making such a comparison the pH of the virus solutions needs careful control. McFarlane and Kekwick (1938) found that variations in pH between 2 and 9 had no effect on the rate at which the boundary moves during centrifugation. It does, however, greatly affect the rate at which the concentrated virus at the bottom of the tube compacts into a coherent pellet. In our tests 0·15 per cent. virus solutions in M/40 phosphate or acetate buffer were centrifuged in the 7·5 ml. tubes of an air-driven centrifuge of the type described by Masket (1941). The fluids were centrifuged for 30 minutes at 40,000 r.p.m. (90,000 R.C.F.), followed by 30 minutes during which the rotor was allowed to coast and the speed fell to 30,000 r.p.m. This degree of centrifugation at the isoelectric point (pH 4·2) packs all the virus into a compact pellet, which remains in the tube when the fluid is decanted. By contrast, at pH 8 there is only a trace of a compacted pellet, and almost all the virus lies at the bottom of the tube as a dense, mobile fluid ; more prolonged centrifugation at this pH compacts more of the virus. With increasing pH from 4 to 8, the ratio of pellet to dense fluid falls steadily. Below pH 4·2 the boundary between supernatant fluid and sediment is less definite, and the sediment, although easily visible, is so limp that it is apt to slide out of the tube when the fluid is decanted.

Comparisons have been made at pH 6 of the ratio of compacted to uncompacted virus in purified virus preparations made from sap and mill extracts of fibre. No differences were found, and the activities of the compacted and uncompacted virus did not differ. .

Virus as a non-precipitating antigen.

Mill extracts of washed fibre from different batches of infected plants vary in their behaviour in precipitin tests, but they usually give no specific precipitate with virus antiserum until the chromoprotein has been removed. This can be removed satisfactorily in any of five ways : by incubation with " trypsin " ; by centrifuging at 12,000 r.p.m. ; or by low speed centrifugation after the extracts have been frozen, heated to 60° C. or acidified to pH 4. The untreated extracts fail to precipitate with virus antiserum because of the presence of virus combined with chromoprotein to form a non-precipitating complex, which also interferes with the precipitation of free virus. This complex has serological properties resembling those of the non-precipitating antigens produced by Bawden and Kleczkowski (1941, 1942) by heating bushy stunt virus with serum albumin.

Table V illustrates how the removal of chromoprotein alters the reactions of a deep green mill extract with virus antiserum. The chromoprotein separated by freezing or by centrifugation at 10,000 r.p.m., often resuspends in water to give a stable suspension. If it is resuspended in its own supernatant fluid, the resulting mixture resembles the original mill extract and is not precipitated by virus antiserum. As chromoprotein from infected leaves prevents precipitation of free virus, there is no reason to suppose that all, or even most, of the virus in the mill extract was originally in the form of a complex that was split by freezing or high-speed centrifugation. The inhibition of precipitation is specific, and if the chromoprotein is added to solutions of antigens other than bushy stunt virus, e.g. tobacco necrosis viruses or serum

TABLE V.—*The Effect of Freezing on the Serological Behaviour of Extracts from Milled Fibre.*

Time after mixing with serum.	Mill extract. Dilution.					Extract after removal of chromoprotein by freezing. Dilution.				
	1/1.	1/2.	1/4.	1/8.	1/16.	1/1.	1/2.	1/4.	1/8.	1/16.
30 min.	—	-·-	—	—	—	++	+	-·-	—	—
2 hours	—	—	—	—	—	++	++	+	+	—
8 ,,	—	—	—	—	—	++	++	++	++	+

Plus signs indicate the degree of precipitation; minus signs, no precipitation. 1 ml. of extract at each dilution mixed with 1 ml. of virus antiserum at 1/50.

TABLE VI.—*Inhibition of the Serological Precipitation of Free Virus by Virus-chromoprotein Complex.*

Time after mixing with serum.	Amount of purified virus added to mill extract (in μg.).						Amount of purified virus added to saline (in μg.).					
	200.	100.	50.	25.	12.	6.	200.	100.	50.	25.	12.	6.
10 min.	—	—	—	—	—	—	++	+	—	—	—	—
20 ,,	++	—	—	—	—	—	+++	++	+	—	—	—
1 hour	+++	+	+	—	—	—	+++	++	++	+	—	—
4 hours	+++	+++	++	+	—	—	+++	+++	+++	++	+	+

Description in text.

TABLE VII.—*Inhibition of Serological Precipitation in Extracts from Fibre Incubated with " Trypsin."*

Time after mixing with serum.	Trypsin extract. Dilution.						Extract after centrifugation at pH 4. Dilution.					
	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.
1 hour	—	—	-·-	—	—	—	+++	+++	++	+	—	—
2 hours	—	—	—	+	++	—	+++	+++	+++	++	+	—
6 ,,	—	—	—	++	++	+	+++	+++	+++	+++	++	+

1 ml. of extract at each dilution mixed with 1 ml. of virus antiserum at 1/50.

globulin, these precipitate normally when the homologous serum is added. Also, chromoprotein from healthy tomato leaves does not inhibit the precipitation of bushy stunt virus by its antiserum. Hence the phenomenon is not simply a result of chromoprotein interfering in some mechanical and unspecific way with the separation of floccules.

If the chromoproteins that separate when mill extracts are frozen, acidified or centrifuged at 12,000 r.p.m. are suspended in water, no more virus is extracted than would be contained in their free water. After thorough washing, however, the protein from a non-precipitating extract still contains bound virus. Incubation with " trypsin " liberates this virus in a form that is precipitated by virus antiserum ; the amount liberated varies with different lots of chromoprotein, but 0·1 mg. from 10 mg. of chromoprotein is an average value. The virus content of chromoprotein derived from later mill extracts is always less than that of chromoprotein from the first extracts.

The washed precipitates of chromoprotein from infected plants combine specifically with virus antibodies. This can be shown by mixing suspensions with diluted antiserum, centrifuging for 15 minutes at 12,000 r.p.m. after incubation, and then testing the supernatant fluid for precipitating antibodies ; 5 mg. of washed chromoprotein is usually sufficient to absorb all the precipitating antibodies from 0·2 ml. of bushy stunt virus antiserum. Heterologous antisera still precipitate strongly after such absorption, and there is no comparable absorption of bushy stunt virus antibodies by chromoprotein from uninfected fibre. Washed chromoprotein from infected plants is antigenic and produces antibodies that precipitate free bushy stunt virus ; a rabbit injected with 15 mg. of a non-precipitating complex of chromoprotein and virus gave a serum with a precipitin titre of 1/80 when tested against purified bushy stunt virus at 0·05 mg. per ml.

We have no evidence that the virus occurs naturally in the fibre as a complex with chromoprotein, for extracts with serological properties similar to those of mill extracts from infected leaves can be made by milling fibre from healthy leaves to which purified bushy stunt virus has been added. For example, 4 g. of washed fibre from healthy tomato leaves was mixed with 1·5 ml. of pH 7 0·3 M phosphate buffer containing 2 mg. of purified virus, passed through the mill twice and extracted with 10 ml. of water ; this green extract did not precipitate with virus antiserum, but after freezing it precipitated to give an end-point of 16. An extract from an identical mixture that was passed through the mill four times likewise only precipitated after freezing ; it then gave an end-point of 8. By contrast, when 4 g. of the uninfected fibre was milled twice with phosphate buffer and then allowed to stand for an hour mixed with 10 ml. of water containing 2 mg. of virus, the green extract was precipitated by virus antiserum and gave an end-point of 32 both before and after freezing. Thus the non-precipitating complex is not formed merely by mixing virus with milled fibre, but only when the two are passed through the mill together. A comparison of the titres also shows that the recovery of virus from the milled mixtures is incomplete, and that repeated milling reduces the amount recovered. This is comparable to the effect already illustrated in Table III. Some, but by no means all, of this missing virus can be liberated from the residual fibre and the separated chromoprotein by incubation with " trypsin " at pH 6·5. Incubating infected fibre with " trypsin " has already been shown to increase the amount of virus liberated by milling. Experiments were therefore made with uninfected fibre to see whether a previous incubation with " trypsin " affected the recovery of purified virus added before milling. There was, however, the same loss with washed fibre and with fibre incubated with " trypsin." These effects are not peculiar to the tomato plant ; when bushy stunt virus is milled with fibre from healthy leaves of French bean (*Phaseolus vulgaris*, var. Canadian Wonder) there is a

similar loss of virus, and the extracts do not precipitate with virus antiserum until the chromoprotein is removed.

The behaviour of mill extracts from different lots of infected plants depends, not on the concentration of chromoprotein, but on the ratio of free virus to virus combined with chromoprotein as a non-precipitating complex. Extracts made from plants grown in the winter often contain little chromoprotein but much virus, and these precipitate with virus antiserum, though more slowly than after the removal of chromoprotein. With much chromoprotein and little free virus, extracts not only fail to precipitate with virus antiserum, but additional free virus can be added without causing precipitation. This is shown in Table VI. A deep green non-precipitating extract was used, which after removing the chromoprotein complex had a precipitation end-point of 16, indicating a free virus content of about 0·05 mg. per ml. 1 ml. samples of the extract were mixed with 1 ml. of virus antiserum at 1/50 and, after incubation for 1 hour at 50° C., varying amounts of purified virus were added to the mixtures. A control series of tubes was also used in which virus was added to mixtures of saline and antiserum. It will be seen that the presence of chromoprotein did not prevent precipitation, but that the concentration of free virus had to be doubled before precipitation occurred, and then it was slower and smaller than in the control tubes containing equivalent amounts of virus.

The results of Table VI show that after mixtures of chromoprotein and antiserum have been incubated, precipitation will occur if the content of free virus is increased sufficiently. This suggests that although the virus-chromoprotein complex combines with antibody, it is not preventing precipitation by leaving no antibody available for combination with free virus. Also, precipitation does not occur if the antiserum is increased to many times the amount that can be absorbed by the virus-chromoprotein complex present. Thus the inhibition of precipitation cannot be attributed to failure of the free virus to combine with antibody, but rather to some factor affecting the normal processes that intervene between combination of antigen and antibody and the separation of visible floccules. The virus-chromoprotein complex is readily sedimented by centrifugation at 12,000 r.p.m., so that the particles to which the virus is bound are much larger than the virus itself, although they are still too small to settle under gravity. Probably only a small part of the surface of these large particles is involved in combination with virus, and so most of it will remain unaffected when the virus combines with antibodies. Most of the hydrophylic groups will remain uncovered, and its failure to precipitate with virus antiserum is not unexpected. Presumably these complexes prevent the precipitation of free virus by antiserum either by preventing the formation of a regular lattice structure of antigen-antibody particles, which is believed to be the first phase of precipitation, or else they diminish the tendency of these aggregates to combine with one another so that they do not aggregate further to give the second stage that leads to the separation of visible floccules.

Only a small part of the total virus need be in the form of a non-precipitating antigen to inhibit precipitation completely. From mill extracts that failed to precipitate with virus antiserum, more than 7/8 of the virus has been found in the supernatant after freezing, and less than 1/8 has been recovered from the chromoprotein by incubation with " trypsin."

Non-specific inhibition of precipitation.

Extracts made from infected fibres that have been incubated with " trypsin " usually behave anomalously when used in precipitin tests with virus antiserum. Although they contain so much virus that they would give a visible precipitate in normal conditions, they may either fail to precipitate, or they precipitate only after

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long incubation with antiserum, and then only at high dilutions. ·If the extracts are brought to pH 4 and centrifuged, the supernatant fluids after neutralization precipitate normally with antiserum. This phenomenon is illustrated in Table VII.

This type of inhibition does not depend on the presence of virus in a non-precipitating form, for extracts with similar inhibitory effect can be made from healthy tomato leaves or from leaves of *Phaseolus vulgaris* and tobacco. Also, the extracts can inhibit the precipitation of more than one antigen-antibody system. The inhibition is therefore not type-specific, but is caused by the presence of material that acts as a " protective colloid " and stabilizes the system. With mill extracts inhibition depends on the ratio of non-precipitating virus complex to free virus, and if an extract fails to precipitate when concentrated it will not precipitate after dilution. Table VII shows that the non-specific inhibition depends more on the actual concentration of the inhibitor, for equal dilution of virus and inhibitor leads to precipitation. There is, however, a critical amount of virus that can be stabilised by a given concentration of inhibitor, and a virus-antibody system that is giving no precipitate will precipitate if the virus content is increased.

The amount of inhibitor that can be extracted from washed fibre depends on the treatments given. Very little is obtained unless the fibre is incubated with " trypsin," and the amount is further increased if it is milled before incubating with " trypsin." This is shown in Table VIII. The precipitate that separates at pH 4 from inhibiting

TABLE VIII.—*The Effect of Different Treatments in Producing Inhibitor from Fibre.*

Treatment.	Inhibition titre.
Milled	1
Trypsined	4
Milled, then trypsined	8
Milled, trypsined and milled again . .	8
Trypsined, then milled	4

The inhibition titre is the highest dilution at which the solution completely prevented precipitation of added virus. After each treatment the fibre was extracted and precipitated at pH 4 as described in the text. The acid precipitates from the successive treatments on each sample of fibre were mixed, washed, dissolved, and reprecipitated before testing.

extracts made from infected fibre contains some virus. Part of this can be removed by washing with water at pH 4, but the removal is more complete if the resuspended precipitate is brought to pH 7 and again precipitated with acid. The materials used for the comparison given in Table VIII were all prepared in this way, and the second precipitate at pH 4 was taken up in pH 7 M/100 phosphate buffer, using 1·5 ml. for each gramme of washed fibre, and centrifuged at 3000 r.p.m. before testing serologically. 1 ml. of the extracts at different dilutions were mixed with 1 ml. of bushy stunt virus antiserum at 1/100, and incubated for 1 hour at 50° C. before adding 0·05 mg. of bushy stunt virus.

We have made some preliminary attempts to purify this inhibitor. After several precipitations with ammonium sulphate and acid, 0·2 mg. of the best preparations have inhibited the precipitation of 0·05 mg. of bushy stunt virus with antiserum. These preparations contain both protein and carbohydrate.

DISCUSSION.

The phenomena described in this paper are not peculiar to tomato plants suffering from bushy stunt. Washed fibre from tomato and tobacco plants infected with tobacco mosaic virus, from tobacco plants infected with tobacco ringspot virus, and

from tobacco and bean plants infected with tobacco necrosis viruses, contains virus that is set free by fine milling or by incubation with " trypsin." Mill extracts from all such infected plants contain some virus combined with chromoprotein to give complexes that do not precipitate with homologous virus antiserum, and extracts from plants suffering from tobacco ringspot or tobacco necrosis usually fail to precipitate with virus-antiserum unless the chromoprotein is removed.

Virus is liberated from washed fibre by other means of grinding than the one described in this paper. A large mill with three rollers 29 cm. long and 15 cm. in diameter and running at 18, 45 and 180 r.p.m. respectively behaved similarly to the smaller mill. Some virus was also liberated from washed fibre by grinding for 10 minutes in an end-runner mill and in a " Waring Blendor," but not by passage through a Latapie mincer. Freezing leaves and passing them through a meat mincer damages most of the cells sufficiently to allow the sap to escape. The further effects of fine grinding probably result from the mechanical destruction of cell components that have withstood mincing, so that virus previously held by them is allowed to diffuse away in solution. During milling there is a continuous release of starch and chromo-protein, which suggests that one of the main components being affected by the milling is the plastids.

Incubation with " trypsin " increases the amount of virus set free by milling, and releases further virus after milling has become ineffective. This suggests that virus is held in the fibre in two different ways, and that some is attached to materials, or contained within structures, which are disintegrated by milling but are not affected by " trypsin," and that other virus is attached to materials which are destroyed by " trypsin " but not by milling. The second type of bound virus would resemble that found in the mill extracts in combination with chromoprotein. We have no grounds for suggesting that virus in the fibre occurs combined with chromoprotein, for combination between the two can be brought about by milling fibre to which free virus has been added. However, this fact does not exclude the possibility that there is some combination between the two in the infected plant.

It is clear that milling has at least two actions on the virus. First, virus is liberated from structures or materials from which it did not previously diffuse at all readily. Secondly, virus is being attached to chromoprotein. It is possible that virus is also being attached to other materials, for as the extent of the milling is increased the amount of virus that can be released by subsequent incubation with " trypsin " falls. This may be due to actual destruction of the virus, perhaps by local, intense over-heating between the rollers. There is no appreciable rise in the general temperature of the fibre during grinding, but it is well known from work on the rubbing together of unlubricated, or slightly lubricated, surfaces that very high local temperatures exist transiently (Bowden and Ridler, 1936). Complexes with serological behaviour similar to those of the virus-chromoprotein complex can be made by heating bushy stunt virus with serum albumin (Bawden and Kleczkowski, 1941), and this could be advanced as an argument in favour of the observed effects being caused by heating. On the other hand, bushy stunt virus is very sensitive to heat (Bawden and Pirie, 1943a), and the virus isolated after milling is as infective as virus isolated from the sap. Neither argument, however, is conclusive, for in the earlier tests the virus was exposed to temperatures of below 100° C. for some minutes, whereas during milling it is reasonable to postulate temperatures much higher but lasting for very short periods, and nothing is known about the behaviour of proteins in such conditions.

Although destruction of virus during milling cannot be excluded, it seems more likely that the loss is due to the attachment of virus to materials not destroyed by milling or by " trypsin." If this is so, it also suggests that virus may be bound to the

fibre by linkages that are not affected by any treatments yet used, and that other methods of extraction may liberate still more virus from infected plants. Other fractions of the minced leaves may also be sources of virus. The deep green precipitate, consisting largely of fragments of chloroplasts, which separates when sap is centrifuged at low speed, is one obvious possibility. Milling has never liberated any virus from this material, but incubation with " trypsin " has occasionally liberated considerable quantities.

Information about the different ways that virus occurs in infected plants is of interest because of its bearing on the site of virus multiplication. Previous work had suggested that virus was limited to the sap, and attempts to culture plant viruses have usually been made with media containing sap. The fact that the solid residues contain virus raises the possibility that synthesis of virus occurs in the insoluble fraction of cells, from which it passes into the sap, and that any " factors " essential for virus multiplication should be sought for in this fraction rather than in the sap. Bushy stunt virus from sap seems to be identical with that from the solid residues, and the experiments described in this paper do not indicate which is the more likely to be the first formed. However, experiments with tobacco mosaic virus, which will be described later, suggest that the virus obtained from the fibre is a precursor of that found in the sap.

SUMMARY.

After the sap has been expressed from minced tomato leaves infected with tomato bushy stunt virus, the solid residues contain approximately as much virus as the sap. This virus is most effectively liberated by incubating the residues with a commercial trypsin preparation and then passing them through a roller mill ; some virus is liberated by either treatment alone. Incubation with " trypsin " greatly increases the amount of virus liberated by milling, whereas extended milling reduces the amount liberated by " trypsin."

Purified preparations of virus from sap and from the solid residues have similar properties. Extracts of milled fibre contain some virus combined with chromoprotein to form a non-precipitating antigen ; such extracts do not precipitate with virus antiserum until the chromoprotein has been removed. Non-precipitating complexes of virus and chromoprotein can be formed by milling fibre of uninfected plants to which purified virus is added.

Extracts of fibre from healthy and infected leaves, which has been incubated with " trypsin," contain material that inhibits the precipitation of bushy stunt virus by its antiserum.

REFERENCES.

BAWDEN, F. C., AND KLECZKOWSKI, A.—(1941) *Brit. J. exp. Path.*, 22, 208.—(1942) *Ibid.*, 23, 169.

BAWDEN, F. C., AND PIRIE, N. W.—(1943a) *Biochem. J.*, 37, 66.—(1943b) *Ibid.*, 37, 70.

BOWDEN, F. P., AND RIDLER, K. E. W.—(1936) *Proc. Roy. Soc. A.*, 154, 640.

MCFARLANE, A. S., AND KEKWICK, R. A.—(1938) *Biochem. J.*, 32, 1607.

MASKET, A. V.—(1941) *Rev. Sci. Inst.*, 12, 277.