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THE VIRUS CONTENT OF PLANTS SUFFERING FROM TOBACCO MOSAIC.

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THE method generally used for extracting plant viruses is to macerate infected leaves in a meat mincer and express the sap through cloth. In previous papers we (Bawden and Pirie, 1944, 1945) have shown that only a part of the virus present in the leaves is contained in the sap; at least an equal amount is retained in the fibrous residues, from which it can be released in a soluble form by special treatments. Fine grinding in a triple roller mill has released several different viruses, including tobacco mosaic and tomato bushy stunt. With tomato bushy stunt virus, however, there was evidence that not all the virus in the fibre was released by this method, and that some was being destroyed (Bawden and Pirie, 1944). Incubation of the fibre with commercial trypsin preparations also released tomato bushy stunt virus, and more was obtained if the fibre was incubated with trypsin before milling than if the fibre was first milled and then incubated with trypsin. The experiments described below show that the fibrous residues of plants suffering from tobacco mosaic virus also contain more virus than can be set free by fine grinding, and that other enzymes are more effective than trypsin in bringing this virus into solution.

MATERIALS AND METHODS.

Most of the work has been done with tobacco (*Nicotiana tabacum*, var. White Burley) as a host plant, but tomato plants have also given similar results. The plants were grown in a heated glasshouse, but received no extra illumination, so that at different times of the year they varied greatly in their appearance and constitution. Nevertheless, the results of experiments done at different seasons have been reasonably constant; the same treatments have always been effective in releasing virus from the fibre, and the amount of virus in the fibre has always exceeded that in the sap. Similarly, the ratio of virus in the sap to that in the fibre is not greatly affected by the length of time the leaves have been infected, though the total amount of virus increases up to 4 weeks after infection. Leaves from 2 to 3 in. broad and 4 to 6 in. long were picked about a month after the plants were inoculated, and when they were showing well-developed symptoms. The midribs were cut out and discarded to avoid the presence of unnecessary fibre with a low virus content. The laminae were then minced, the sap extracted and the fibre washed and neutralized, as previously described (Bawden and Pirie, 1944). The washed fibre, squeezed as dry as possible by hand, has a water content of about 70 per cent, and forms the material for most of the extractions described. The technique used in passing the fibre through the roller mill was the same as that described previously.

The two main enzyme preparations used were from commercial trypsin and the alimentary tract of snails. Solutions of commercial trypsin were dialysed and clarified by low-speed centrifuging, and used at a final concentration of 2 g. of dry matter per litre. Although the effective constituent of this mixture has not been identified, similar results have been obtained with solutions of crystalline chymotrypsin. The mixture of snail enzymes was obtained from *Helix aspersa*. The snails were kept for some days without food, then killed with chloroform and removed from their shells. A slit was made in the left-hand side of the snail near the edge of the mantle, when the distended crop usually protrudes. It was pulled out gently with forceps, and the intestine and oesophagus were cut so that the crop falls into a beaker. When several crops were obtained, they were cut up roughly with scissors, diluted with water, so that there was 1 ml. of final suspension for each snail, and clarified by centrifuging. The fluid retains its activity, apparently unchanged, for some months at 0° C.

Samples of 0.5 g. (wet weight) washed fibre were mixed with 1 ml. of enzyme preparation and 0.3 ml. of 0.3 M pH 6.8 phosphate buffer and incubated for 48 hours at 37° C. with chloroform as disinfectant. Mixing is difficult because of the porridge-like consistency, and there are probably considerable variations in pH, but dilution with water or buffer slows down the reaction. During incubation the pH falls and it is necessary to add NaOH to keep the pH between 6 and 7, where there is the maximum effect with both trypsin and the snail enzymes. After incubation the volume of the mixture was made up to 10 ml. with water and clarified by centrifuging for 20 minutes at 3000 r.p.m. Before proceeding to any other treatment, the fibre was washed by allowing it to soak for about 20 minutes in 10 ml. of water and then centrifuging.

The virus content of the different extracts of infected leaves was estimated serologically, by determining the precipitation end-point of the fluids when titrated against tobacco mosaic virus antiserum. The sera used were all prepared by Dr. A. Kleczkowski, by the intravenous injection of rabbits with purified tobacco

mosaic virus ; they give no reaction with any extracts of healthy plants. The precipitin tests were made at 50° C. by methods previously described, 1 ml. of antiserum at a dilution of 1 in 400 being added to 1 ml. lots of test fluids diluted serially with a factor of 1 in 2. The greatest dilution to give a precipitate visible to the naked eye after 3 hours' incubation with serum was taken to be the precipitation end-point.

The use of serological methods for estimating quantities of tobacco mosaic virus is complicated by the fact that the virus exists in different physical states, which behave differently in precipitin tests (Bawden and Pirie, 1945). These differences seem to depend on the degree of aggregation of the virus particles, and the same weight of virus in an aggregated state may give a precipitin titre more than four times as great as when unaggregated. Thus a change in the degree of aggregation can readily be interpreted as an increase in virus content. To reduce this uncertainty, before extracts were tested serologically, they were all subjected to treatments that cause considerable aggregation. This can be done by heating to 60° C. at pH 5.7, but with solutions of low virus content this method is not entirely reliable and incubation with trypsin is to be preferred. All extracts that had not been incubated with trypsin (or with phosphate and chloroform, which have a similar effect) in the course of their preparation were therefore incubated at 37° C. and pH 7 for 10 hours in the presence of 0.1 per cent commercial trypsin. After such treatment, the virus behaves like a typical bacterial flagellar antigen, precipitating rapidly with antiserum and giving a fluffy, open type of precipitate, with an end-point that is a satisfactory measure of the virus content of different extracts. There is, however, no information on the original state of aggregation of the virus in the different types of extracts, nor on the proportion of serologically active virus that is also infective.

Both trypsin and the mixture of substances in the snail enzyme are strong inhibitors of infectivity. When they are added to virus solutions there is an immediate reduction in the number of lesions produced. This effect is reversible, for infectivity is increased if the virus and inhibitors are separated by sedimenting the former in the ultracentrifuge or precipitating it with acid. The interpretation of infectivity tests on previously unaggregated virus, however, is rather uncertain because of the complication of aggregation ; incubation with snail enzymes or trypsin usually leads to a relatively small, irreversible fall in infectivity, presumably because of the aggregation caused. Infectivity tests were made by the local lesion method on leaves of *Nicotiana glutinosa*, using a Latin square or similar design to reduce errors arising from variations in susceptibility between different leaves.

RESULTS.

Liberation of virus by milling and trypsin.

The release of tobacco mosaic virus from washed fibre by passage through the roller mill, and the ultracentrifugal fractionation of the resulting extract, has already been described (Bawden and Pirie, 1945). One passage through the mill does not give all the virus that can be liberated by milling, and further diminishing quantities can be obtained by successive millings and extractions with water. As with bushy stunt virus, there is evidence that the milling is either destroying some of the virus or converting it into some unextractable form.

TABLE I.—*The Effect of Varying the Sequence of Milling and Incubation with Trypsin on the Liberation of Tobacco Mosaic Virus.*

Sample 1.		Sample 2.		Sample 3.		Sample 4.	
Treatments.	Titre.	Treatments.	Titre.	Treatments.	Titre.	Treatments.	Titre.
Mill	1000	Mill	1000	Trypsin	500	Trypsin	500
"	100	Trypsin	600	Mill	2000	"	100
Trypsin	500	"	100	Trypsin	1200	Mill	2000
"	30	Mill	50	"	20	Trypsin	1400
Trypsin	30	—	—	Mill	40	"	40
						Mill	100
Totals	1660		1750		3760		4140

Each column deals with a separate sample from the same lot of washed fibre, which was subjected successively to the treatments listed. The serological titres given are the observed precipitation end-points of the 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.

This is shown in Table I, where the quantities of virus obtained from samples of one lot of fibre by four different sequences of extraction are compared. It will be seen that the greatest yield is obtained when the fibre is first incubated with trypsin and then milled, and that it is much reduced if the order of treatments is reversed. Also, the more extensive the milling, the less is the virus that can subsequently be recovered by trypsin.

To explain a similar loss of tomato bushy stunt virus we suggested that the virus was being attached to some constituent of the fibre during passage through the mill. As evidence in support of this, we showed that there is a similar loss of virus when purified preparations are mixed with fibre from healthy plants and the mixture milled, and that some of this virus could then be recovered by incubating the fibre with trypsin. We have now made similar experiments with tobacco mosaic virus; there is little or no absorption of virus when purified preparations are mixed with fibre from healthy plants, but there is considerable loss if the mixtures are milled. Only a small part of this lost virus can be recovered by incubating the milled fibre with trypsin or snail enzyme. It seems, therefore, either that the virus is being attached to some substrate unaffected by the enzymes, or that there is actual destruction of virus during the milling. Similarly, there are two possible interpretations of the increased liberation of the virus when milling follows incubation of infected fibre with trypsin. The enzymic digestion may destroy material with which the virus combines during milling, or it may so soften the fibre that there is less intense rubbing and local heating on passage through the mill. Digestion with the trypsin preparation does cause a very obvious softening, and after this treatment the fibre passes through the mill very rapidly. The digestion brings about one-third of the fibre into solution; most of this is protein, but carbohydrate, amounting to about 10 per cent of the dry weight of the fibre, also goes into solution. After the digestion, however, the fibre still contains some nitrogen, presumably in the form of protein.

In the mill extracts of infected fibre, some tobacco mosaic virus is in combination with chromoprotein. When the deep green extracts are clarified by freezing solid, thawing and centrifuging, the precipitate of chromoprotein contains virus that is not brought into solution on extraction with water. In this condition the virus combines with its antiserum, but is not precipitated as a result of such combination. The presence of this non-precipitating complex of virus and

chromoprotein can prevent the precipitation of free tobacco mosaic virus if this is not greatly aggregated. This combined virus can be released, as previously described for similar complexes of tomato bushy stunt virus (Bawden and Pirie, 1944), by hydrolysing the chromoprotein with trypsin. The virus is then obtained in an aggregated form and precipitates normally with antiserum. The precipitation end-points obtained from preparations made in this way suggest that about 3 per cent of the weight of the chromoprotein complex which separates from frozen mill extracts may be tobacco mosaic virus.

Liberation of virus by snail enzymes.

Even after preliminary digestion with trypsin there is no reason to suppose that milling has no deleterious effects, or that it releases all the virus contained in the fibre. Alternative methods have therefore been sought. More gentle methods of grinding, such as a Latapie mincer or a Waring Blendor or shaking with sand, were relatively ineffective in releasing virus. So were many enzyme preparations. Incubation with lysozyme from egg white, papain : HCN, pepsin, "Takadiastase," and another commercial extract made from moulds, "Luizyme," were all less effective than trypsin. The mixture of enzymes obtained from snails, however, was more effective both in disintegrating the leaf fibre and in liberating the virus. Incubation with this showed that the fibre contains more virus than can be set free even by trypsin and milling combined.

Liberation of all the virus cannot be brought about by one incubation with the snail enzymes. Increasing the time of incubation beyond 48 hours gives no increase of virus in the extract. Apparently not all the virus set free during the incubation comes into solution in the extract, for if the fibre is extracted again with water, or with a mixture of phosphate and the snail enzyme, there is often as much virus in this as in the first extract. On further extraction the amount of virus obtained falls, but more can be liberated if the fibre is again incubated with fresh enzyme. Usually three such incubations, each followed by one or two extractions of the fibre with water, are needed to liberate all the virus that can be obtained by this method. These phenomena are illustrated in Table II, where the precipitation end-points of successive extracts of infected fibre are

TABLE II.—*The Liberation of Virus from Washed Fibre by Incubation with Enzymes.*

Fibre 1.		Fibre 2.		Fibre 3.		Fibre 4.	
Treatments.	Titre.	Treatments.	Titre.	Treatments.	Titre.	Treatments.	Titre.
Snail	1000	Snail	5000	Snail	2000	Trypsin	320
Wash	1000	Wash	1300	Wash	600	Wash	80
"	300	Snail	2000	Snail	1000	Trypsin	600
Snail	2500	Wash	1000	Wash	1000	Wash	40
Wash	2000	Snail	600	Snail	1000	Trypsin	30
"	1300	Wash	300	Wash	600	Snail	1000
Snail	600	Trypsin	200	"	100	"	1000
Wash	160			Snail	160	Wash	1000
"	80			Trypsin	100	Snail	1000
Snail	300					Wash	600
Wash	100						
"	100						

Each column deals with a different lot of washed fibre, which we subjected successively to the treatments listed. The serological titres are the observed precipitation end-points of the extracts multiplied by a factor to bring them to the standard ratio of 1 ml. of fluid from 1 g. wet weight of fibre.

set out. It also shows that the snail enzymes liberate considerable amounts of virus that are not liberated by trypsin.

An estimate of the amount of virus liberated by different procedures can be obtained by totalling the precipitation end-points given by successive extracts. This has been done many times, and the amount obtained from the fibre by incubating with snail has always exceeded that present in the sap or that obtained from the fibre by other methods. A more precise comparison of the efficacy of different procedures can be made by mixing aliquots of successive extracts made from one sample of fibre, so that the final pool corresponds to a known weight of fibre, and then determining the quantity of virus obtained. This has been done both by comparing the precipitin titres of such pools, and by isolating the virus from them by ultracentrifugation and precipitation with acid and salts. Table III shows the results of two such experiments. From this it is clear that most virus is obtained from the fibre by snail enzyme and least by milling alone, whereas milling after previous incubation with trypsin is intermediate. The virus that is lost during milling is not recovered by subsequent incubation with snail enzyme, and the total amount liberated is much reduced if milling is the first treatment given to the fibre. Thus, if this loss is a consequence of attachment to some constituent of the fibre rather than of actual destruction of virus, the attachment is one that is apparently unaffected by snail enzymes and trypsin. Table III also shows that less than one-third of the total virus content of infected leaves is obtained in the sap.

We have shown previously (Bawden and Pirie, 1945) that the virus in mill extracts is, weight for weight, less infective than that in sap. By ultracentrifugation, the virus in both types of extract can be separated into fractions with widely different infectivities, but the mill extracts contain a greater proportion of virus with small particles and low infectivity. The significance of this is difficult to assess, because of the possibility that milling is inactivating the virus. The infectivity results in Table III, however, suggest that the virus obtained from the fibre is less infective than that found in the sap, for the purified preparation made from virus liberated either by snail or by trypsin are no more infective than virus purified from the mill extract. The whole problem is, however, com-

TABLE III.—*Yields and Infectivities of Tobacco Mosaic Virus Extracted by Different Methods.*

Type of extract.	Yield of virus in mg. per g. dry weight of washed fibre.	Infectivity. Average number of lesions per leaf at—	
		10 ⁻⁵ .	10 ⁻⁶ .
Sap	10.4	118	35
Milled fibre	22	72	14.5
Fibre incubated three times with snail enzymes	93	58	14
Virus from sap after incubation with trypsin	—	81	21.5
Sap	27	173	75
Fibre incubated three times with snail enzymes	72	89	17.5
Fibre incubated with trypsin, milled, then incubated with snail enzymes	41	91	18
Fibre milled, then incubated twice with snail enzymes	24	95	21

plicated because of the uncertainty as to the effects of aggregation on infectivity at high dilutions. It will be seen that the infectivity of the virus obtained from sap is reduced after still further aggregation by incubation with trypsin, but even then is still higher than that of virus liberated from the fibre. It seems likely, therefore, that virus particles of varying infectivity do, in fact, occur in the leaf, and that the virus contained in the sap has a higher proportion of highly infective material than that bound to the fibre.

Except for their aggregating effect, we have no reason to think that trypsin or the snail enzymes have any irreversible action on the infectivity of tobacco mosaic virus. There seems to be no destruction of the virus in any part of the digestive tract, for the faeces of snails that have fed on washed infected fibre are rich in infective virus. The enzymes responsible for the disintegration of the fibre and the liberation of the virus are largely confined to the crop, and extracts made by grinding the stomach wall or the hepatopancreas have little action. We have called the action enzymic, but the only evidence we have for this is that the activity of the preparations is destroyed by boiling. We have no information on the particular enzyme involved, and attempts to fractionate the preparations have failed. When ultracentrifuged at 70,000 r.c.f. some material sediments, but the activity remains in the supernatant fluid. None of the fractions separated with ammonium sulphate has been active, and although active material can be precipitated with alcohol or acetone, this leads to little or no fractionation.

The optimum pH for liberation lies between 6 and 7. This suggests that cellulase (or lichenase), the best known constituent of the mixture of enzymes contained by snails, is not responsible for the liberation of the virus, because cellulase has an optimum at pH 5.2 (Karrer, Joos and Straub, 1923). No certain conclusions can be drawn, however, for after an apparently ineffective incubation at pH 5.2, there is liberation of virus if a second extraction is made of the fibre at pH 7.0. A similar effect was noticed when fibre of tomato plants suffering from bushy stunt was incubated with trypsin (Bawden and Pirie, 1944), but it has not been studied in detail.

During digestion with the snail enzymes, from 60 to 70 per cent of the solid matter of the fibre goes into solution. The distinct leaf fragments disintegrate into a histologically formless mass in which nothing but a few cellulose fibres remain distinguishable. About 25 per cent of the residue after incubation is carbohydrate, as estimated by the orcin method (Pirie, 1936), and about 10 per cent is nitrogen. As the original fibre contains from 3.5 to 5 per cent nitrogen, two-thirds of the original nitrogen remains associated with the insoluble material. This is not unexpected, because the snail digestive fluid contains little or no protease. Most of this protein goes into solution on subsequent digestion with trypsin, but this treatment liberates no more virus.* This protein does not seem to impede the action of the snail enzymes, for they do not liberate any more virus or cause any more extensive digestion of fibre that has previously had its nitrogen content lowered by incubation with trypsin. Similarly, the action is not impeded by the presence of pectin and lipoids, for it is not facilitated if these are first removed by treating the fibre with 5 per cent ammonium oxalate solution or with a mixture of alcohol and ether. The addition of 10 parts of a mixture

* This is not true of all other viruses. Snail enzymes also liberate tomato bushy stunt virus, but only incompletely. Subsequent incubation with trypsin liberates some more of this virus after the residues have thoroughly incubated with snail enzymes.

of equal volumes of alcohol and ether to neutral solutions of purified tobacco mosaic virus precipitates the virus without causing any loss of serological activity, whereas in slightly acid conditions there is some denaturation. Precipitation from neutral solutions causes some loss of infectivity, though this is not readily interpretable, as the tests have not been done with solvents rigorously freed from peroxides, and loss of infectivity without loss of serological activity is known to result from oxidation.

DISCUSSION.

The two main points that call for discussion are the quantity of virus contained in infected leaves, and the reasons for variation in infectivity of virus preparations obtained in different ways. It is clear that the virus content of infected plants is much higher than was previously demonstrated, and that only a small proportion is obtained in the sap that has been the starting material for previous work. The virus that remains behind is an appreciable fraction of the insoluble protein of the infected leaf. The presence of considerable quantities of an abnormal protein in infected leaves was demonstrated by Martin, Balls and McKinney (1938, 1939), for they found an increase in the leaf nitrogen undigested by trypsin. After boiling the tissue this extra nitrogen became digestible. They pointed out that this behaviour was similar to that of the virus, and suggested that their figures set upper limits to the total amount of virus in the leaf. Depending on the susceptibility of the variety of tobacco used and on the duration of infection, they found from 16 to 170 mg. of this type of protein per g. dry matter in the leaf. Although for many reasons our results are not directly comparable with theirs, the yields of virus that we have obtained by actual isolation agree well with the middle of the range given by Martin *et al.* This is clear from Table III, which shows that 7.2–9.3 per cent of the total dry weight of the fibre can be isolated as virus after incubation with snail enzymes. As the virus has a nitrogen content of 16.6 per cent, these two figures correspond respectively to 1.2 and 1.5 per cent of nitrogen, or approximately one-third of the total nitrogen that remains in the washed fibre. In addition to this there is the virus in the sap, and the combined yield is about 100 mg. per g. of original leaf dry matter.

The occurrence of so large a percentage of the total nitrogen of the infected leaf in the form of an abnormal protein raises several interesting metabolic questions. We have made no systematic comparisons of the ratio of virus nitrogen to total nitrogen in the fibre during the course of infection, but the nitrogen content of healthy and infected plants has been studied by other workers. Stanley (1937*a, b*) stated that the total nitrogen of the plant was increased by infection, but this is not borne out by his data. His published figures show a slight increase in the percentage of nitrogen in the washed residue from minced leaves, but the infection caused such stunting of the plants that the weight of the residue was greatly reduced. As a result, healthy plants averaged 1.3 g. of nitrogen in this fraction and infected ones only 0.7. The protein and non-protein nitrogen that remained soluble after the addition of dipotassium phosphate* increased from 0.19 to 0.20 g. The normal leaf protein that is precipitated by phosphate was presumably included in the leaf residue, but this is not stated

* In this, as in many other papers published between 1937 and 1939, Stanley says he used disodium hydrogen phosphate, but his statements on the manner of use make it probable that the potassium salt was, in fact, used.

explicitly. Martin, Balls and McKinney (1939) and Takahashi (1941) likewise found a slight increase in the percentage of nitrogen in the infected leaf; in neither paper is the total dry weight of the leaf given, so that there is no evidence for an increase in nitrogen rather than a decrease in the other leaf components. It seems likely that the virus is synthesized at the expense of some normal leaf protein, and there is evidence for this. Martin *et al.* (1939) find that during the first few days of infection the increase in virus is accompanied by a comparable decrease in normal protein, and under conditions of nitrogen starvation Woods and DuBuy (1941) state that the infected leaf makes virus instead of normal chromoprotein. Similarly, Takahashi (1941) finds that the synthesis of virus proceeds in detached leaves kept in the dark when normal protein is autolyzing.

It is obvious that "normal leaf protein" is a mixture of very many proteins, and there is no reason to think that even the chromoprotein is a chemical individual. The multiplication of the virus is unlikely to affect all of these proteins similarly, so that conclusions drawn from gross chemical analysis may be equivocal. This is illustrated by the amino-acid analyses published by Lugg and Best (1945). They point out that tobacco mosaic virus and normal leaf protein differ in their amino-acid composition, and they give figures for the amide nitrogen, tyrosine, tryptophan, cystine and methionine respectively, in whole leaves, the soluble protein and the residue of healthy and infected plants. The tryptophan and methionine figures are the most significant, because the residue from healthy leaves has 1.9 and 1.4 per cent of its nitrogen in these forms, whereas the virus has 2.9 and 0. The figures for the infected residue are 1.7 and 1.1. If the virus in their leaf residue is simply added to the normal leaf protein, or replaces part of all the components equally, their methionine value is reasonable, but their tryptophan value is too low. The most logical interpretation of the results obtained by Lugg and Best is that virus infection specifically depresses the synthesis of components of the normal protein mixture that are richest in tryptophan.

We have already shown that extracts of infected leaves made in different ways contain virus with different infectivities (Bawden and Pirie, 1945). Because of the suspected deleterious effect of milling, and the possibility that it might be destroying the infectivity of some virus without destroying serological activity, the significance of these differences could not be assessed. However, as the virus in sap as well as that in mill extracts could be separated into fractions with widely different infectivities, it seemed probable that virus particles with different abilities to cause infection actually occur within the infected plant and are not artefacts. The work with the snail enzymes supports this view. There is no reason to believe that these enzymes have any destructive action on the virus, although they do inhibit infectivity and cause aggregation. The fact that the virus obtained from the fibre by incubation with snail enzymes is less infective than that from sap, even after the latter has been incubated with trypsin (Table III), suggests that there is a real difference between them. How to interpret these differences is not at all clear. Our methods of testing infectivity are reasonably good for comparing the relative infectivities of different virus preparations, but there is no method of measuring the amount of infective virus in any one preparation. To get infection with the most active preparations that we have made, even when transmission is facilitated by the use of abrasives, about 10^{-10} g. must be used, a weight equivalent to at least 10^7 virus particles. Many reasons can be advanced to explain this: There is probably considerable loss of

inoculum on the surface of the leaves, and it may be that a large number of virus particles are needed to cause infection. It is, however, equally possible that a single infective virus particle can cause infection, and that most of the particles are not infective. From the fractionations we have carried out on sap, it is clear that all previous preparations must have contained mixtures of particles with different degrees of infectivity; it is probable that in our most infective fractions this is also true.

In measuring infectivity we are merely assessing the ability of virus particles to establish themselves, and initiate the chain of events that leads to virus multiplication and the development of symptoms. It is generally assumed that all virus particles that have not been inactivated are capable of causing infection, but there is no positive evidence for this, and it is possible that there are particles unable to do so which nevertheless may have other activities in the cells in which they are produced. The systemic infection of uninoculated cells is brought about by the movement of virus particles from the inoculated cells. This mobile virus is likely to be that occurring in solution in the cell sap rather than that tied to the fibre in insoluble forms, and it is perhaps significant that, although this is a minor part of the total virus, it is the most infective.

Our results provide no positive evidence as to the manner in which the virus is held by the fibre. The methods we have used may liberate it either because they destroy substances with which the virus was combined to give insoluble complexes, or because they disrupt structural elements in the leaf so that the relatively large virus particles can more easily pass into the extracting fluids. There is also no evidence about the site of virus multiplication, for it is equally possible that the virus in the sap is derived by the gradual solution of originally anchored virus, as that originally soluble virus from the sap became anchored to the fibre as infection proceeds.

SUMMARY.

Virus can be released from the leaf residues of plants suffering from tobacco mosaic by fine grinding, or by incubation with either trypsin or the mixture of enzymes from snails' crops. Grinding causes loss of virus, and more virus is obtained if incubation with trypsin precedes grinding than if the operations are reversed. Most virus is released by incubation with the snail enzymes. Successive incubations are needed to release all the virus from the residues; this may amount to one-third of the total insoluble nitrogen of the leaf. The total virus in the leaves accounts for 10 per cent of their dry matter; less than a third of the virus is obtained in the sap, but this is the more infective.

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