Proc. R. Soc. Lond. B. **182**, 297–318 (1972)
Printed in Great Britain

Factors affecting the amount of tobacco mosaic virus nucleic acid in phenol-treated extracts from tobacco leaves

By Sir Frederick Bawden, Treas.R.S.†
and N. W. Pirie, F.R.S.
Rothamsted Experimental Station, Harpenden, Herts

(*Received* 1 *June* 1972)

Extracts from the lower leaves of tobacco plants infected with tobacco mosaic virus and pulped in the presence of phenol are less infective than extracts from leaves pulped and then treated with phenol. Extracts from uninfected leaves mixed with purified virus behave similarly. The difference becomes progressively smaller as leaves are taken from further up the plant.

The difference is smaller when the interval between pulping and adding phenol is short, and it is still smaller if air is rigidly excluded during the interval.

Fixation of virus nucleic acid to the leaf fibre is the main factor responsible for this difference. Part of the normal nucleic acid of the leaf is fixed similarly. Fixation is partly prevented by including yeast nucleic acid in the extraction fluid, or by excluding Ca^{2+} from it by adding citrate. Part of the nucleic acid is held tenaciously by the fibre.

The possible significance of this fixation *in vivo* in controlling the apparent susceptibility to infection of plants in different physiological states is discussed.

Introduction

Leaf physiology can be so greatly affected by changes in age, nutrition or environment, that the same virus inoculum may produce 100 or more local lesions on one leaf and few or none on another. Leaves that produce only few lesions, usually also produce smaller lesions than leaves that produce many, suggesting that conditions inimical to the initiation of infection on a leaf may also restrict the spread of infection between cells. Differences in physiological state have a considerable effect on the susceptibility to infection by tobacco mosaic virus (TMV) when inocula consist of nucleoprotein particles, but a much greater one when inocula consists of nucleic acid (TMV:RNA) (Bawden & Pirie 1959).

TMV probably survives outside plants, and usually initiates infection in them, when in the form of a rod approximately 300 nm long and containing 5% of RNA – but this may be a sequestration product rather than the agent that moves between cells in infected tissues. Although it is remarkably stable, there is much evidence that it is dismantled during the process of initiating infection and some reason to think it spreads between cells in a leaf in a more evanescent form. In the hope of getting evidence about the states in which the virus exists in leaves and moves between cells, we examined extracts, made in different ways, from leaves at various stages of infection. The infectivity of extracts containing TMV dismantled by exposure to phenol was influenced, not only by the period for which

† Sir Frederick Bawden, Treas.R.S. died on 8 February 1972 while this paper was in final preparation.

the leaves had been infected, but also by the sequence of events during extraction, the precise conditions of extraction, and the method used to remove the phenol so that the extracts would not damage the test plants. Engler & Schramm (1959) claimed that extracts made by pulping leaves with phenol were more infective than those made by treating sap with phenol. They suggested that the difference was caused by phenol preserving free infective TMV:RNA from inactivation by sap. We find that when there is a difference between the infectivities of the two types of extract, it is the other way round, and that the explanation is more complex. Some facets of the process may have physiological significance.

Many different phenomena interact to produce the differences in infectivity between different types of phenol-containing extract: Ca^{2+} in the concentration range typical of tobacco sap inhibits infection by TMV:RNA; the extent to which the predominant group of ribonucleases (RNase) in tobacco is destroyed or removed by phenol depends on other components in the system; other leaf RNases are stabilized and in some conditions diffuse through dialysis membranes; TMV:RNA is also absorbed on leaf fibre and dialysis membranes to an extent that depends on the pH and on the presence of other substances. The combined effects of these and other factors frustrated our many attempts to get consistent results by extracting a mixture of leaves from all positions on systemically infected tobacco plants. Part of the reason for the inconsistency became evident when leaves from different positions on the stems were extracted separately. Whereas the upper leaves gave infective extracts regardless of the method of extraction, lower leaves gave non-infective extracts when pulped in the presence of phenol but infective ones when supplements were added to the extraction fluid or when the leaves were pulped thoroughly before adding phenol. Because of the bearing this difference may have on susceptibility of leaves to infection, we have tried to unravel its mechanism.

The differences are not a consequence of infection, because they occur when leaves from different positions on uninfected plants are pulped with added TMV. The total RNase activity of sap increases with increasing resistance to infection, but we concluded (Bawden & Pirie 1959) that this increase was inadequate to account for the differences in susceptibility. Although differences in the amounts, or location, of some of the individual RNases of the leaf, may explain part of the difference in susceptibility, this could not be established without a detailed study of the individual RNases. In the accompanying paper, we (Bawden & Pirie 1972) present some observations on RNase relevant to the infectivity of extracts.

Phenol-saturated leaf extracts damage leaves rubbed with them and can be used in infectivity assays only when diluted so much that they usually produce inconveniently few lesions. Removing the phenol in different ways gives extracts that differ in infectivity. Thus, a dialysed inoculum is more infective than one from which the phenol is removed by extraction with diethyl ether. To a great extent this is because Ca^{2+} is not extracted by ether. This phenomenon is discussed in the following paper.

Other leaf components, e.g. aldehydes, nicotine and spermine, can diminish the infectivity of TMV:RNA solutions – especially when they are extracted with phenol. The effect of these substances, described in the accompanying paper, is greatest in solutions containing little salt and we conclude that, at physiological concentrations and in the presence of the other components of leaf extracts, they are probably not an important cause of the infectivity differences.

The main factor influencing the infectivity of phenol-containing extracts, made in different ways from leaves in different physiological states, is the extent to which TMV:RNA remains or becomes attached to the leaf fragments, not its inactivation after being extracted.

Materials and methods

Virus and host plants

All the work was done with the type strain of TMV that has long been maintained at Rothamsted. Plants were raised and kept in the fluctuating conditions of a glasshouse, heated to keep a mean temperature near 20 °C; during winter young plants were given supplementary artificial lighting.

The tobacco (*Nicotiana tabacum* L.) variety Java was the most used systemic host, providing infected leaves from which infective TMV:RNA was extracted in various ways. The bottom two leaves were inoculated and the systemically infected leaves were picked 4 to 6 weeks later when symptoms were well developed. Java tobacco was also used in most experiments in which TMV was added to uninfected leaves.

The tobacco variety Xanthi n.c. was mainly used for infectivity assays. Usually eight inocula were compared on eight plants, each trimmed to four leaves, and kept in darkness for 24 h before use to increase their susceptibility and uniformity. Each inoculum was rubbed over eight half-leaves, distributed so that each occurred equally often at each leaf position and on left and right halves of leaves.

An interest in the differences in the resistance to infection of plants, in different physiological states was the main reason for undertaking this research. Even when seemingly uniform plants are selected for assays, there can be large and inconvenient differences in response to TMV:RNA. In most sets of eight half-leaves, there is a tenfold difference in the number of lesions on individual leaves and still larger differences are common. We have no explanation for this and, because of it, attach little importance to total numbers of lesions on eight half-leaves that differ by less than a factor of 3.

Preparation of TMV

Early accounts of the nucleic acid prepared by disrupting TMV with phenol stress its instability; this was almost certainly caused by the RNase in the preparations, because treating them with bentonite made them more stable. Our preparations retain their infectivity for years when frozen and for weeks at 0 °C but unfrozen. To get such stable preparations we make use of the exceptional stability of intact TMV to ensure that the starting material is as free from RNase

as possible. We do not prepare TMV by ultracentrifugation only, because it is then contaminated with much RNase, but precipitate it repeatedly with acid and ammonium sulphate, incubate it with citrate and $CHCl_3$, and finally sediment it by ultracentrifugation (Pirie 1956*a*). Such purified TMV contains little or no RNase and the RNA prepared from it after disruption with phenol is stable without need to remove contaminants with bentonite or other absorbents. Complete removal of contaminants is obviously unnecessary when the TMV is only to be used in experiments in which it is added to whole uninfected plants. It could be argued that, for such experiments, TMV in a more 'native' state would be preferable. However, even minimal purification produces TMV that is no longer in the original state (Bawden & Pirie 1945; Pirie 1956*a*) and it seems preferable to work with a preparation in which these changes have gone to the limit compatible with retention of infectivity, rather than with one in an indefinite intermediate state.

Labelled TMV was made in 30 leaves, 8 to 10 cm wide, floated in dishes containing 1 l of Hoagland solution to which 1 millicurie of ^{32}P in the form of KH_2PO_4 had been added. The dishes were covered with Polythene sheet to prevent evaporation and the leaves were inoculated after a day in the medium. Four days later they were pulped with twice their weight of 30 mM K_2HPO_4, pressed, and the fibre pulped again with an equal volume of phosphate. The combined extracts were shaken briefly with $CHCl_3$ and centrifuged. To ensure that very little of the P in the final product was in a form other than TMV:RNA, the TMV was thoroughly purified. It was precipitated at pH 3, dissolved neutral, precipitated with $(NH_4)_2SO_4$ at 40 % of saturation, re-dissolved, precipitated again at pH 3 and dissolved in 0.1 M trisodium citrate. After 24 h incubation at 36 °C, brown contaminants were removed by centrifuging at 10000 rev/min. The virus was then precipitated again with $(NH_4)_2SO_4$, dissolved and precipitated at pH 3. After washing twice or thrice on the centrifuge, it was dissolved neutral and sedimented by centrifuging for 2 h at 60000*g*.

From 90 g wet weight, of leaves 50 mg of TMV was usually recovered. Its freedom from ribosome fragments and other P-containing materials, that commonly contaminate preparations of TMV, was demonstrated by incubating part of it again with citrate and $CHCl_3$ and ultracentrifuging. Less than 1 % of the radioactivity was in the supernatant fluid. Between 1 and 3 % of the original millicurie of ^{32}P is recovered in the TMV and 0.1 mg of virus gives 1000 to 1500 counts per minute (counts/min) when measured with a thin window Geiger–Müller counter with about 25 % efficiency. This is a greater initial activity than is needed to follow the movement of TMV:RNA. When fresh, the labelled TMV was used mixed with 7 times its weight of unlabelled TMV; the ratio was diminished as the ^{32}P in the labelled TMV decayed.

Pretreatment of leaves

Systemically infected Java tobacco plants were used when about 1 m high and not yet forming flower buds; uninfected plants were 1.5 to 2 m high and buds were

usually forming. Leaves of White Burley tobacco also show all the main phenomena described here, but they were seldom used because their squat and deformed habit when infected makes it impractical to separate the leaves into uniform groups of contrasted age.

Only leaves more than 5 cm wide, and neither senescent nor otherwise damaged, were taken. The midribs were cut out and the leaf blades were cut transversely into strips about 2 cm wide, suitable groups of strips were mixed thoroughly in a bucket to ensure that samples taken from the mass were as uniform as possible. For most experiments, 8 or 10 g samples were used within an hour and the remaining slices were kept at −20 °C. Fresh material and material frozen for several weeks behaved similarly.

Cell components of differing molecular size can be separated by using the cell structure as a dialysis membrane (Bawden & Pirie 1945; Pirie 1956*b*). In some experiments leaves were frozen at −20 °C to destroy their osmotic control and then soaked in 10 to 20 times their volume of water after thawing. Most of the sugars and salts are removed by 2 h freezing and 2 h soaking, but both treatments must be prolonged for at least 24 h to remove other components with which we are here concerned. It is often more convenient to freeze the pieces of leaf in the water used for their extraction than to unravel the limp mass produced when the leaves are frozen alone. Very little virus comes into the extract, from infected leaves handled in this way, provided they are not rubbed or sheared. A press (Davys, Pirie & Street 1969) in which 1 ton can be applied, without shear, over a 450 cm^2 grooved platen is suitable but not essential. Leaves treated in this way will be referred to as 'freeze-washed' (F-W).

Preparation of phenol extracts

Analar phenol was used. So that it could be conveniently pipetted, a stock solution liquefied by adding 10 % water was kept, protected from light, and was discarded at the first sign of discoloration. This solution will be called phenol. For some extractions, 10 g samples of leaf were pulped for 2 min in an M.S.E. 14000 rev/min 'blender' with 25 ml water, containing whatever other agents were to be present initially, and 5 ml phenol. In many experiments, the phenol was not added initially. After it had been added, mixing was completed by 10 s more pulping.

Contact between disintegrated leaf and the components of the extraction medium is immediate in the 'blender'. To avoid the intense aeration in the 'blender' and to be able to subdivide a pulp and treat parts of it in different ways, many batches of leaf were forced through a narrow slot by means of a hydraulic press. The unit (Pirie 1961) is designed to be flushed with N_2 before pressure is applied and the pulp can be kept in N_2 while phenol and/or any other agent is added. To facilitate subdivision the stainless-steel ports on the latest model have the same standard taper as interchangeable glass joints so that glass-ware can be attached. Material pulped in this manner will be referred to as 'pressed'.

For different purposes, different methods were used to separate extract from

fibre. When the fibre was needed as dry as possible, the pulp was poured into a bag made by fastening a 14 cm square of thin finely woven cloth on to the mouth of a centrifuge tube, 3.5 cm in diameter, with a rubber band, and centrifuged for 5 to 10 min at 1000 rev/min. Some residual liquid can then be squeezed from the packed fibre and the cloth in a small hand press (the type sold to get juice from garlic is suitable). It is difficult to make this set of operations quantitative. In quantitative work or when successive extracts were being made, the fibre was separated by centrifuging at 2000 rev/min after removing air from the pulp by exposing it to vacuum. With this procedure, so much more extract remains with the fibre that it was usually re-extracted with water before extracting with any other agent. With a suitable arrangement of 12 mm bore tubing, 'pressed' pulp was also degassed, transferred to a centrifuge tube and stoppered without allowing access of air.

Usually, the extracts were re-extracted with 1/10 volume of phenol, centrifuged, and the phenol layer added to the fibre if that was being further extracted. The aqueous layers were dialysed in separate vessels, usually against 25 times their volume of water for 12 h and 25 volumes of 5 mM K_2HPO_4 for a further 12 h. All these operations of 'blending' 'pressing', centrifuging and dialysing were done at or near 0 °C. The fluids were assayed for infectivity immediately after dialysis and the excess was stored at −20 °C to allow further assays.

Some extracts produced enough lesions on Xanthi to be assayed without further treatment except dilution with water to protect the assay plants from damage by phenol. During summer, diluting 1 in 5 is enough, but not during winter when plants are more sensitive. Conclusions drawn from extracts diluted as little as this are confused by the effects of the Ca^{2+} that remains in the inoculum.

Results

The infectivity of extracts from infected leaves

Our early results comparing the infectivity of extracts made in the presence of phenol in different ways, but all dialysed, showed an invariable trend. Extracts made from F-W leaves gave most lesions, those made by adding phenol after pulping gave fewer, and those made by pulping in the presence of phenol gave fewer still. However, using mixed batches of leaves of all ages, the differences between extracts differed greatly in different experiments. Table 1 shows the reason. For this test, leaves from four positions on the stems of systemically infected plants were taken. Part of each lot was pulped to get sap in the conventional manner, and another part was pulped with phenol. Sap from the lowest leaves contained less TMV than sap from the others, but the differences were small. By contrast, the amount of infective TMV:RNA in the four phenol extracts differed greatly. Parallel experiments show that the difference between the infectivity of an extract made in the presence of phenol (Ph-1), and one made by adding phenol to pulp that has been allowed to stand at 18 °C for 30 min (Ph-2) or to F-W leaves

TABLE 1. BEHAVIOUR OF LEAVES FROM DIFFERENT POSITIONS ON THE STEM

		lesions on eight half leaves					
		sap at		extracts made in the presence of phenol		extracts made in the presence of phenol from freeze-washed leaves	
type of leaf	precipitation end point of sap with antiserum	1:10000	1:100000	undiluted	diluted 1:5	undiluted	diluted 1:5
top	1:600	1140	222	1370	198	—	—
upper middle	1:400	1475	249	17	4	—	—
lower middle	1:600	1890	397	5	1	2140	1376
bottom	1:200	1165	78	0	0	1595	547

Sap was pressed out from 10 g of infected leaf after grinding in a mortar with 0.7 ml M K_2HPO_4 and 0.7 ml M KOH. It was frozen, thawed, diluted with its own volume of water, adjusted to pH 5.5, heated to 60 °C for 10 min and centrifuged. 10 g lots of leaf, or freeze-washed leaf, were pulped in a 'blender' with 25 ml water, containing 0.7 ml M KOH, 0.7 ml M K_2HPO_4, and 5 ml phenol. After centrifuging the extracts were dialysed to remove phenol.

becomes smaller as leaves are taken from successively higher positions on the plant. The two or four leaves immediately above those that were inoculated are usually yellower than the others by the time of harvest. Ph-1. extracts from these fail to infect the test plants. The next eight or ten leaves are all green, but Ph-2 extracts from the lower three or four may give 10 times as many lesions as Ph-1 extracts. The infectivities of the two types of extract from upper members of the group differ little.

These differences are greatest during winter or overcast summer weather. After a period of bright sunshine, Ph-1 and Ph-2 extracts from leaves only 20 cm from the inflorescence, and only 6 cm wide, may both be non-infective, whereas extracts from F-W leaves in this position and physiological state are infective. Shading for 3 or 4 days changes these plants to the 'winter' condition. All the experiments described in the remainder of this section were done on the four to six lowest leaves of Java plants infected for 4 to 5 weeks, about 1.5 m high, and shaded for a few days when they were harvested during bright weather.

The environment TMV:RNA moves into when a leaf is pulped is unphysiological even without added phenol, and it is reasonable to explore the effects of slight changes in it. Changing pH or adding substances that sequester Ca^{2+} has a great effect. For the tests on phenol-containing extracts recorded in table 1, the leaves were pulped in 30 mM K_2HPO_4 which combines both effects. Another experiment compared the effect of pH on the infectivity of extracts from leaves pulped in conditions Ph-1 and Ph-2. Table 2 shows that the difference between Ph-2 and Ph-1 extracts is diminished when the extracts are made slightly alkaline, and that much, but not all, the infectivity lacking in Ph-1 is released by later extraction with citrate.

There are similar differences between Ph-1 and Ph-2 extracts when some of the conditions of extraction are altered. Thus: the volume of phenol used was chosen

TABLE 2. EFFECT OF pH ON THE SEPARATION OF TMV:RNA FROM LEAF FIBRE

10 g of infected leaf was pulped with 25 ml water and 5 ml phenol, after withdrawing one-third of the pulp, 0.2 ml M KOH was added, another third was withdrawn and 0.2 ml M KOH was added to the remainder. All three were then centrifuged and dialysed. Another 10 g batch of leaf was pulped with water and left for 30 min at 20 °C before adding phenol. Thereafter the treatment was the same. The fibre was extracted again with water and yet again with 50 mM trisodium citrate.

		lesions on eight half leaves with 2 ml of		
initial conditions	pH	1st extract	2nd extract (water)	3rd extract (citrate)
leaves pulped with phenol				
no KOH added	6.1	0	13	181
1st KOH addition	7.0	1	0	618
2nd KOH addition	8.5	5	0	282
leaves pulped and phenol added after 30 min				
no KOH added	6.1	770	163	750
1st KOH addition	7.0	40	17	612
2nd KOH addition	8.5	23	1	1495

because it is slightly in excess of that needed to saturate the water phase and the moist fibre, and so ensures uniform conditions in the extracts, and complete disruption of the TMV remaining in the fibre. This excess is unneccessary; the effects are similar when water saturated with phenol is used so that there is no free phenol phase. TMV is disrupted by phenol solutions that are less than half saturated (Bawden & Pirie 1940). The two right-hand columns in table 1 show that extracts made in the Ph-1 manner from lower leaves that have been freeze-washed are very much more infective than Ph-1 extracts made from the same batch of leaves without freeze-washing. This cannot be directly interpreted because, although leaves retain their original character during a period of freezing, they slowly lose it when thawed before adding phenol. A period of thawing is obviously inseparable from the F-W procedure. Table 1 shows that the system that diminishes the infectivity of Ph-1 extracts no longer operates after freeze-washing. An experiment in a later section (table 7) shows that F-W leaves can be partly restored to their original condition by restoring to them the reconcentrated diffusate.

The duration of blending is not critical; results are similar when it is shortened to 1 min or extended to 6 min. But extraction is more complete when leaves are 'pressed' to a smooth paste by forcing them through a narrow slot; 'press' extracts, may contain 2 to 4 times as much TMV as blender extracts.

There are other points of difference in the two methods of disintegration. In a 'blender', pulp is exposed to phenol at the instant when the leaf is pulped, whereas in a 'press' it is not feasible to get such immediate contact, though the interval can be diminished to a few seconds by having the phenol in the chamber into which pulp is extruded. In a 'blender' aeration is intense, whereas in a 'press',

TABLE 3. THE INFECTIVITY OF EXTRACTS MADE WITH RESTRICTED ACCESS OF AIR IN THE INTERVAL BETWEEN PULPING AND ADDING PHENOL

8 g batches of infected leaves were pressed through a narrow slot into 20 ml of water, 28 mM with respect to KOH and Na_3PO_4. Two were pressed in air and two with exclusion of air, 4 ml of phenol were already mixed with the aqueous solution in one, in the other three the phenol was added after 40 min at 20 °C in the conditions stated. All were then centrifuged at 0 °C and the aqueous layers re-extracted with 0.5 ml of phenol. Second extracts were made by suspending the fibres and phenol layers in 20 ml of water and centrifuging after 30 min, 3rd extracts by suspending in 20 ml of 30 mM trisodium citrate and centrifuging after 3 h, all at 0 °C. The same set of test plants was used to assay the 2nd and 3rd extracts, a different set for the first. All extracts were dialysed before assay.

	lesions on 8 half leaves			
	1st extract		2nd extract (water)	3rd extract (citrate)
	2 ml	0.4 ml	2 ml	0.4 ml
immediate addition of phenol under N_2	474	11	0	98
phenol added after 40 min under N_2	985	40	—	—
phenol added after 40 min in air unshaken	2010	314	22	382
phenol added after shaking 40 min in air	1880	630	39	331

even without N_2 flushing, it is minimal if the pulp is left as an undisturbed mass. Table 3 shows that the extract from 'press' pulp, to which phenol was added quickly, had little infectivity; when the addition was delayed to 40 min in N_2 the extract was more infective. Extracts from leaves 'pressed' in air were still more infective, especially when the pulp was shaken rather than being left undisturbed. Table 3 also shows that, in experiments of this type, the third extract, made with citrate, is much more infective than the intermediate water extract. When all extracts, unlike those used in the experiment shown in table 2, have nearly the same pH, the final extract in citrate is usually most infective when the original extract was also most infective. Hence, initial extracts that lack infectivity seem not to do so merely because TMV:RNA is held back in a form susceptible of later extraction.

The conclusion from these experiments seems to be that old leaves contain a system that is impaired by oxidation after the leaf structure has been damaged, or by removing diffusible components. This system either prevents TMV:RNA from separating from the leaf fibre or destroys it in the aqueous extract. It does not completely destroy the TMV:RNA within the fibre because at least part survives in such a form that it can be extracted later by citrate.

Dialysed non-infective Ph-1 extracts do not inactivate TMV:RNA during 24 h. This suggested that, if there is an inactivator, it is either used up or can diffuse

away. Diffusates, after some concentration and fractionation, inactivate TMV: RNA (Bawden & Pirie 1969). One cause of this inactivation is RNase that is phenol-stable and to some extent diffusible through 'Cellophane'. However, Ph-1 and Ph-2 extracts have similar RNase activity, so this group of enzymes cannot be invoked to explain the initial difference in the infectivity of extracts. Differences in RNase content probably explain differences in the extents to which extracts lose infectivity when stored frozen for long periods: infectivity persists longer in those stored at pHs greater than 8, or saturated with phenol, conditions in which leaf RNases are inhibited. Jervis & Pirie (1972) describe some properties of the enzyme concentrated from extracts containing phenol and the inactivation of TMV:RNA by it is described in the accompanying paper (Bawden & Pirie 1972).

Having concluded that the sole causes of loss of infectivity are not destruction in the fibre, or in the extract after separation from the fibre, the possibility remains that it is fixation to the fibre. Measurement of the total RNA content of extracts, by precipitation with acid or Ca^{2+}, and measurement of light absorption at 260 nm by the redissolved precipitate, is compatible with this explanation. Most of this absorption is caused by the normal RNA of the leaf and there is more RNA in Ph-2 than Ph-1 extracts from mature uninfected leaves. Conditions that hinder separation of TMV:RNA from the fibre, also hinder separation of normal RNA.

The effects of citrate suggest that Ca^{2+} is involved in fixation, although it does not precipitate TMV:RNA from environments resembling those in leaf extracts. A further complication in the simple involvement of Ca^{2+} in the phenomenon is that a series of measurements of the Ca^{2+} precipitable from acid solution by oxalic acid showed no inverse correlation between infectivity and Ca^{2+} content in extracts made from leaves from different positions on the plant, or made in different ways. Nevertheless, as table 4 shows, infectivity is affected by the Ca^{2+} content of the extract. To make the point clear, an illustrative experiment is chosen in which

TABLE 4. CONTRASTED EFFECTS OF CALCIUM AND CITRATE ON THE INFECTIVITY OF LEAF EXTRACTS

Eight 10 g leaf samples, four middle and four lower, were pulped with 25 ml water, 0.7 ml M KOH and the supplement at 24 mM. In four (Ph-1), 5 ml phenol was present during the pulping; in the other four, phenol was added 30 min after pulping. The extracts were all then dialysed as described in the text.

leaf position	supplement		lesions on eight half-leaves
middle	phosphate	Ph-1	644
		Ph-2	1245
	calcium	Ph-1	3
		Ph-2	163
lower	phosphate	Ph-1	240
		Ph-2	1385
	citrate	Ph-1	1360
		Ph-2	1213

there is less difference than usual between the Ph-1 and Ph-2 extracts from middle leaves. Batches of leaf that give Ph-1 extracts with little infectivity would not demonstrate the infectivity-diminishing effect of Ca^{2+} but, for the same reason, the diminution in the difference between Ph-1 and Ph-2 extracts from the lower leaves is much less than in some experiments. EDTA behaves similarly to citrate. In addition to preventing fixation on the fibre, citrate, as shown in tables 2 and 3, partly releases TMV:RNA that has become fixed. More evidence on this is given later. After dialysis the Ca^{2+} content is less than 3 mM; this is the minimum concentration at which Ca^{2+} in the inoculum affects the number of lesions produced (Bawden & Pirie 1972).

TABLE 5. EFFECT OF ADDING YEAST NUCLEIC ACID TO INFECTED LEAF PULP BEFORE AND AFTER DISRUPTING TMV WITH PHENOL

Four 8 g lots of leaf were 'pressed' into 20 ml of water, 28 mM with respect to KOH and K_2HPO_4. With three of the samples this was done anaerobically. In one of these, 4 ml phenol was mixed with the water so that pulp was exposed to it within a few seconds. In the other two it was added after 10 min. Each lot was split after opening the press unit, about 3 min elapsed before the addition of phenol to the first lot, the YNA to the fourth, and the further quantity of YNA to the sixth and eighth. Each fibre was extracted with 10 ml water and re-extracted with 10 ml 50 mM trisodium citrate.

treatment		number of lesions on eight half leaves: 0.4 ml 1st extract	2 ml 2nd extract (water)	0.4 ml 3rd extract (citrate)
pulped in air	phenol added quickly to half	20	16	194
	phenol added after 2 h aeration	565	180	362
pulped with phenol in N_2	no addition	20	5	305
	0.2 g/l polymeric YNA	133	16	208
pulped in N_2 with 0.2 g/l polymeric YNA and phenol added after 10 min in N_2	no addition	506	331	220
	further 3.3 g/l polymeric YNA added after the phenol	1158	1450	418
pulped in N_2 with 0.2 g/l crude YNA and phenol added after 10 min in N_2	no addition	567	290	479
	further 3.3 g/l crude YNA added after the phenol	1135	161	810

Measurements of the absorption of u.v. light show, as already mentioned, that less of the normal RNA of the leaf is extracted in Ph-1 than in Ph-2 conditions. If the capacity of the insoluble part of the leaf for binding RNA is limited, it should be possible to protect TMV:RNA from being bound by adding another RNA. 50 mg of yeast nucleic acid (YNA) in the 25 ml of solution used to make a 'blender' Ph-1 extract, more than doubles infectivity. This is not a large enough difference for the effect to be unequivocal. Furthermore, water that has lain for

a few minutes on even the undamaged surface of a tobacco leaf contains so much RNase that it can be recognized by conventional means. The presence of 1 to 2 g/l of YNA in an inoculum similarly increases the number of lesions given by TMV: RNA – presumably because the two nucleic acids compete for the RNase on the leaf surface. This point is treated more fully in the accompanying paper (Bawden & Pirie 1972).

The effect of YNA shows more clearly when infected leaves are pulped by pressing through the slot under N_2, for then the YNA can be allowed to react with leaf components anaerobically before TMV:RNA is separated from TMV by adding phenol. Table 5 shows one such experiment. One lot of leaves was 'pressed' in air, phenol was added to half of it at once and to the other half after 2 h; the difference in lesions (20:565) with that pair shows that the batch of leaves was behaving in the usual manner. The other three lots were 'pressed' in N_2, one of them directly into extraction fluid containing phenol, and the other two into fluids containing polymeric or crude YNA. Phenol was added to this last pair after standing for 10 min in N_2. After opening the press, each of the pulps made anaerobically was split and more YNA added to half. All eight were then centrifuged and dialysed in the usual way. The extract from pulp exposed to phenol before adding YNA was less infective than those mixed with YNA before being exposed to phenol (20:506 and 567) and the infectivity of extracts from these pulps is enhanced by adding more YNA after the phenol has acted (table 5). The infectivities of the 2nd and 3rd extracts from the fibres show that, as with other systems of extraction, much infective material remains in the fibre in a form released by citrate; the table also shows that the more infective 3rd extracts tend to come from fibres that have already released the more infective first extracts.

Other experiments suggest that the state of aggregation of YNA, the duration of contact (in the range 1 to 30 min) between pulp and YNA before adding phenol, and the concentration of YNA (in the range 0.2 to 2 g/l) are not critical. The precise effects of duration and concentration will, presumably, depend on the amounts of leaf RNase and normal leaf RNA present, and these will differ in different batches of leaf. Other experiments also show that when a Ph-1 pulp is split, adding YNA after phenol gives a less infective extract than adding citrate.

More direct evidence that RNA is being bound in the conditions of Ph-1 extraction, rather than being hydrolysed so that it is able to diffuse out of dialysis sacs, is given by experiments in which infected leaves are pulped with TMV labelled with ^{32}P. It is not certain that TMV added in this way behaves in the same manner as TMV in the infected leaf. These experiments are therefore described among those in which healthy leaves were pulped with purified TMV.

The behaviour of extracts from uninfected leaves with added TMV

A systemically infected tobacco plant is stunted and has discoloured leaves. The chronological age of its leaves and their positions up the stem are easily defined, but their physiological age is indeterminate. Uninfected plants are larger

and have more uniform leaves. The changes undergone by TMV in the presence of phenol and leaf can be more minutely analysed when TMV is added to uninfected leaves than with infected leaves because the TMV or TMV:RNA can be added at any stage in the treatment without having been subjected to that treatment. Also, by using isotopically labelled TMV, the behaviour of TMV:RNA can be distinguished from that of normal leaf RNA. In most of the experiments described here, 20 mg of TMV was added to 10 g of leaf because this is the amount usually extracted from systemically infected tobacco plants.

TABLE 6. INFECTIVITY AND ^{32}P CONTENT OF FRACTIONS MADE IN DIFFERENT WAYS FROM UNINFECTED LEAVES MIXED WITH LABELLED TMV

Three 10 g samples of uninfected leaves along with 20 mg of labelled TMV were pulped with 25 ml water, 28 mM with respect to KOH and K_2HPO_4 or trisodium citrate. In the first two, 5 ml phenol was present during the pulping; in the third it was added afterwards. The second extracts were made with 30 mM trisodium citrate. In the control, TMV in 32 ml water with 0.2 ml M phosphate was shaken with 5 ml phenol. All extracts were then dialysed.

treatment		lesions on eight half-leaves	counts/min on the extract	counts/min on the fibre or phenol layer
pulped with phenol and alkaline phosphate	1st extract	8	244	
	2nd extract	479	1188	3700
pulped with phenol and alkaline citrate	1st extract	2570	3518	
	2nd extract	1095	1222	1070
pulped with alkaline phosphate and phenol added after 30 min	1st extract	485	900	
	2nd extract	209	892	4650
control		753	3500	1900
control diluted 1:5		221		

Table 6 compares the infectivity and ^{32}P content of extracts made by pulping uninfected leaves with phenol and TMV containing ^{32}P, either in alkaline phosphate on in citrate, with an extract made by pulping in phosphate and adding the phenol later. After separating the extract, the fibre was re-extracted with citrate without the intermediate water extraction used in the experiments recorded in tables 2 and 3. The ^{32}P content of samples from each extract and from the fibre was measured; the figures given in table 6 are the total for the fraction calculated from this. Clearly, a mixture of TMV and uninfected leaf behaves similarly to infected leaf. The Ph-2 extract contains more TMV:RNA than the Ph-1 extract, and the extract made with citrate in the initial mixture contains still more, whether the TMV:RNA content is judged by measurements of infectivity or ^{32}P. The deficits are only partly made up later by extraction with citrate. Experiments with labelled TMV show that the missing TMV:RNA accompanies the fibre and make it probable that the same process accounts for the differences between the infectivity of extracts made from infected leaves in different ways.

All these extracts were dialysed before assaying infectivity, so some TMV:RNA may have been hydrolysed enough to diffuse through, or into, the dialysis membrane. Measurements of ^{32}P in evaporated diffusates and incinerated dialysis tubes show that the former never contain more than a tenth of the total and the latter a negligible amount. Measurements of the diffusible ^{32}P were made solely to check the recovery of ^{32}P in the various fractions: they do not give precise information about the changes undergone by TMV:RNA. There are two extreme possibilities: the appearance of 10% of the ^{32}P in the diffusate could result from extensive hydrolysis of 10% of the TMV:RNA with no change in the remainder, this would have no measurable effect on infectivity; alternatively, it could result from removing 10% from the end of every particle, which would probably destroy all infectivity. The actual state of affairs is probably intermediate so that evidence on the factors controlling the infectivity of extracts must come from the study of processes other than the formation of diffusible ^{32}P. Whenever comparisons were made, there was more diffusible ^{32}P in Ph-2 than Ph-1 extracts although the Ph-2 extracts were more infective.

The composition of the aqueous phase affects the partitioning of TMV:RNA between water and phenol (Bawden & Pirie 1972); many substances diminished the amount of TMV:RNA in the aqueous phase. However, in about 50 out of 60 experiments in which TMV was pulped with uninfected leaf in conditions in which little was fixed on the fibre, the extract was more infective than the control in which TMV alone was treated with phenol. In the absence of other evidence, we suggest that this apparent activation arises because some leaf components favour the movement of TMV:RNA into the water phase. Table 6 shows this phenomenon; the control, arbitrarily made in phosphate, contains less ^{32}P, and is less infective, than the extract from leaves where fixation was prevented by citrate in the extraction fluid. No difference has been noticed between citrate and phosphate solutions when purified TMV alone is disrupted by phenol. Considering the inaccuracy of infectivity assays, there is reasonable correlation between lesion-count and ^{32}P content. When the correlation is less good we invoke the presence of TMV:RNA that has been damaged by RNase during the extraction.

Uninfected F-W leaves, pulped with labelled TMV and phenol, show the general nature of the fixation phenomenon clearly. The experiment set out in table 7 compares the infectivities and ^{32}P contents of extracts and fibres from fresh leaves, F-W leaves, and F-W leaves to which concentrated diffusate was added to re-establish conditions resembling the initial ones. Re-establishment is incomplete because, unavoidably, the process of freeze-washing partly resembles Ph-2 conditions. To avoid having the ^{32}P distributed among many fractions, the fibres in this experiment were not washed; much of the total of 1130 counts/min in the F-W fibre was therefore contributed by extract that remained in it and most, but not all, of the ^{32}P was removed when part of the fibre was washed twice more with water. This experiment served also as the control for further tests. The diffusate from a separate sample of the same batch of F-W leaves was decalcified with

TABLE 7. INFECTIVITY AND ^{32}P CONTENT OF EXTRACTS AND FIBRES MADE FROM UNINFECTED LEAVES, LEAVES DEPLETED OF THEIR DIFFUSIBLE COMPONENTS, AND DEPLETED LEAVES TO WHICH THE DIFFUSATE WAS RESTORED: ALL WITH LABELLED TMV

Three 10 g samples of uninfected leaf, as harvested or F-W (see text), were pulped with 20 mg labelled TMV and 5 ml phenol in 25 ml water, or reconcentrated diffusate, containing 0.7 ml M KOH and 0.7 ml M K_2HPO_4. Control as in table 6.

	lesions on eight half-leaves		counts/min	
	extract	extract at 1:5	on the extract	on the fibre or phenol layer
no pretreatment	86	16	40	3400
freeze-washed	2360	1135	2820	1130
freeze-washed with return of diffusate	105	36	65	4100
control	1690	618	3500	1900

ammonium oxalate, concentrated, and added to the F-W leaves before they were pulped with labelled TMV and phenol. A separate comparison showed that this extract had an infectivity and ^{32}P content similar to that from F-W leaves. Further evidence for the role of Ca^{2+} in fixation, comes from experiments in which the Ca^{2+} was added during the extraction of F-W leaves; at 30 mM the behaviour resembled that of unwashed leaves closely; nine-tenths of the ^{32}P can then be retained by the fibre. Although much of the RNase diffuses away during freeze-washing, some remains firmly attached to the fibre even after the pulp is finely dispersed and washed repeatedly on the centrifuge. This enzyme is destroyed by phenol; its presence makes the study of fixation on fibre that has not been treated with phenol impracticable.

Ph-1 extracts from uninfected leaves plus TMV are made more infective by including YNA in the mixture, as already described with infected leaves. Adding the YNA after phenol is less effective. In one such experiment the Ph-1 extract gave three lesions, the extract from part of the pulp to which YNA had been added at 2 g/l gave 116 lesions, and the extract made by pulping in the presence of the same amount of YNA gave 855 lesions.

The composition of the fluid through which infection spreads from cell to cell is unknown but it may be more dilute than 'sap' made by crushing leaves in a mortar. All the pulps described so far were diluted because dispersion is then more uniform than that attainable in a mortar and the necessary two- or three-fold dilution does not seem, of necessity, to be objectionable. A few 'blender' extracts were compared with extracts made with pestle and mortar in the amount of diluent used in the 'blender', and extracts made in a mortar without diluent. The last method yields Ph-1 extracts that are more infective than those made in the other ways, but less infective than Ph-2 extracts or Ph-1 extracts, also made with

pestle and mortar, with minimal dilution with citrate. Qualitatively therefore, the phenomena do not depend on the precise technique used for making leaf pulp. The ^{32}P contents of these extracts and residual fibres agree with the infectivity measurements. Hence this change in the conditions does not alter the general conclusion that differences in infectivity are caused less by differences in the extent to which RNase has attacked TMV:RNA, than by the extent of fixation on the fibre, which depends on the concentrations of various components in the extracts. The differences between leaves in different positions on the plant are as definite when extracts are made with pestle and mortar, and when ^{32}P is used to measure the amount of TMV:RNA in an extract, as when the infectivity of 'blender' extracts is measured.

TABLE 8. THE FIXATION OF ^{32}P FROM LABELLED TMV BY INFECTED LEAVES EXTRACTED IN DIFFERENT WAYS

Two 10 g lots of infected leaves were pulped with 1.5 mg of labelled TMV in 25 ml water containing 0.7 ml M K_2HPO_4 and 0.7 ml M KOH either with 5 ml phenol during the pulping or added 30 min later. Fibres re-extracted with 15 ml water and then with 15 ml 30 mM trisodium citrate. All extracts dialysed.

		lesions on eight half-leaves		counts/min	
		extract	extract at 1:5	on the extract	on the fibre
leaves pulped with phenol and alkaline phosphate	1st	0	—	96	
	2nd	31	0	192	
	3rd	765	—	2200	6600
leaves pulped with alkaline phosphate and phenol added after 30 min	1st	775	343	680	
	2nd	218	—	272	
	3rd	280	—	1330	8000

We earlier commented that the more infective third extracts of infected leaves with citrate usually come from fibres that gave the more infective first extracts. This does not happen with uninfected leaves pulped with TMV, as shown in table 8, and by the two extractions made initially with phosphate shown in table 6. That infected leaves and uninfected leaves plus virus should differ is not surprising: it is indeed more surprising that the two types of starting material give such similar results.

The initial stimulus for our work was the observation that the infectivity of extracts from infected Java tobacco leaves depended on how they were prepared, but a detailed study was made because it seemed that the phenomenon may shed light on physiologically controlled differences in susceptibility to infection. This is almost always measured on a local-lesion host, such as Xanthi, but little could be learned by studying this host in which effects are soon confused by the products of necrosis. Uninfected Xanthi leaves, taken when they are very susceptible, mixed with TMV and extracted by the various methods we describe, behave similarly to the upper leaves of Java and give infective extracts regardless of the

sequence of the extraction operations. Leaves from Xanthi plants older and less susceptible than those used for assay, behave in essentially the same way as the lower leaves from Java.

The behaviour of extracts from infected leaves with added TMV

In the experiments just described, the ^{32}P in fibres and extracts is not necessarily all in the form of infective TMV:RNA: some may have been modified by enzymic and other reactions in the leaf pulp, and some may have come from initially non-infective particles of TMV. But it was all present initially as TMV:RNA,

TABLE 9. THE INFECTIVITY OF EXTRACTS FROM THE SOLUBLE AND INSOLUBLE COMPONENTS OF INFECTED LEAF PULP

16 g of infected leaves were pulped in N_2 and dispersed in 40 ml of water, 30 mM with respect to KOH and K_2HPO_4. Two 6 ml samples were withdrawn and treated with phenol, with and without aeration. The remainder of the pulp was centrifuged with exclusion of air. The supernatant was treated with phenol, with and without aeration. The sediment was suspended in a volume of water equal to the volume of supernatant fluid and divided into four parts with exclusion of air. Citrate was added to two of the suspensions. One water and one citrate suspension were aerated before adding phenol. All eight extracts were re-extracted with phenol and dialysed.

treatment	lesions on eight half-leaves with 2 ml of extract
pulp treated with phenol while still in N_2	209
pulp aerated for 30 min before adding phenol	753
solution treated with phenol immediately	880
solution aerated for 30 min before adding phenol	1070
sediment treated with phenol immediately	1
sediment treated with phenol after 30 min aeration	372
sediment in 50 mM citrate treated with phenol immediately	1000
sediment in 50 mM citrate treated with phenol after 30 min	960

and every particle had an equal chance of being labelled. Nothing could be learnt from experiments on infected plants grown on labelled phosphate because normal RNA, phospholipids, and other phosphorus compounds would all be labelled. Experiments on infected leaves mixed with labelled TMV are a compromise and were included to see whether the infected state introduced any striking anomalies. For the experiment in table 8, 1.5 mg of labelled TMV was added to each 10 g sample of infected leaf. Ph-1 and Ph-2 extracts were made in phosphate, a second extract was made with water and a third with citrate. Infectivity measurements

(made at two concentrations with Ph-1 second extract and Ph-2 first extract) show the usual trend. The ^{32}P measurements correlate well on the Ph-1 extracts but not on the Ph-2 extracts – especially not on the third. In spite of their infectivity, third extracts seem to contain much ^{32}P that is no longer in the form of infective TMV:RNA. Other similar experiments showed a similar lack of correlation between ^{32}P and infectivity in successive extracts: this suggests that there is some RNase action within the fibre when the processes of extraction are prolonged.

The behaviour of fractions from infected leaf pulp

Results of attempts to fractionate pulp from infected leaves were variable and inconclusive until the importance of oxidation was recognized. Table 9 shows a simple fractionation of pulp 'pressed' anaerobically. It is clear from the first two lines that the pulp gave the usual Ph-1: Ph-2 difference. The next two show that aerating the soluble components from the pulp has little effect on infectivity after treatment with phenol. Aeration or extraction with citrate has, however, a striking effect on the infectivity of extracts made by adding phenol to the centrifuge sediment. Experiments on the more detailed fractionation of the components of the sediment, and on the use of reducing agents such as ascorbic acid and dithiothreitol in place of exclusion of air, have not yet given systematic results.

Fibre-bound TMV:RNA

Unless citrate or some similar agent is added to the initial extraction fluid, two-thirds or more of the ^{32}P becomes attached to the fibre of either infected or uninfected leaves that have not been freeze-washed. This attachment may be related to the attachment to inoculated leaves noticed many years ago (e.g. Jeener & van Rysselberge 1955) as a prelude to infection. In mixtures of the type we use, so much of the TMV:RNA seems to become attached to the fibre, that slight differences in the completeness of attachment would suffice to explain most of the observed differences in the infectivity of extracts. Fixation is easily prevented: this is shown by the relatively small amount of ^{32}P held by the fibre of F-W leaves or leaves pulped initially with citrate. Some TMV:RNA that has become attached to the fibre can be dislodged by citrate: this is shown by the greater infectivity of third extracts, made with citrate in experiments such as the ones set out in tables 2, 3 and 8, but recovery is incomplete. If pools are made of aliquots from successive extracts from batches of leaves treated in different ways, the treatment that gives the most infective first extract usually gives the most infective pool. This suggests that the extraction of RNA can be made more complete by preventing initial fixation to the fibre rather than by releasing it from the fibre later. Even when ^{32}P is used as the criterion of extraction, so allowing the use of techniques that destroy the infectivity of TMV:RNA the ^{32}P is unexpectedly difficult to dislodge.

Fibres such as those described in tables 6 and 8 yield successive further extracts that diminish in infectivity and, less abruptly, in ^{32}P content. After four or five

extractions with citrate at pH 8, more than half the ^{32}P initially present in the washed fibre is still bound. Little of it will come off on exposure to mild extracting agents for a few minutes at 0 °C; TMV:RNA is probably extensively degraded by agents that can dislodge it from the fibre. Because of its possible connexion with the initial stages of infection, and because of the stability of the link, the nature of the attachment between TMV:RNA and fibre deserves detailed study, but some preliminary observations are worth recording. They were all made on fibre that was not freeze-washed or exposed to citrate during the initial pulping, and that had been extracted with citrate at least four times to remove the more labile ^{32}P-labelled TMV:RNA. Fibres from infected and uninfected plants, each pulped with labelled TMV, behaved similarly.

When fibre that has been extracted several times to remove the more labile ^{32}P is disintegrated for a further 2 or 3 min in a 'blender' with 10 to 20 times its (moist) weight of water and then fractionated centrifugally, the more readily sedimented particles have the least specific activity. Thus the sediments at 1000, 3000 and 10000 rev/min gave 140, 250 and 350 counts/min per mg dry matter respectively, but there is so much more material in the first fraction that it carries four-fifths of the total activity. This observation merely suggests that the veins and supporting materials of the leaf are less involved than the more easily dispersed parts. About one-fifth of the activity is dislodged by 0.1 M KOH during a few hours, but half is dislodged when the extraction is preceded by a brief extraction with citric, acetic or hydrochloric acids at pH 3. By alternating acid and alkaline treatments, extraction can be made nearly complete. Boiling ammonium oxalate dislodges almost all the ^{32}P into the pectin jelly. Unexpectedly, incubation with added leaf RNase in pH 5.7 citrate is no more effective than incubation in citrate alone; the RNA seems to be partly protected from enzyme attack. More than half the ^{32}P is extracted from moist fibre by 20 volumes of 50:50 ethanol:ether containing 1% HCl. After evaporation to dryness *in vacuo* the ^{32}P is no longer soluble in lipid solvents; the ^{32}P remaining in the fibre is now more readily extracted at pH 9.

Discussion

Every method of extracting a tissue as heterogeneous as a leaf is an uneasy compromise between retaining conditions approximating to those characteristic of a specific part of the tissue, and allowing the metabolic processes of the tissue to continue during extraction. We made extracts in the presence of excess phenol because this is an extensively used system, it disrupts TMV within the tissue and so releases TMV:RNA uniformly throughout it, and it inactivates or extracts most of the leaf RNase, thus partly protecting TMV:RNA from destruction. As work proceeded it became apparent that phenol treatment also prevented, or at least delayed, other relevant changes in leaf pulp. What could have been dismissed as a study of one particular set of artefacts, could then be regarded as a study of the potentialities of a tissue when oxidative and other processes are controlled.

Obviously, the system studied may also be an artefact – all happenings in a leaf pulp may be so interpreted – but at least two types of change are minimized: during the extraction, both RNase and oxidative enzymes are effectively controlled. Similarly, there is an element of artificiality in the use of alkaline phosphate as the standard dispersing medium for the pulp. But different parts of the leaf have different pHs and it would be equally artificial to expose neutral cell contents to acid vacuolar sap.

We suggest that the fixation of TMV:RNA, whether made *in situ* or added to uninfected leaf during extraction, is controlled by the composition of the medium, especially its Ca^{2+} and RNA content, and by the presence of a structure on the stroma that is so easily damaged that it is partly or completely inactivated when some other methods of extraction are used. Several other processes could diminish the RNA content of a leaf extract, but we think them less important. Thus different conditions of pulping release TMV from cells to different extents, but the relative infectivities of extracts to which phenol was added at different stages are similar regardless of the method of pulping. Further, freezing, exposure to ether and exposure to phenol all make leaves limp and soft so that they disintegrate more easily than untreated leaves, but the effect of phenol contrasts sharply with the others. The strongest evidence against an explanation, based on failure of TMV to be released, comes from experiments with TMV added to uninfected leaf, for release is obviously not then involved. If attention were confined to infectivity measurements in these experiments with added TMV, an explanation based predominantly on destruction of TMV:RNA by RNase would be tenable, but it seems to be excluded by the experiments with ^{32}P. Finally, the labile agent could be soluble and not part of the stroma. That explanation would be incompatible with the ^{32}P results unless the resulting complex with TMV:RNA then associated with the phenol-treated stroma. The accompanying paper (Bawden & Pirie 1972) describes several instances of increased precipitability of TMV:RNA in the presence of leaf components, and it may be that some such process is implicated, but there is no evidence that the concentration of any of these components is affected by brief periods of oxidation. However, the possibility needs remembering, and the position or state of ionization of Ca in pulp may change during oxidation, and affect the fixation of TMV:RNA.

The strongest reason for thinking that the change that takes place between pulping the leaf and adding phenol is an oxidation, is that excluding air during the interval largely prevents it. Extracts made by pulping in the presence of phenol, or when air is excluded during any interval between pulping and adding phenol, are colourless; those made from the lower leaves of tobacco plants pulped in air without phenol are brown. The upper leaves of tobacco give colourless extracts when pulped with phenol and green extracts when there is an interval between pulping and adding phenol. The green pigment is not chlorophyll, or a derivative of it, because it is not extracted into the phenol layer, it reddens on acidification and it has a different absorption spectrum. It is presumably the pigment found in

several plant extracts by Kozlowski (1950) and named allagochrome by Habermann (1963). It seems probable that this is a macromolecular oxidation product of chlorogenic acid, made by one of the enzymic processes surveyed by Pierpoint (1971). Even when oxidative changes are prevented by excluding air, there may be autolytic changes.

RNase will attack the normal RNA of the leaf, though not readily in the slightly alkaline conditions we used. Incompletely controlled RNase action, by changing the integrity and distribution of normal RNA, probably accounts for some inconsistencies in the behaviour of different batches of leaf. There are also obvious differences in texture between Ph-1 and Ph-2 pulps. Metabolic products arising from the action of leaf mitochondria on substrates such as malate, inactivate TMV:RNA (Bawden & Pirie 1959); we have no evidence that this type of inactivation is important in Ph-2 extracts.

In analytical work, tissue RNA is usually extensively degraded by exposure to acid or alkali to ensure complete extraction. Fixation of the type we describe may have been overlooked although common. But a few instances of fixation have been investigated: thus Artman & Roth (1971) concluded that the RNA often found associated with chromatin is bound during the process of preparation, and Stepanov, Voronina, Ovchinnikov & Spirin (1971) separated a protein fraction from liver that binds RNA from *E. coli*; Ca^{2+} was not implicated in either. TMV, and therefore TMV:RNA arising from it by phenol fission, probably occurs at several different sites in an infected leaf, as does the RNA in an uninfected leaf. There is no reason to assume that all these RNAs behave similarly for they may have different spatial relations with whatever structure is responsible for fixation. Nevertheless, the methods we used to extract infective TNV:RNA might be worth trying in work on normal RNA.

The large difference between upper and lower leaves is unexplained. Young leaves contain more ribosomal RNA (Pirie 1950) and this may be even more effective than yeast RNA in competing with TMV:RNA for fixation. Extracts from leaves in different positions on the plant contain similar amounts of total Ca, but the amount present as Ca^{2+} may vary. Reducing agents such as –SH and ascorbic acid may be more abundant in extracts from young than old leaves. We have no evidence on these points. Detailed work was restricted to mature leaves because they give the most consistent results.

The complete resistance of some hosts to infection by some viruses can be plausibly explained by postulating an inability of the virus-synthesizing mechanism of the host to respond, by copying, to the stimulus of the invading virus. However, in other hosts there may be response, but the newly formed virus, or RNA produced from it or as a stage in its formation, may get bound by a process analogous to the one we describe; this could prevent it spreading far enough from initially infected cells to allow its presence to be recognized visually.

References

Artman, M. & Roth, J. S. 1971 Chromosomal RNA: an artefact of preparation? *J. molec. Biol.* **60**, 291.

Bawden, F. C. & Pirie, N. W. 1940 The effects of alkali and some simple organic substances on three plant viruses. *Biochem. J.* **34**, 1278.

Bawden, F. C. & Pirie, N. W. 1945 The separation and properties of TMV in different states of aggregation. *Br. J. exptl. Path.* **26**, 294.

Bawden, F. C. & Pirie, N. W. 1959 The infectivity and inactivation of nucleic acid preparations from tobacco mosaic virus. *J. gen. Microbiol.* **21**, 438.

Bawden, F. C. & Pirie, N. W. 1969 Ribonucleases. *Rothamsted Experimental Station Report for* 1968 **1**, 114.

Bawden, F. C. & Pirie, N. W. 1972 The inhibition, inactivation and precipitation of tobacco mosaic virus nucleic acid by components of leaf extracts. *Proc. R. Soc. Lond.* B, **182**, 319.

Davys, M. N. G., Pirie, N. W. & Street, G. 1969 A laboratory-scale press for extracting juice from leaf pulp. *J. Biotechnol. Bioengng.* **11**, 528.

Engler, R. & Schramm, G. 1959 Infectious ribonucleic acid as precursor of tobacco mosaic virus. *Nature, Lond.* **183**, 1277.

Haberman, H. M. 1963 Allagachrome 1 Properties, purification and assay procedures. *Plant Physiol.* **38**, 381.

Jeener, R. & Rysselberge, C. van 1955 Un essai d'infection directe des cellules du parenchyme de la feuille de tabac à l'aide de virus de la mosaïc marqué par le ^{14}C et le ^{32}P sort du virus. *Biochim. biophys Acta* **17**, 233.

Jervis, L. & Pirie, N. W. 1972 The ribonucleases in tobacco leaf extracts treated with phenol. (In the Press.)

Kozlowski, A. 1950 Formation of a green oxidation-reduction indicator in plant extracts. *Nature, Lond.* **165**, 495.

Pierpoint, W. S. 1971 Formation and behaviour of o-quinones in some processes of agricultural importance. *Rothamsted Experimental Station Report for* 1970, **2**, 199.

Pirie, N. W. 1950 The isolation from normal tobacco leaves of nucleoprotein with some similarity to plant viruses. *Biochem. J.* **47**, 614.

Pirie, N. W. 1956*a* Some components of tobacco mosaic virus preparations made in different ways. *Biochem. J.* **63**, 316.

Pirie, N. W. 1956*b* General methods for separation; making and handling extracts. In *Modern methods of plant analysis* (eds. K. Paech and M. V. Tracey) **1**, 26. Berlin: Springer.

Pirie, N. W. 1961 The disintegration of soft tissues in the absence of air. *J. agric. Engng. Res.* **6**, 142.

Stepanov, A. S., Voronina, A. S., Ovchinnikov, L. P. & Spirin, A. S. 1971 RNA-binding protein factor of animal cell extrcts. *FEBS Letters* **18**, 13.