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Some Factors Affecting the Activation of Virus Preparations Made from Tobacco Leaves Infected with a Tobacco Necrosis Virus

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SUMMARY: Preparations of the Rothamsted tobacco necrosis virus were made by the ultracentrifugation of sap from infected tobacco leaves after a preliminary concentration by freezing. Not all the anomalous nucleoprotein in these preparations was infective, and the products were fractionated by differential ultracentrifugation at lower speeds and by precipitation at pH 4 in the presence of sedimentable protein from uninfected leaves. The more readily sedimentable and precipitable material carried with it most infectivity, whereas the other material had the greater serological activity.

Preparations made quickly from freshly expressed sap were less infective than those made from sap that had been frozen or allowed to age for a few days. The extent of the activation produced by these treatments depended on the physiological condition of the infected leaves.

As much virus could be extracted from the leaf residues as occurred in the sap. The infectivity of this residual virus depended on the medium used for its extraction.

It is suggested that much of the infectivity of this virus in sap is acquired during or after extraction from the leaf, but the relationship between the particles with different sizes and properties remains uncertain.

Smith & Bald (1935) identified tobacco necrosis as a virus disease and, since then, several causative viruses have been differentiated by their serological reactions (Bawden, 1941) and by their different properties *in vitro* (Bawden & Pirie, 1942, 1945*a*). A crystalline nucleoprotein has been isolated from plants infected separately with each virus, and with all except one there is much evidence identifying the protein with the virus, although the precise relationship between the two remains unestablished. The virus we have called the Rothamsted culture, however, is exceptional, and the status of the crystalline nucleoprotein obtainable from plants infected with it is very uncertain. All highly infective preparations were demonstrably inhomogeneous, and there was little positive evidence about the relationships between the various components. Increasing the homogeneity of the preparations of nucleoprotein decreased the infectivity, but the significance of this was uncertain as the virus readily loses infectivity during treatments that appear not to affect other tobacco necrosis viruses.

When preparations of the Rothamsted tobacco necrosis virus made in 1945 were ultracentrifuged they gave pellets that were crystalline; when these pellets were washed quickly with successive small lots of water, the material extracted in the successive washes was, weight for weight, progressively less infective. The most slowly dissolving material was the most highly crystalline and usually had the highest precipitin titre, but was the least infective. This phenomenon has three equally plausible interpretations: infective and non-

infective virus particles may resemble one another and crystallize together, but dissolve at different rates; infective particles may not crystallize, and the crystalline material may all be non-infective, though derived by some modification of virus particles; the crystalline material may all be non-infective and, although specific to infected plants, may be distinct from the virus particles and not derived from them. Our recent experiments have not decided between these various interpretations, but they have given further information on the conditions that lead to variations in infectivity, with the result that much more infective preparations can now be made. As other viruses may behave similarly, it seems worth recording some of the anomalous phenomena we have encountered with the Rothamsted tobacco necrosis virus, although few are definitely interpretable and many are not consistently reproducible.

MATERIALS AND METHODS

The virus used was derived by repeated subculture from the stock we used previously under the name Rothamsted. Transfers were usually made with a bulk inoculum, but at irregular intervals fresh stocks were built up from virus derived from single local lesions. There is no proof that we have worked continually with the same virus or virus strain, but there is no evidence to the contrary; nor is there any evidence that our stocks contained more than one tobacco necrosis virus, although this also cannot be disproved. The most that can be said is that all the inocula produced material that was serologically related and which shared the same general properties and crystalline characters. The virus was propagated in tobacco plants, *Nicotiana tabacum* var. White Burley, which were dusted with celite before the upper surfaces of their leaves were rubbed with infective sap. We (Bawden & Pirie, 1945*a*) have previously described large seasonal differences in the susceptibility of plants and stated that during summer yields are low and purification difficult. These seasonal differences are correlated with variations in light intensity (Bawden & Roberts, 1947, 1948). Susceptibility in summer is much increased when the plants are raised under shade and placed in the dark for 2-3 days immediately before they are inoculated. By doing this, high yields of virus have been obtained throughout the year and purification has been greatly facilitated.

Infectivity tests were made by the half-leaf method using French bean, *Phaseolus vulgaris* var. Prince, half-leaves being rubbed as evenly as possible with the forefinger wet with the inoculum. Preparations to be compared were tested at two dilutions, usually differing by a factor of ten, and each dilution was inoculated to at least six half-leaves. The inocula were systematically varied over the half-leaves so that none appeared twice on the same plant, all possible combinations occurred on the same plant, and each was applied to an equal number of left- and right-hand halves.

Precipitin titres were determined by adding 1 ml. of virus preparation at various dilutions to each of a series of tubes containing 1 ml. of antiserum at a constant dilution, usually 1/50. The tubes were immediately placed in a water-bath held at 50°, with the fluid columns half-immersed so that convection currents kept the mixtures continuously moving. The precipitin titre was taken

as the smallest amount of antigen that produced a precipitate visible to the eye after 3 hr. in the water-bath. Antisera were prepared in rabbits by the intravenous injection of virus preparations in various stages of purification and possessing various degrees of infectivity. Fully-crystalline preparations with little infectivity were most active antigenically, equal weights of these producing antisera with higher precipitin titres than those obtained by injecting more infective but less homogeneous preparations, but no qualitative differences have been noted between antisera produced against the various types of antigen.

Preliminary treatments

The Rothamsted tobacco necrosis virus loses infectivity more rapidly in sap than when purified, and it seemed possible that the heterogeneity of purified preparations might be, at least in part, an artefact caused by the exposure of virus particles to the conditions obtaining in sap. Some sap components of small molecular weights cause particles of tobacco mosaic virus to aggregate, and *in vitro* changes with this virus can be diminished by removing such components before the infected leaves are macerated (Bawden & Pirie, 1945*b*). In preparing unaggregated tobacco mosaic virus, convenient treatments are to use the leaf cells as dialysis membranes after destroying their osmotic control by freezing or exposure to chloroform. In preparing this tobacco necrosis virus, however, such treatments are inappropriate as they themselves cause much loss of infectivity. In other attempts to lessen deleterious changes, sap was cooled, the various treatments were made with as little delay as possible and the preparations kept as cold as possible throughout. So far from increasing infectivity, this treatment gave a less infective product than that made from sap kept at room temperature and fractionated over a longer period. It is obvious that particles of the virus are affected in various ways in sap, but the changes that predominate at first increase rather than decrease infectivity. Our most infective preparations were made by using ultracentrifugation to sediment the virus and delaying its application for some time after the sap had been expressed from macerated leaves.

Small quantities of the virus are readily prepared by the direct ultracentrifugation of clarified sap, but the virus content of sap is small and this method is laborious when quantities sufficiently large for chemical study are needed. In our previous work, the virus in sap was first concentrated by a preliminary precipitation with ammonium sulphate, but this is undesirable as the required concentration of salt causes considerable inactivation. Attempts at ultra-filtration on cellophan by the method described by Paterson, Pirie & Stableforth (1947) were unsuccessful, because the sticky precipitate that separates from sap clogs the filtering surface. Although the removal of other sap components would be advantageous, it is not essential at this stage. The primary purpose of the treatment is simply to decrease the volume of fluid so that more virus can be ultracentrifuged. The method we found most suitable was to remove most of the water by freezing.

The removal of water by freezing is used in several technical processes (apple-jack is one well-known product) and it has occasionally been used to

concentrate proteins (Mellanby, 1908; Palmer, 1934; Bawden & Pirie, 1937), but it has been less used than it merits as a routine method for concentrating unstable substances. The process has been long known and probably originated from accidental observations by arctic travellers; thus, Gerart de Veer (1596), who wintered with Barents on Nova Zembla, remarks 'wee were forced to melt the Beere, for there was scant any unfrozen Beere in the Barrell, but in that thicke Yeaste that was unfrozen lay the strength of the Beere, so that it was too strong to drinke alone, and that which was frozen tasted like water'. Glauber (1658) recognized that only the water went into ice when dilute acetic acid was frozen and that stronger acid could be made by separating the unfrozen part. Boyle (1688) concentrated leaf extracts, in addition to beer and sea water, and concluded that the flavoured and coloured constituents were concentrated in the unfrozen part. His experiments refuted the statements of Aristotle and some medieval writers that the unfrozen part had the physical form of the plant from which the extract came; on all these issues our results confirm those of Boyle.

Preparation of infective virus in bulk

Leaves are picked 6–10 days after inoculation when they are well covered with lesions, and the main veins are cut away to avoid dilution with juice of a low virus content. The laminae are minced in a domestic meat mincer, and the sap expressed by squeezing through madapollam; the fibrous residue is minced and pressed again. After clearing by centrifuging for 10 min. at 7000 r.p.m., the sap is poured into a tray and placed in the freezing chamber of a refrigerator. Freezing proceeds from the surfaces, and the fluid collects in the centre and in spaces between ice crystals. These spaces remain connected until about 90 % of the water is frozen. Freezing is then discontinued, for otherwise the separation of the frozen and unfrozen parts becomes more difficult. The ice-block is broken by pounding through a grid with 1 cm. holes mounted in a metal funnel, and centrifuged immediately. With quantities of 300 g. or more, a basket centrifuge is convenient, but the separation can be done readily with a bucket-type centrifuge, by fitting perforated containers into the buckets. We have used household plastic cups without handles and with ten to twenty 1 mm. holes drilled in and around the bottom of each cup. The cups fit into the metal buckets or glass centrifuge tubes but are prevented by their conical shape or a flared rim from slipping right in. A few minutes' centrifugation at 3000 r.p.m. separates the fluid from the ice, and about one-sixth of the original volume of sap is obtained at the first run. This will usually contain about nine-tenths of the total virus, but a second run is advisable to ensure that this is so. If more than 90 % of the water has been allowed to freeze, much of the fluid will be contained in isolated pockets within the ice and will not be liberated until there has been extensive thawing during centrifugation. This condition can be recognized by the presence of coloured droplets within the ice at the end of centrifugation. With infective sap, virus and colour concentrate together, and the diminution in the colour of successive thawings is a reliable guide to virus content. If a second extract contains

appreciable quantities of virus, it can be concentrated by repeating the freezing.

All the constituents of the sap are concentrated by the freezing, and the density and viscosity are so increased that sedimentation in the ultracentrifuge is delayed. The fluid is therefore dialysed for a few hours against distilled water, when much of the low molecular-weight protein precipitates and can be removed by low-speed centrifugation. The clarified supernatant fluid is then centrifuged for 30–40 min. at 40,000 r.p.m. (80,000 *g*) in a centrifuge of the type described by Masket (1941), which sediments all the virus into compact pellets. In summer the pellets at this stage are usually brown and opaque, but in winter they may be yellow and almost transparent, with a crystalline fringe. The pellets are collected into one tube, mixed intimately with a volume of water equal to about one-hundredth that of the original sap, and centrifuged at 7000 r.p.m. (6000 *g*) after standing for a few hours. Some virus usually remains in the insoluble residue, which is therefore extracted a second time with water. The pooled extracts are again ultracentrifuged, when the pellets are clear, pale brown or yellow, with a definite crystalline fringe. The pellets are suspended in water and left for a few hours before insoluble material is removed by centrifuging at 7000 r.p.m.

The preparations at this stage are only faintly opalescent at concentrations around 5 *g./l.*; if they are not colourless, sedimentation for a third time will make them so and increase both their infectivity and serological activity. Additional centrifugations at 40,000 r.p.m., however, produce no useful fractionation, as all the material present sediments and redissolves in water. Nevertheless, the preparations are far from homogeneous, and they can be separated into fractions containing materials that have different ratios of infectivity to serological activity. Some separation is produced by centrifuging at 20,000 r.p.m. (22,000 *g*), even though our centrifuge is not well adapted to such separations because the tubes are inclined at only 10° to the axis. The results of one experiment, in which 7.5 ml. of a 1 *g./l.* virus solution was kept at 20,000 r.p.m. for 30 min. and then slowed to a stop in 15 min., will illustrate the kind of separation achieved. The contents of the tube were separated into three parts: the top 6.5 ml. of fluid, which was siphoned off immediately the centrifuged stopped; the bottom 1 ml. of fluid, near to or in contact with the pellet, which was decanted; and the pellet, which was dissolved in water. The three fractions contained 3, 1.7 and 2.8 mg. of solid matter respectively, and did not differ appreciably in serological activity, all three precipitating with antiserum to a dilution of 9 *mg./l.* Their relative infectivities, however, differed considerably; the mean numbers of lesions produced per half bean leaf by the materials in the three fractions were respectively 54, 79 and 110 at 2 *mg./l.*, and 14, 30 and 41 at 0.2 *mg./l.*

Similar results were obtained in many other experiments, although sometimes the material that compacted into a pellet from preparations centrifuged for 30 min. at 20,000 r.p.m. was less active serologically than that in the supernatant fluid, despite its greater infectivity. When the material in the pellets was dissolved and centrifuged at 20,000 r.p.m. it again partitioned, but there

was little fractionation; the two parts had similar infectivities and serological activities. However, by repeating the centrifugation on the more slowly sedimenting material, this could be separated into fractions with differing infectivities. When the uncompacted material from several tubes centrifuged at 20,000 r.p.m. was pooled, concentrated by sedimentation at 40,000 r.p.m., and then again centrifuged at 20,000 r.p.m., the material remaining in the upper fluid was sometimes less than a tenth as infective as that in the pellet compacted in the first centrifugation at 20,000 r.p.m., although less was needed to give a visible precipitate with antiserum. When the material that has failed to compact twice at 20,000 r.p.m. is sedimented at 40,000 r.p.m. it gives pellets that are crystalline throughout, whereas pellets from the more infective material that compacts at 20,000 r.p.m. are largely amorphous with a crystalline halo. Pellets of both kinds of material can be fractionated by washing rapidly, with successive small lots of water, in the manner previously described (Bawden & Pirie, 1945*a*) when the most rapidly dissolving part from each type of pellet is, weight for weight, more infective than the more slowly dissolving parts.

After the most infective fractions have been separated by centrifugation at 20,000 r.p.m., they are still not homogeneous. This is shown by acidifying to pH 3.5-4.5, when part of the material precipitates and serological activity and infectivity partition unequally between the precipitated and soluble parts. If the suspension is quickly neutralized, the precipitate dissolves and infectivity is little affected, but a few hours' exposure to pH 4 at room temperature causes much inactivation. The partitioning of infective and serologically active material between the precipitate and fluid when preparations were centrifuged around pH 4, depended on the time of exposure, the pH value, the virus concentration, salt concentration, and on the amount of extraneous leaf protein present in the preparation. In some conditions, serological activity and infectivity could be almost completely separated, the precipitate containing most of the infectivity, whereas the neutralized soluble material was poorly infective but precipitated strongly with antiserum. Tables 1 and 2 give the results of experiments made in 0.01 M phthalate and show the effects of different times of exposure to acid and of different pH

Table 1. *Effect of acid on ratio of infectivity to serological activity*

(Virus preparation in pH 3.95 0.01 M phthalate buffer and left for specified time before either neutralizing or centrifuging and neutralizing.)

Treatment	Precipitation end-point (mg./l.)	Mean lesions per half-leaf at		
		100 mg./l.	10 mg./l.	1 mg./l.
Control at pH 7	12	127	32	4
Neutralized after 30 min. at 16°	12	105	17	3
Separated after 15 min. at 16°:				
Neutralized fluid	12	115	31	6
Neutralized precipitate	> 100	24	2	0
Separated after 14 hr. at 0°:				
Neutralized fluid	12	72	16	2
Neutralized precipitate	> 100	42	12	0.5

Table 2. *Differential separation of infective material on precipitates formed at different pH values*

(Virus preparation in 0.01M phthalate buffer, centrifuged after standing for 15 min. supernatant fluids and resuspended precipitates neutralized before testing.)

pH	Fraction	Precipitation end-point (mg./l.)	Mean lesions per half-leaf at	
			10 mg./l.	1 mg./l.
4.4	Fluid	12	51	10
	Solid	> 100	5	0.2
3.9	Fluid	12	56	11
	Solid	> 100	31	3
3.5	Fluid	12	11	2
	Solid	> 100	68	8

values. In the almost complete absence of salts, for example, using dialysed virus preparations and adjusting the pH with 0.01M-HCl, and with more concentrated virus preparations, infectivity and serological activity could be separated still more sharply. The material soluble at pH 4 may precipitate with antiserum at concentrations down to 6 mg./l., whereas the acid-precipitable material may precipitate only at concentrations above 25 mg./l., yet the latter may be a hundred times as infective. With virus preparations made so that they contain little normal plant protein, such dramatic dissociations of infectivity from serological activity are unusual, and much of the infective part of the preparation remains soluble between pH 3-4.5. With such preparations, however, the separation can be brought about by the addition of an unstable sedimentable protein that can be isolated from uninfected tobacco leaves (Pirie, 1950). Table 3 records an experiment showing

Table 3. *The removal of infective material by normal plant protein precipitated at pH 4*

(Virus preparation, or virus preparation plus an equal weight of normal leaf protein, in 0.01M phthalate buffer, centrifuged after 15 min. and precipitate and supernatant fluid neutralized before testing.)

Treatment	Precipitation end-point (mg./l.)	Mean lesions per half-leaf at		
		100 mg./l.	10 mg./l.	1 mg./l.
Control at pH 7	12	77	13	4
Preparation at pH 4:				
Fluid	12	67	8	5
Precipitate	100	4	1	0
Preparation plus normal protein	12	51	7	4
Preparation plus normal protein at pH 4:				
Fluid	12	2	0	0
Precipitate	50	49	5	1

the removal of the infective component from a virus preparation on the precipitate of this protein produced at pH 4. In this experiment the simple presence of the normal protein reduced the number of lesions; this was not usually observed and in controls in which the ratio of virus to normal protein was varied in the range 2.5 : 1 to 1 : 50 no systematic influence on the in-

fectivity was found. To get sharp separation, the protein must be freshly prepared and ultracentrifuged sufficiently often to free it from acid-precipitable proteins of low molecular weight, for these prevent the separation of the infective part of a virus preparation when the weights of normal protein and of the virus preparation are approximately equal. When much larger amounts of the normal protein were added, parts of the virus preparation precipitated in acid solution only when the salt concentration was less than 0.02M. Infective material does not therefore separate on the precipitate that forms when infective sap is acidified, but it does if the sap is first dialysed. Sedimentable proteins from tomato and French-bean leaves can also be used to fractionate the virus preparations; our previous (Bawden & Pirie, 1945*a*) failure to demonstrate this phenomenon probably occurred because the bean protein then used was too old and insufficiently freed from contaminants with low molecular weights. We have added other materials, for example, nucleic acid and hyaluronic acid, to acid solutions of the acid-soluble component of virus preparations, and we have also attempted to separate the components by differential absorption with charcoal, kaolin, kieselguhr, and leaf fibre, but none has behaved like the leaf protein and produced fractions with widely differing ratios of infectivity to serological activity.

The procedure of separating an acid precipitate, either with the help of normal plant protein remaining in the virus preparations or with added normal protein, has obvious potentialities for obtaining material that is, weight for weight, more infective than that separated by differential centrifugation. Most of the infectivity may be concentrated in the one-tenth of the original virus preparation that precipitates together with the normal plant protein, but we have not been able to devise a method whereby these two can subsequently be separated without destroying infectivity. We do not know whether the infective component reacts specifically with the antisera that precipitate the crystalline component. Only when it separates together with an excess of acid-precipitable plant protein have we any reason to consider that it is reasonably free from the crystalline component, and in this condition the neutralized preparations either fail to precipitate with antisera or do so only at concentrations of 100 mg./l. or greater. This is not evidence that free infective virus does not precipitate with the antiserum; it may simply mean that precipitation is prevented by the presence of large amounts of serologically unspecific protein.

Separation from sap of virus in its least modified state

We have already mentioned that the infectivity of purified preparations of this tobacco necrosis virus depends on the initial treatment given to sap, the final product being more infective if the sap is aged or frozen before it is ultracentrifuged than if it is centrifuged when freshly expressed. It seems from this that the preparations we have so far described are not only demonstrably inhomogeneous, but also contain material that is artefact, derived from some precursor by changes brought about during the treatment. It may well be that some of the components of these preparations occur in the more necrotic

parts of an infected leaf but it would seem reasonable to assume that the state of the virus in infected cells adjacent to necrotic areas more nearly resembles its state in fresh sap. Knowledge of the properties of virus in these cells, rather than in the necrotic ones, is likely to shed light on the physiology of virus multiplication. Attempts have therefore been made to isolate the virus in its least modified form by working with sap kept cold and making fractionations with as little delay as possible.

The sap from freshly minced leaves is expressed into a cylinder cooled with ice and is immediately clarified by centrifuging at 7000 r.p.m.; the supernatant fluid is centrifuged for 30 min. at 40,000 r.p.m. in a rotor chilled with ice. The pellets are evenly suspended in water, using about one-tenth the volume of the original sap, left for an hour at 0° and then centrifuged for 10 min. at 7000 r.p.m. The residue is extracted a second time with water, and the pooled clarified extracts are ultracentrifuged again in a chilled rotor. The pellets are suspended in water and freed from insoluble material at 7000 r.p.m. A third ultracentrifugation may give a further fractionation, but it usually does not do so when the supernatant fluids have been thoroughly drained away after the first two runs. Such preparations are more opalescent than those from frozen sap and they are less stable. After 5–10 days at 3°, about a third of their dry matter coagulates and can be removed by low-speed centrifugation. The coagulation is promoted by the presence of chloroform as an antiseptic and is delayed by 1 g./l. azide; it is not affected by toluene and thymol.

Table 4. *Yields and activities of virus preparations made from sap receiving different initial treatments*

Treatment of sap	Yield (mg./l.)	Precipitation end-point (mg./l.)	Mean lesions per half-leaf at	
			2 mg./l.	0.2 mg./l.
Centrifuged immediately	350	15	17	2
Centrifuged after 2 days at 3°	320	15	133	23
Centrifuged after freezing	180	12	156	40

Table 4 shows the yields, precipitation end-points and relative infectivities of preparations made from samples of the same batch of sap by the method described in the previous paragraph, by the same method but using sap that was left at 3° for 48 hr., and by the method described earlier in this paper. The increase in infectivity, without any commensurate decrease in yield, obtained by the second and third methods is obvious. Increases of this magnitude are achieved only with certain leaves, and the increase in activity produced by ageing sap depends on the age of leaf, the duration of infection and the extent to which the lesions cover the leaf surface. With leaves infected for the same lengths of time, the increase in activity is greatest with those that are yellowest and most completely covered with lesions. This is illustrated by Table 5, which shows the results of an experiment in which leaves from one batch of plants were separated into three groups showing different grades of chlorosis and necrosis. Sap from each group was treated in two ways; one lot was chilled and ultracentrifuged immediately and the other was aged for

Table 5. *Differential activation of virus by ageing in sap from different leaves*

Leaves	Sap	Precipitation end-point (mg./l.)	Mean lesions per half-leaf at	
			1/1*	1/10*
Youngest, crisp and green	Fresh	25	92	17
	Aged	12	47	5
Intermediate	Fresh	18	67	18
	Aged	6	91	24
Oldest, limp and chlorotic	Fresh	12	88	17
	Aged	6	105	26

* The preparations made from the fresh lots of sap were tested in infectivity tests at four times the concentration of those from the aged ones; actual concentrations were 4 and 0.4 mg./l. for the fresh lots and 1 and 0.1 mg./l. for the aged. The ageing was for 2 days in sap at 3° before ultracentrifugation.

48 hr. at 3° before being ultracentrifuged. The yields of purified virus from the three lots of aged saps were similar, and with all three kinds of leaves larger yields, with lower precipitation end-points, were obtained from the fresh than from the aged samples. All the preparations made from fresh lots of sap had similar infectivities, but those from the aged saps differed. From the most yellowed and from the intermediate leaves, the virus from aged sap was more than four times as infective as that from fresh sap, whereas ageing had little effect on the infectivity of the virus from the greenest leaves. A further portion of sap from the yellowest leaves was frozen before being ultracentrifuged and the virus prepared from this, as in the experiment shown in Table 4, was still more infective than that prepared from aged sap. Experiments were also made in which batches of leaves from comparable positions were picked from one set of infected plants at 4, 7 and 10 days after inoculation. All the samples of virus prepared immediately from chilled sap were again about equally infective, whereas the samples prepared from sap allowed to age for 48 hr. at 3° differed, those from the leaves taken soonest after inoculation being less infective than the later ones. Here again the difference lies, not in the virus as it is first obtained from the leaves, but in the conditions obtaining in sap; sap from extensively necrotic leaves gave increased infectivity on ageing and that from other leaves failing to do so.

Although we can gauge from the appearance of infected leaves whether or not ageing in sap will increase infectivity, we do not know the processes involved. Ultracentrifuged unactivated virus has been exposed to various salt solutions and to sap, both fresh and aged, from old and young uninfected leaves, and to the supernatant fluid from ultracentrifuged infective sap, but none of these treatments has produced unequivocal activation. Because of this failure, it has been possible to study the mechanism of activation only in infective sap. At 3° maximum infectivity is reached after 2-4 days, and infectivity then gradually declines; at room temperature, inactivation is so much more rapid that the initial activation may be obscured. Oxygen does not seem to be needed, for infectivity increased in samples of sap from which

the air had been largely removed by exposure to vacuum. Freezing for 1–5 hr. at -5 to -10° caused activation, and prolonged freezing did not decrease infectivity. When sap is dialysed, a large precipitate separates and virus prepared from the supernatant fluid is sometimes more and sometimes less infective than that prepared from fresh sap. Factors that we are not controlling probably affect the extent to which infective particles are adsorbed by this precipitate. Heating sap at 80° for short periods, and ageing pellets ultra-centrifuged from fresh sap in their supernatant fluids at 3° , have also sometimes increased infectivity. These phenomena are illustrated by the experiment recorded in Table 6, but they do not always occur and we cannot explain the frequent failures we have had in experiments intended to serve as repetitions of this one.

Table 6. *Effects of different treatments in increasing infectivity*

Treatment	Mean lesions per half-leaf at	
	5 mg./l.	0.5 mg./l.
Virus from fresh sap	8	1.5
Above at double concentration	15	2.5
Pellet from fresh sap exposed to supernatant fluid	22.5	4.2
Sap heated for 4 hr. at 27°	29	4
Sap aged for 2 days at 3°	26	6

After being subjected to the specified treatment, the virus was twice sedimented at 40,000 r.p.m. and resuspended in water before testing.

The treatments that increase the infectivity of virus in sap all precipitate some of the normal protein and polysaccharide, suggesting that some component of this mixture may inhibit infectivity and that its removal explains the phenomenon. The precipitation proceeds spontaneously even at 3° but it can be diminished by adjusting the pH to 6.5 or 7.5, or by adding 1 g./l. of sodium azide, but these treatments do not prevent the activation. Apparently similar precipitates also separate from samples of sap in which ageing at 3° produces little or no increase in infectivity. Precipitation, therefore, is probably not significant. Most of the components that separate during ageing can be precipitated from sap at pH 4. Because of the salt content of sap, little infective or serologically active material separates with this precipitate, and the treatment usually increases the infectivity of the supernatant fluid, but always to a smaller extent than ageing or freezing. We have already mentioned that precipitates separate from virus preparations made from fresh sap, but this produces no greater increase in infectivity than would be expected from the removal of contaminating inert material and it leaves the ratio between infectivity and serological activity unaffected. It seems unlikely that the difference between activated and other preparations can be explained by the presence of inhibitors in the latter which are destroyed during the processes of activation. When small quantities of fresh tobacco sap, or the sedimentable components from it, are added to activated virus there is no striking reduction

in infectivity, and when the virus has been quickly re-isolated from mixtures with fresh sap by ultracentrifugation in the cold, it has been recovered with its full infectivity.

Properties of activated and unactivated virus preparations

Unactivated preparations made by isolating the virus from fresh cold sap contain some sedimentable normal leaf protein. This can be removed by precipitation at pH 4, when, as already stated, it usually carries with it most of the infective part of the virus preparation. This protein denatures in a few days at room temperature in the presence of chloroform or in a longer period at 0°. The infectivity of the fluid is unaltered by this precipitation. If there is any activation, it is compensated by an equal removal of infective material on the precipitate. The coagulation of the normal protein is associated with the production of low molecular weight components, which can then be separated from the virus by a further ultracentrifugation. When this has been done, comparisons between such preparations and the more infective ones made from aged or frozen sap have revealed no significant or systematic differences in chemical and physical properties. When they are centrifuged at 20,000 r.p.m., both kinds of preparation separate into pellets and uncompact material, and with both the material in the pellet is, weight for weight, more infective. Thus, there are no gross differences in particle weights between the components occurring in the two kinds of preparation.

All preparations made by the methods described in this paper have had higher carbohydrate contents than those described in 1945. Then, 7-8.5% was the usual range, and this is what we now find for preparations that are sedimented again after lying for some weeks at 3°. The carbohydrate content of fresh preparations varies from 14 to 22%; the phosphorus content varies between 1.7 and 2.0%, as in our earlier preparations. Fresh preparations, therefore, do not contain all their carbohydrate in the form of a nucleic acid. We (Bawden & Pirie, 1938*a, b*) found extra carbohydrate associated with incompletely purified preparations of potato virus X and tomato bushy stunt virus, but it seemed most probable that this was simply mixed with these viruses. The fact that the carbohydrate in preparations of the Rothamsted tobacco necrosis virus becomes unsedimentable after a time suggests that, in fresh preparations, it is combined with the virus but that the linkage is labile. The breaking of this linkage may control the susceptibility of the virus to the inactivation produced by exposure to citrate, which is described in the accompanying paper (Bawden & Pirie, 1950). Sufficient material has not yet been obtained for a thorough study of the carbohydrate that occurs in the supernatant fluid when old virus preparations are centrifuged.

When examined in the electron microscope even preparations that have been fractionated by sedimentation at 20,000 r.p.m. contain particles of two sizes, one about 37 m μ and the other about 17 m μ in diameter; these are presumably the components previously described that give sedimentation constants of 285 and 49 (Ogston, 1942). The smaller particles appear to be spherical but the larger, after drying and metal shadowing, have an apparently mamillated

surface compatible with the idea that they are built up by the coalescence of smaller particles. Preparations that consist exclusively of the small particles can be made from the crystals that separate slowly from concentrated virus solutions in water, and the particles in them have a tendency to adhere regularly in sheets similar to those found by Wyckoff (1949) in the crystals themselves. These preparations are almost devoid of infectivity. Preparations consisting almost exclusively of the larger particles may also be of low infectivity. Comparisons have been made between quickly prepared unactivated preparations and aged activated preparations from the same batch of sap. The ratio of large to small particles in these may be the same. This is in agreement with the similar behaviour of the two types of preparation when fractionated ultracentrifugally. The fact that the more readily sedimenting fraction is the more infective naturally suggests that the larger particles are the vehicle of infectivity, but it is clear that not all of them carry infectivity and that the difference between an infective and a non-infective particle is not at present perceptible on an electron-microgram. We hope to deal more thoroughly with these relationships in a later paper.

Release of virus from the residues of infected leaves

Tomato bushy stunt and tobacco mosaic viruses can be extracted in large amounts from the fibrous residues remaining after infected leaves have been minced and squeezed to express the sap, either by fine grinding or by digesting the residues with enzymes from the crop of the snail (Bawden & Pirie, 1944, 1945*b*). These treatments also liberate, from leaves infected with the Rothamsted tobacco necrosis virus, material that reacts specifically with the virus antiserum. Fine grinding disperses normal leaf components into a form in which they are not readily separated from the virus preparations by ultracentrifugation, and the incubation necessary for liberation by the snail enzymes in itself largely destroys infectivity. Hence these methods are not suitable for studying the infectivity of the virus that remains in the leaf residues. Of the other methods we have tried, only simple washing will be discussed.

When fibre that has been minced twice and pressed by hand is suspended in water, additional material that reacts with virus antiserum is extracted. Extraction is slow and the precipitin titre of the extracts increases for periods of extraction up to 2 days. The total yield of serologically active material that can be extracted in this manner and recovered by sedimentation from the extracts is about half that prepared from the sap from the same sample of fibre. The yield is slightly increased by additional passages of the fibre through a mincer. The nature of the extracting fluid has little effect on the yield; thus the precipitin titres of extracts made with water, 20 g./l. or 2 g./l. sodium chloride, or with the supernatant fluid from ultracentrifuged infective sap, have not differed by a factor of two. The infectivities of extracts in the different fluids have, on the other hand, differed greatly. The interpretation of these differences is complicated by our ignorance of the extent to which differences arise from different abilities of the fluids to extract different amounts of the

infective particles, or from their differing abilities to affect infectivity after the virus has been extracted from the fibre. Successive extracts with water give material that shows a progressively smaller ratio of infectivity to serological activity. This phenomenon is illustrated by the experiment given in Table 7. The successive extracts were made with diminishing volumes of water,

Table 7. *Infectivity of material obtained from minced leaves in successive extracts*

Extract	Antigen content (precipitation end-point × volume in ml.)	Mean lesions per half-leaf at	
		1/1*	1/10*
Sap	1860	115	46
1st water	320	85	52
2nd water	200	44	14
3rd water	160	12	2
4th water	150	4	2
5th water	100	4	0.2

Infectivity tests were made with all fluids diluted to equal antigen contents, these were approximately 1/1 = 5 mg./l. and 1/10 = 0.5 mg./l.

so that the virus contents of the extracts should not differ too widely. The virus in each extract was concentrated by ultracentrifugation, the precipitin titres were determined and then infectivity tests were made at dilutions corresponding to equal antigen contents. It can be seen that, after the first extract, in which residual sap was present, the infectivity fell sharply.

This phenomenon again does not seem to be explicable by postulating that inhibitors of infectivity are extracted in increasing amounts in successive extracts, for when the virus from sap, from the first and from successive extracts, has been purified by repeated ultracentrifugations, the differences in infectivity have persisted unchanged. When salt solutions are used as extractants, the ratio of infectivity to serological activity in successive extracts differs less than with water, and using the supernatant fluid from ultracentrifuged infective sap, there may be no difference. This is shown in Table 8. This experiment was done with the fibrous residues from the leaves used for the

Table 8. *Relative infectivities of virus obtained in successive extracts from leaves of different ages*

Leaves	Mean lesions per half-leaf from virus in					
	Sap		Residue extracted with water		Residue extracted with supernatant fluid	
	1/1	1/10	1/1	1/10	1/1	1/10
Young, green	114	51	21	4	74	30
Intermediate	70	29	43	9	74	29
Old, yellow	126	45	122	31	146	60

Infectivity tests were made with all fluids diluted to equal antigen contents, these were approximately 1/1 = 5 mg./l. and 1/10 = 0.5 mg./l.

experiment given in Table 5, and the residues were extracted successively with water and the supernatant fluid. The virus in each extract was concentrated by ultracentrifugation, precipitin titres were determined and infectivity tests made at comparable antigen contents. The young and intermediate groups of leaves show clearly the failure of water to give extracts as infective as those subsequently obtained from the same fibre with clarified sap. There was no such phenomenon with the yellowest leaves, sap from which gave the greatest activation on ageing, and this suggests that the phenomenon may result from the failure of activation to occur in water, rather than from a differential extraction of already infective particles into the different ionic environments. This idea receives some further support from the fact that fibre from healthy plants does not preferentially adsorb infective particles from virus preparations whether in water or in sap.

DISCUSSION

Our results do not simplify knowledge of viruses; rather the reverse. They show that changes in infectivity may occur in the initial extract and in fluids that are usually regarded as bland; they seem inexplicable on the basis of a single unit particle in which changes can only cause loss of infectivity. Leaf sap, made by mincing fresh leaves and pressing lightly, is an environment in which this tobacco necrosis virus does not maintain its properties constant. In this respect it is not unique, for many others lose infectivity rapidly in sap, and tobacco mosaic virus, which retains its infectivity for long periods in sap, changes its physical state (Bawden & Pirie, 1945*b*). The tobacco necrosis virus is unusual in that, in addition to changes that decrease infectivity, others are demonstrable that increase it. Also unusual is the occurrence in infective sap of at least two kinds of particles differing in size, neither of which occurs in sap from healthy leaves. Turnip yellow mosaic virus may be analogous, for sap from infected plants contains two specific particles that differ in chemical constitution but have similar sizes (Markham & Smith, 1949). So many variables influence the activity of the Rothamsted tobacco necrosis virus that, even in freshly expressed sap, it may not necessarily be in the form in which it occurs in intact cells, nor may it have the activities it has there. Sap is not a physiological fluid and viruses are not exposed to it until leaves are minced. There is no *a priori* reason why it should be suitable for maintaining viruses with their original properties, and it may be that the properties of preparations of other viruses made without precautions designed to prevent changes are also not those of the viruses in their original habitats. This is not a plea for limiting work to unfractionated extracts; that would not solve the problem, because as soon as cells are damaged changes begin. These may be unavoidable, but an awareness of them should not lead to a vitalist defeatism about the possibility of gaining information about the intrinsic properties of viruses. It should simply lead to caution in attributing the properties of purified material to the virus in the cell.

Ultimately the components of the leaf that cause changes in the extracted virus may be removed without exposing the virus to them, or it may be

possible to infiltrate substances into intact leaves that will protect the virus from change. At present the best that can be done is to release the virus into sap at a low temperature and then isolate it as rapidly as possible. This obviously does not ensure that changes have been avoided, but there will most likely be less change and the virus will be more nearly in its original state than when prepared by more dilatory methods. If this assumption is justified, then a possible, although extreme, interpretation of the increased infectivity caused by ageing in sap, or by freezing sap, is that undamaged cells contain no virus particles capable of causing infection and that infectivity is a property acquired only when particles are liberated into a suitable medium. A similar change might occur without experimental intervention in necrotic and withered parts of leaves, but infectivity may not be a property of any particles in undamaged cells.

We have called the preparations of materials sedimented from infective sap, virus preparations, but it is uncertain which of the components of the preparations most warrants the name virus. There appear to be at least four substances in the preparations of sedimentable material we have made from extracts of infected leaves:

A. The crystallizable nucleoprotein that we have described earlier (Bawden & Pirie, 1945*a*). This appears to be the antigen specific to infected plants and it occurs in species as remotely related as tobacco, French bean and tulip when they are infected with the Rothamsted tobacco necrosis virus. It is the major constituent of most purified preparations but we have no evidence that it is ever infective. If it is, infectivity is a transient phase, for by the time homogeneous preparations are obtained infectivity is almost wholly gone.

B. The infective material that is made by freezing or ageing in sap; this sediments more rapidly than A.

C. The precursor of B, which is either less infective than B or is wholly non-infective.

D. Unstable normal leaf protein.

The only reason we have for postulating a relationship between A and B is that antisera made against non-infective preparations of A specifically neutralize the infectivity of preparations of the virus (Kassanis, 1943). Its occurrence in infected hosts of unrelated species, and its absence from plants showing identical symptoms caused by other tobacco necrosis viruses, suggest that it is a product specific to the activities of the Rothamsted virus and not simply a consequence of necrosis. It may be a stage in the development of virus particles, but this is not established and there is nothing to suggest whether it is more likely to be a stage in synthesis or degeneration. Antisera made against substance A precipitate infective preparations, but it is unknown whether this is because they combine with substance B. Our most infective preparations give lower precipitation end-points than do preparations of substance A, suggesting that precipitation may occur because of contamination with substance A. Pellets centrifuged from the most infective preparations contain some scattered crystals and electron-micrograms also show the presence of particles about 17 $m\mu$. in diameter mixed with larger ones. This is

evidence of contamination with substance A, but A and B may be serologically related and the lower precipitin end-points of infective preparations may merely be a result of the larger particle size of B.

The relationships between B and C are wholly unknown, except that differential centrifugation and electron microscopy suggest that they do not differ widely in size. An activation of the type found might occur from the addition, removal, or rearrangement of groups, and we have no observations to favour one possibility rather than another. Substance D is a contaminant of all freshly made preparations, but occurs especially in those made without delay from chilled sap. Only traces occur in preparations that have been frozen or have been sedimented again after lying for some weeks. The only connexion of which we are aware that it has with the virus is its ability, when fresh, to precipitate component B to a greater extent than A at pH 4.

Our results are most simply explained by the assumption that D is an irrelevant contaminant, that B and C are aggregates containing approximately the same number of particles physically comparable to A. The mechanism that brings about the transition between C and B is of great interest, but much valuable information will be lost if attention is exclusively directed towards infective components. At many stages in the development of symptoms the greatest part of the anomalous nucleoprotein of the leaf appears to be in non-infective states and any picture of the development of a virus disease must include them.

If phenomena similar to those described here are at all widespread, it is clearly dangerous to assume that any anomalous material that can be ultra-centrifuged from an infective extract, or any anomalous particle that can be seen in an electron-microgram, is an entity capable of infecting a new host. We have already described the separation of several different nucleoproteins from the sap of plants infected with tobacco mosaic virus and it seems probable that other viruses, if studied intensively, will be found to be associated with a comparable complex range of substances.

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REFERENCES

- BAWDEN, F. C. (1941). The serological reactions of viruses causing tobacco necrosis. *Brit. J. exp. Path.* **22**, 59.
- BAWDEN, F. C. & PIRIE, N. W. (1937). The isolation and some properties of liquid crystalline substances from solanaceous plants infected with three strains of tobacco mosaic virus. *Proc. roy. Soc. B*, **123**, 274.
- BAWDEN, F. C. & PIRIE, N. W. (1938*a*). Liquid crystalline preparations of potato virus X. *Brit. J. exp. Path.* **19**, 66.
- BAWDEN, F. C. & PIRIE, N. W. (1938*b*). Crystalline preparations of tomato bushy stunt virus. *Brit. J. exp. Path.* **19**, 251.
- BAWDEN, F. C. & PIRIE, N. W. (1942). A preliminary description of preparations of some of the viruses causing tobacco necrosis. *Brit. J. exp. Path.* **23**, 314.
- BAWDEN, F. C. & PIRIE, N. W. (1944). The liberation of virus, together with materials that inhibit its precipitation with antiserum, from the solid leaf residues of tomato plants suffering from bushy stunt. *Brit. J. exp. Path.* **25**, 68.

- BAWDEN, F. C. & PIRIE, N. W. (1945a). Further studies on the purification and properties of a virus causing tobacco necrosis. *Brit. J. exp. Path.* **26**, 277.
- BAWDEN, F. C. & PIRIE, N. W. (1945b). The separation and properties of tobacco mosaic virus in different states of aggregation. *Brit. J. exp. Path.* **26**, 294.
- BAWDEN, F. C. & PIRIE, N. W. (1950). Some effects of freezing in the leaf, and of citrate *in vitro*, on the infectivity of a tobacco necrosis virus. *J. gen. Microbiol.* **4**, 482.
- BAWDEN, F. C. & ROBERTS, F. M. (1947). The influence of light intensity on the susceptibility of plants to certain viruses. *Ann. appl. Biol.* **34**, 286.
- BAWDEN, F. C. & ROBERTS, F. M. (1948). Photosynthesis and predisposition of plants to infection with certain viruses. *Ann. appl. Biol.* **35**, 418.
- BOYLE, R. (1688). *New experiments and observations touching cold.*
- GLAUBER, J. R. (1658). *Miraculi Mundi Continuatio*, p. 215. *Opera Chymica*, Franckfurt.
- KASSANIS, B. (1943). Neutralization of some plant viruses by rabbit sera. *Brit. J. exp. Path.* **24**, 152.
- MARKHAM, R. & SMITH, K. M. (1949). Studies on the virus of turnip yellow mosaic. *Parasitology*, **39**, 330.
- MASKET, A. V. (1941). A quantity type rotor for the ultracentrifuge. *Rev. sci. Instrum.* **12**, 277.
- MELLANBY, J. (1908). Diphtheria antitoxin. *Proc. roy. Soc. B*, **80**, 399.
- OGSTON, A. G. (1942). Examination in the ultracentrifuge. *Brit. J. exp. Path.* **23**, 328.
- PALMER, A. H. (1934). The preparation of a crystalline globulin from the albumin fraction of cow's milk. *J. biol. Chem.* **104**, 359.
- PATERSON, J. S., PIRIE, N. W. & STABLEFORTH, A. W. (1947). Protective antigens isolated from *Br. abortus*. *Brit. J. exp. Path.* **28**, 223.
- PIRIE, N. W. (1950). The isolation, from normal tobacco leaves, of nucleoprotein with some similarity to plant viruses. *Biochem. J.* (In the Press.)
- SMITH, K. M. & BALD, J. G. (1935). A description of a necrotic virus disease affecting tobacco and other plants. *Parasitology*, **27**, 231.
- VEEB, G. DE (1596). *The third voyage Northward to the Kingdoms of Cathaia, and China, in Anno 1596*. Quoted from (1906) *Purchas his Pilgrimes*, 13, 91. Glasgow: MacLehose and Sons.
- WYCKOFF, R. W. G. (1949). *Electron microscopy*. New York: Interscience Publishers.

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