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FURTHER STUDIES ON THE PURIFICATION AND PROPERTIES OF A VIRUS CAUSING TOBACCO NECROSIS.

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In the course of a preliminary survey of some of the viruses causing tobacco necrosis, we (Bawden and Pirie, 1942) found that one culture was sharply differentiated from the others by its small sedimentation constant, its failure to crystallize when precipitated with ammonium sulphate, and by the ease with which it lost infectivity. This Rothamsted culture was derived by bulk transfer from one of the sources of virus distinguished serologically by Bawden (1941), and it is serologically related to one of the components of the mixture of viruses purified by Pirie, Smith, Spooner and McClement (1938). We have now studied a derivative of this culture in more detail and can give a fuller account of its properties. Preparations have still not crystallized when salted out with ammonium sulphate, but when sufficiently concentrated they crystallize readily in the absence of salts. Because of the ease with which this tobacco necrosis virus loses infectivity, the relationship between the crystalline products and the virus as it occurs in the plant is even more uncertain than with the other viruses that we have studied. Using methods that largely destroy infectivity, fully crystalline and apparently homogeneous products are easily prepared. Such products seem to be antigenically identical with infective virus, and antisera prepared against them specifically neutralize infectivity (Kassanis, 1943). Loss of infectivity without loss of serological activity can also be induced with other crystalline viruses that we have studied, and such non-infective preparations crystallize in the same manner as infective preparations. Thus, by analogy with these other viruses, it is likely that infective virus of this Rothamsted culture would crystallize in the same manner as our largely inactivated products, but we do not claim that this is definitely established.

MATERIALS AND METHODS.

The virus used in this work has come from single lesion isolates made from the original Rothamsted culture. Six local lesions were taken, ground up separately and inoculated to healthy plants; the sap from these plants was then tested against a range of antisera prepared against different cultures of tobacco necrosis viruses. Four of the isolates reacted only with antiserum to the Rothamsted culture, and so were

presumed to be the same as the main component present in this culture. One of these four was used as the source for the work now to be described. The results of the tests on the other two isolates showed that our old culture contained a mixture of viruses, and that single local lesions may contain more than one virus ; one reacted with antisera to both the Rothamsted and the potato culture, and the other only with antiserum to the potato culture. Natural infections with tobacco necrosis viruses occur fairly commonly, especially in the winter months ; to reduce the chances of perpetuating contaminants, fresh isolations were made at intervals, from single local lesions, of virus which reacted only with antiserum to the Rothamsted culture.

There is no proof that we have worked continuously with the same virus strain, but there is no evidence to the contrary, and at least all are serologically related. The only suggestion we have that our isolates may not be identical with the main component of the old Rothamsted culture is that purified preparations of the isolates have consistently given lower precipitin titres.

The virus has been propagated in tobacco, *Nicotiana tabacum*, var. White Burley, and French bean, *Phaseolus vulgaris*, var. Canadian Wonder. The yield depends on the number of local lesions produced, and to increase this celite or carborundum (400 mesh) was always incorporated in the inocula. Leaves were picked from 5 to 7 days after inoculation, when the lesions were well developed but before the leaves had become dry and brittle.

The distribution of the tobacco necrosis viruses in the leaf tissues is similar to that of tomato bushy stunt virus (Bawden and Pirie, 1944). When the leaves are passed through a meat mincer, about one-half of the virus is extracted in the sap that can be expressed, but the remainder remains attached to the fibrous residues. This can be liberated either by digestion with commercial trypsin, or by grinding the fibre finely in a triple-roller mill and then extracting with water. Such mill extracts are deep green, and contain some virus combined with the chromoprotein in such a manner that it is not precipitated by virus antiserum. The chromoprotein is easily denatured by freezing in conditions that do not inactivate the virus. Such frozen mill extracts are pale yellow, and afford the most favourable starting material for purification.

For sedimenting the virus we have used an air-driven ultracentrifuge of the type described by Masket (1941) ; 40 minutes at 40,000 r.p.m. (90,000 g.) compacts all the virus in the form of a coherent, rigid pellet, which remains in the tube when the supernatant fluid is decanted. Infectivity and serological tests were made by methods previously described (Bawden and Pirie, 1942). All the antisera used were prepared by Dr. A. Kleczkowski.

PURIFICATION OF THE VIRUS.

Rather different methods of purification are needed with different host plants and at different times of the year. Healthy French beans contain a macromolecular protein which sediments and precipitates with ammonium sulphate along with the virus. This is presumably similar to that found by Loring, Osborn and Wyckoff (1938) in other legumes. It precipitates at pH 4·5, and low-speed centrifugation in these conditions is a necessary step in preparing the virus from beans. This leads to some loss of virus on the precipitate, and to some reduction in the infectivity of that remaining in the supernatant fluid. There is less of this interfering protein in the trifoliate leaves than in the first formed bean leaves, and there is less in the mill extract than in the sap. The ratio of virus to other materials in both tobacco and bean extracts is greater in the dull winter months than in better growing conditions, so that less fractionation is needed with preparations made during the winter.

The leaves are minced, as much sap as possible is squeezed out by hand through madapollam and the residue is minced again. If the leaves are so dry that the weight of sap is less than half the original weight of the leaves, the mince is extracted with a volume of water equal to that of the sap. The fibre is then passed through the triple-roller mill, and the resulting smooth paste is thoroughly mixed with four times its weight of water; after a few minutes' soaking the fluid is expressed. Additional virus can be obtained by a second milling and extraction of the fibre. The sap and extracts are mixed, frozen for 10 to 20 hours at — 8° C. and then thawed. After centrifuging, the virus is precipitated from the supernatant fluid by the addition of 280 g. of ammonium sulphate to each litre. The mixture is centrifuged as soon as the ammonium sulphate is dissolved, for a few hours' exposure to the strong salt solution reduces infectivity. This inactivation cannot be avoided by working at low temperatures, for cooling greatly delays the precipitation and so increases the necessary time of exposure. The precipitate is packed tightly by centrifuging for 45 minutes at 3000 r.p.m.; it is drained well before being suspended in a volume of water equal to one-tenth the volume of the original extracts. Any insoluble material is removed by centrifuging at 10,000 r.p.m. If necessary, the virus in the supernatant fluid can be further fractionated and concentrated by another precipitation with ammonium sulphate, but usually it is now in a suitable state for purification by high-speed centrifugation, and it is centrifuged for 40 minutes at 40,000 r.p.m.

If the original extract is rich in virus, so that the ratio of virus to other sedimentable components is high, the pellets look fibrous to the naked eye and, when examined under the microscope, have the serrated crystalline edges shown in Fig. 1. Fig. 2 is a photomicrograph of the middle of such a pellet. The pellets are suspended in water and left for 1 to 2 hours at 0° C. for the virus to dissolve, when they are centrifuged at 10,000 r.p.m. to remove any insoluble material. The supernatant fluid should now be pale yellow, and when ultracentrifuged again should give pellets which appear to be wholly crystalline and which dissolve completely in water after an hour at 0° C. If there is an insoluble residue, it is removed and the ultracentrifugation is repeated until the pellets dissolve completely.

When small volumes of leaf extracts are handled, more infective products can be made if precipitation with ammonium sulphate is omitted, and fractionation of the frozen leaf extracts is done solely by ultracentrifugation. The pellets that separate from either frozen sap or mill extract from severely affected leaves grown in the winter are often crystalline on the first sedimentation, but sometimes they are brown and apparently amorphous. When resuspended in a volume of water equal to one-tenth of the original volume, much of the pellet does not redissolve. The insoluble material is removed by centrifuging at 10,000 r.p.m., and the supernatant fluid is again ultracentrifuged. The pellets are now fully crystalline and dissolve completely in water. As is shown below, however, these properties are not criteria of homogeneity, because preparations can be fractionated further by precipitating part of the material at pH 4·5 and by making use of the different rates at which different components dissolve.

The infectivity of preparations at this stage is variable, but if the purification has been carried through speedily, 1 ml. of inoculum containing from 10^{-7} to 10^{-9} g. of protein is usually sufficient to produce infection in beans, and at a dilution of 10^{-5} between 50 and 100 lesions per leaf are usual. With its antiserum the preparations give a dense granular precipitate and precipitin titres of from 1/200,000 to 1/300,000. It is necessary to check the precipitin tests with control tubes containing saline instead of antiserum, for if the preparations have not been fractionated with acid, especially if made from infected beans, they are liable to precipitate unspecifically during incubation at 50° C.

Yields of Virus.

The susceptibility of both bean and tobacco plants to tobacco necrosis varies greatly with the season. From November to February plants are extremely susceptible, and inoculated leaves become completely necrotic without the addition of any abrasive to the inoculum. Sap and mill extracts from such leaves give precipitin titres up to 1 in 64 with virus antiserum ; at this time the fragile leaves contain only small quantities of other proteins that interfere with purification, and yields of from 100 to 200 mg. of virus are regularly obtained from a litre of extract. In the spring and autumn large numbers of local lesions are obtained only when abrasive is incorporated in the inoculum. However, the virus content of extracts from leaves well covered by lesions is smaller and precipitin titres in excess of 1 in 8 are rare. The better growing conditions also lead to the production of large quantities of soluble and sedimentable proteins, so that purification is more difficult and yields rarely exceed 10 mg. of virus per litre. During the summer susceptibility is even less, and satisfactory preparations have not been made from plants infected between June and September. No great differences have been found between the yields of virus obtained from bean and tobacco plants grown under comparable conditions and bearing comparable numbers of lesions, but tobacco leaves form the better material for use as inoculum. This is probably because bean leaves contain some inhibitor of infectivity, as more lesions are often obtained from bean sap diluted 1 in 50 with water than from undiluted sap.

If the pH of minced bean leaves is raised, by the addition of sodium hydroxide, from its normal value of 5·7–6·0 to 6·5–7·0, the infectivity of extracts is usually increased appreciably. The yield of purified virus, however, is greatly decreased, presumably because much virus is lost during the removal of the other materials extracted from the leaves by this treatment. The quantity of material that sediments on ultracentrifugation but does not resuspend is doubled by such extraction, and the amount of normal component that sediments and resuspends but is insoluble at pH 4·5 may be increased twenty-fold.

PROPERTIES OF THE VIRUS.

Inactivation.

Perhaps the most characteristic property of this tobacco necrosis virus is the ease with which it loses infectivity without losing its serological activity. In necrotic leaves, or in expressed sap, loss of infectivity is rapid ; within a few weeks at 0° C. only a few lesions may be obtained from material that when fresh was giving hundreds of lesions per leaf. Increasing the temperature increases the rate of inactivation, and at temperatures above 60° C. loss of infectivity with sap or with purified preparations is almost complete within a few minutes, though for loss of serological activity 10 minutes' heating at 90° is necessary. The loss of infectivity is least rapid in salt-free, neutral solutions of the purified virus. We have not studied in any detail the many treatments that lead to loss of infectivity with this culture of tobacco necrosis virus, but we have made some experiments on conditions that do not affect other tobacco necrosis viruses or tomato bushy stunt virus. The most striking of these are exposure to acid and salts. Exposure for 10–20 hours to values between pH 4 and 4·5 leads to a reduction of over 90 per cent. in the infectivity, and we have used this acid inactivation as a criterion to determine whether our products have become contaminated with tobacco necrosis viruses other than the Rothamsted culture. Table I shows the effect of exposure to M/100 acetate buffer at pH 4·4 and 4·1 on three different preparations ; short exposures have little effect, so that by working quickly, the acid-precipitable material in partially purified preparations can be removed without reducing

TABLE I.—*The Effect of Exposure to Slightly Acid Conditions on Infectivity.*

Treatment at 18° C.	Infectivity. Average number of lesions per leaf at 1 : 20,000.	1 : 200,000.
Control	150	51
1 hour at pH 4·4	95	9
17 hours at pH 4·4	14	4
Control	127	41
24 hours at pH 4·1	9	1
Control	123	23
24 hours at pH 4·1	1	0

the infectivity greatly. Loss of infectivity as a result of exposure to neutral salt solutions is less reproducible, for in different experiments exposure to the same conditions has given varying results. We have not compared a large range of different salts, but sodium phosphate causes more rapid inactivation than either sodium chloride or ammonium sulphate. Table II shows the effect of exposing the purified virus to phosphate and sodium chloride for various lengths of time.

TABLE II.—*The Effect of Exposure to Salts on Infectivity.*

Treatment at 18° C.	Infectivity. Average number of lesions per leaf at 1 : 20,000.	1 : 200,000.
Control	162	53
0·065 M phosphate for 1 minute	186	63
,, ,, 210 minutes	71	9
,, ,, 73 hours	6	1
0·26 M NaCl for 73 hours	88	29

Even after ageing for some months, or after prolonged exposure to acid or phosphate, purified preparations still retain 1/1000 or 1/10,000 of their original infectivity. When used as inoculum at 1 mg./ml. they may give an average of three or four lesions per bean leaf. We have on several occasions propagated virus from such lesions to see whether this residual infective virus was a contaminant, but it has always given us preparations indistinguishable from the main Rothamsted culture.

Crystallization.

Preparations of the Rothamsted culture made by the methods described give colourless solutions that are rather clearer than similar solutions of other tobacco necrosis viruses or tomato bushy stunt virus. When centrifuged at 40,000 r.p.m. for one hour in the pH range 5–7 all the virus is compacted into a pellet that is apparently uniform and wholly crystalline. Outside this pH range the pellet is incompletely crystalline, and all the virus is not compacted into the pellet, but some remains in a dense layer of fluid overlying the pellet. It seems that the virus has only a limited solubility in water, for the simplest interpretation of the crystallinity of the pellets is that the concentration of the dense layer of independently sedimenting particles, which is the first result of ultracentrifuging, exceeds this solubility so that the virus

crystallizes as it collects. When partially purified preparations are ultracentrifuged the contaminated pellets are incompletely crystalline, and crystals can often be seen to grow after the tubes are removed from the rotor.

All the other viruses we have studied have given on ultracentrifugation clear jellies, either isotropic or birefringent, which disperse in a few minutes when stirred with water. The crystalline pellets from the Rothamsted culture may remain incompletely dissolved in water at pH 5·5–6·5 for some hours at room temperature. At 0° C. they dissolve more rapidly than at room temperature, and there is more rapid solution in the presence of traces of salt or if the pH is raised to 7·5. Fresh preparations are demonstrably inhomogeneous, for pellets that look fully crystalline contain components that dissolve at different rates and have different infectivities. This can be shown by suspending the crystalline pellet in water, and centrifuging immediately at 3,000 r.p.m. to deposit the undissolved fraction. When the two fractions are compared they give the same serological titre, but the more quickly dissolving fraction has the greater infectivity (Table III). The results given in the accompanying paper (Ogston, 1945)

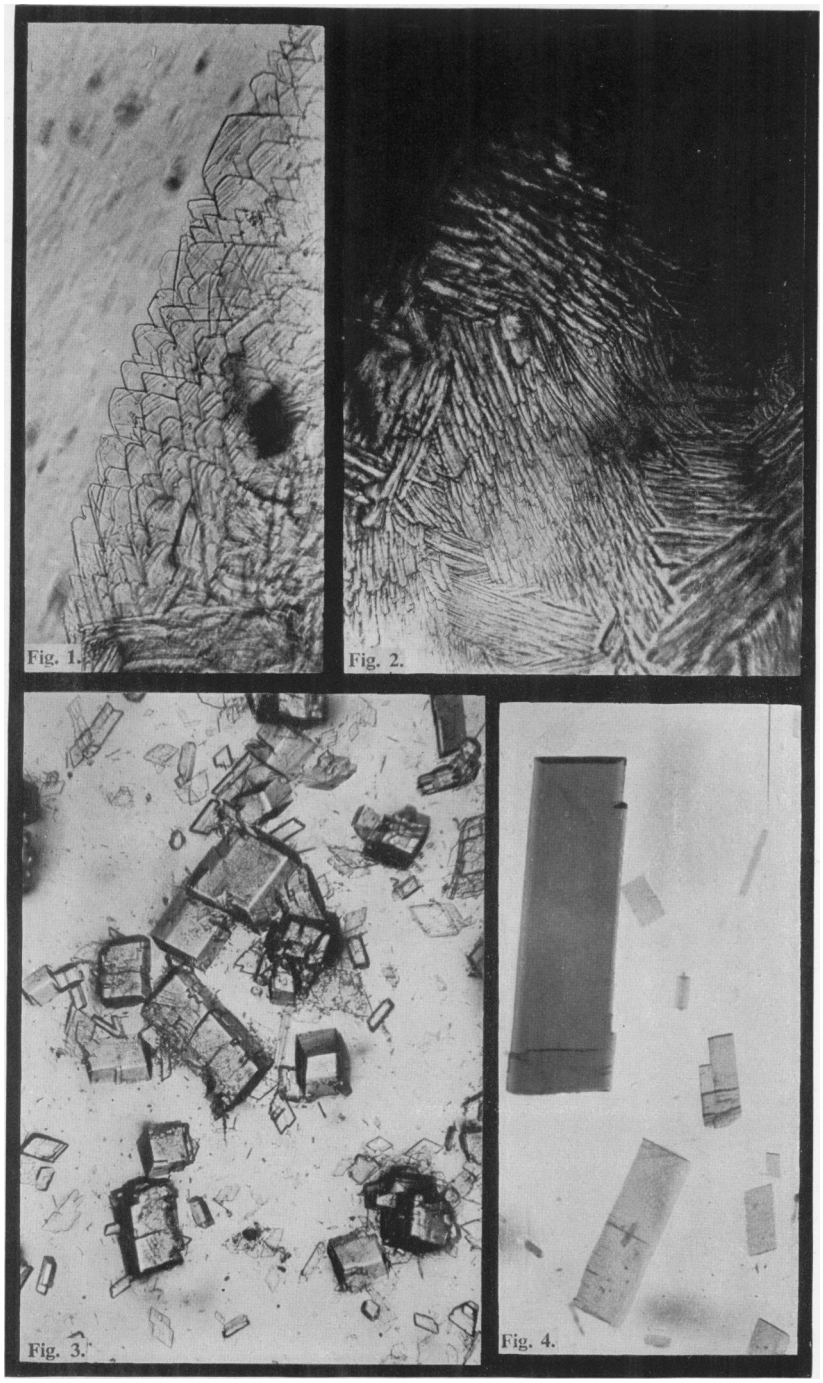
TABLE III.—*Infectivity of Successive Extracts of Crystalline Pellets.*

Extract.	Weight. (mg.)	Average lesions per leaf at 10^{-4}.	Average lesions per leaf at 10^{-5}.
First	11	430	230
Second	5·3	240	63
Third	1·9	89	17
Final	0·3	30	7
First	5·5	170	87
Final	1·7	84	12
First	10	188	34
Final	3	92	7

The results of experiments with three different preparations are given. Four successive extracts of the crystalline pellet were made with the first preparation; the first extract was made by stirring the pellet with water and centrifuging immediately at 3000 r.p.m.; the second and third were each extracted 30 minutes before centrifuging, and the final extract was left for 24 hours. Only two extracts were made of the second and third preparations; the first was made by stirring the pellet in water and centrifuging immediately, and the final was left for 24 hours.

show that both fractions are centrifugally homogeneous, and have the same sedimentation constant. With fresh preparations the rapidly dissolving fraction is the main component, though this itself is not homogeneous, for if it is ultracentrifuged it can usually again be fractionated by washing the crystalline pellet. Parallel with the fall in infectivity as a result of ageing or exposure to pH values between 4 and 5, the proportion of the pellet that dissolves rapidly also falls, and in preparations that are almost non-infective little or nothing goes into solution during such a quick extraction.

The decrease in solubility as a result of ageing probably explains the second type of crystallization given by the Rothamsted culture, for this occurs in stored solutions made by dissolving the crystalline pellets. If such solutions are more concentrated than 10 g./l., after some days or weeks at 0° C. they deposit crystals on the walls of the vessel. Fig. 3 shows their appearance soon after crystallization has begun, but growth continues for several months, and laths up to $1 \times 2 \times 6$ mm. have been produced (Fig. 4). By this time the preparation will have lost substantially all its infectivity. Crowfoot and Schmidt (1945) state that these crystals are of two kinds,



Crystals of the Rothamsted Culture of Tobacco Necrosis Virus.

FIG. 1.—The serrated edge of a crystalline pellet produced by ultracentrifugation. ×45.
FIG. 2.—Centre of the pellet shown in fig. 1. × 45.
FIG. 3.—Crystals separating from a salt-free solution after standing for some days. × 45.
FIG. 4.—Large laths produced after crystal growth has continued for three months. × 9.

thick triclinic prisms and thin hexagonal or pseudo-hexagonal plates, and they have made X-ray crystallographic measurements on them. The part of a preparation that separates as crystals does not differ in any of the properties we have studied from that remaining in solution. Solutions of the crystals and their mother liquors give the same serological titre. When ultracentrifuged, they both give crystalline pellets indistinguishable in appearance from those given by freshly made preparations, though they dissolve much more slowly than these.

Repeated attempts have been made to get crystals by slow precipitation with ammonium sulphate, using the technique that was successful with the other cultures of tobacco necrosis virus, but all have failed. The potato culture resembles the Rothamsted culture in that it also crystallizes from salt-free solutions (Bawden and Pirie, 1942). If the lozenge-shaped crystals produced by precipitating the potato culture with ammonium sulphate are dissolved to give a solution containing more than 5 g. per l., crystals begin to separate after some time at pH 4·5, which resemble those produced by ammonium sulphate precipitation. Crystallization of the potato culture, however, is not associated with loss of infectivity, and there is no difference between the infectivity of the fraction of a preparation that crystallizes in this way and that of the fraction remaining in solution.

When adjusted to pH 4·5 most preparations have given a precipitate, which with those made from infected beans has sometimes been as much as 30 per cent. of the whole preparation. We do not regard precipitability from dilute salt solutions at pH 4·5 as a property of the virus, however, because some preparations made from tobacco leaves, which were as infective as any we have handled, were completely soluble at this pH. If the precipitate and supernatant fluid are separated and neutralized quickly and their serological activities and infectivities compared, the precipitated material gives a smaller serological titre than the soluble part, though it often is more infective. Sometimes indeed nearly the whole of the infectivity is associated with the precipitate, though more than two-thirds of the material precipitating with virus antiserum remains soluble. Similar precipitates were encountered when preparations of tobacco cultures I and II (Bawden and Pirie, 1942) were brought to pH 4·5, and we suggested that the virus was being absorbed during the precipitation of some other leaf component. To explain the behaviour of the Rothamsted culture on this basis involves the assumption that infective virus particles are more readily absorbed by such leaf components than non-infective particles. This seems probable, but there is no positive evidence for it ; when sedimentable protein from healthy bean leaves was precipitated at pH 4·5 in the presence of a virus preparation which was soluble at this pH, there was no such preferential absorption.

Chemical Composition.

The carbohydrate and phosphorus contents of purified preparations of the Rothamsted culture fall within the ranges 7·0–8·5 per cent and 1·7–2·0 per cent respectively, i.e. similar to those described for other viruses causing tobacco necrosis (Pirie Smith, Spooner and McClement, 1938 ; Bawden and Pirie, 1942). No consistent differences have been found between the fractions with different rates of solubility into which a preparation can be separated, or between freshly made, infective preparations and old, non-infective preparations. The ratio of carbohydrate to phosphorus is that characteristic of a nucleic acid, and material with the physical properties of nucleic acid can be separated from the protein by exposure to dilute alkali. After precipitating the denatured protein with 5 per cent ammonium sulphate solution, nucleic acid can be precipitated from the fluid by the addition of strong acid. Colour

reactions show that the nucleic acid contains a pentose. The Rothamsted culture of tobacco necrosis virus, like other plant viruses previously studied, is, therefore, a nucleoprotein of the ribose type, but we have not demonstrated that the sugar is actually ribose.

DISCUSSION.

In this paper the crystalline product we have isolated from plants infected with the Rothamsted culture of tobacco necrosis virus has been referred to as virus. This has been done mainly because of analogies with other viruses with which we have worked, for there is, in fact, no positive evidence that any of the properties of our product are those of the virus itself. Indeed, there is positive evidence that our infective preparations are heterogeneous, and no conclusive evidence that infective virus can be obtained in crystalline form. However, the simplest interpretation of our results is that the virus particles readily lose infectivity without suffering gross changes, and that in the infective and non-infective states they have the same size, crystal forms and serological activity. Other viruses can be made to undergo such losses of infectivity and, in the absence of contrary evidence, it is reasonable to think that treatments which produce loss of infectivity in the Rothamsted culture, but which have no effect on other tobacco necrosis viruses or on tomato bushy stunt virus, cause this type of inactivation. On this assumption, our purified preparations would invariably contain a mixture of infective and non-infective particles, and the variation in infectivity of different preparations is simply a reflection of the different proportions in which infective virus and the modified, non-infective derivative are present.

There is one important difference between the behaviour of the Rothamsted culture and the other viruses. When crystals of partially inactivated preparations of tomato bushy stunt virus or the potato culture of tobacco necrosis virus are dissolved, there is no difference between the infectivity of the material that dissolves rapidly and that which dissolves slowly, whereas the results given in this paper show that when crystalline ultracentrifuge pellets of the Rothamsted culture are suspended in water the infective material dissolves more readily than the non-infective. Thus if these pellets are wholly crystalline, as their appearance suggests, then it seems that loss of infectivity has no effect on crystal form or serological activity, but reduces the rate of solution. The appearance of crystallinity, however, is not trustworthy as an index of homogeneity, for if purified preparations of the potato or tobacco II cultures of tobacco necrosis are added to purified preparations of the Rothamsted culture in the ratio of 1 to 4 by weight, ultracentrifugation gives an apparently homogeneous and wholly crystalline pellet, although neither of these other viruses crystallizes in these conditions, and one-fifth of the " crystalline " pellet is a non-crystalline, invisible gum. An obvious alternative explanation of our results, therefore, is that the crystalline material is wholly non-infective, derived from virus by changes insufficient to affect most properties, and that infective virus is not crystallizable, but occurs in the pellets around the crystals as an invisible gum, which dissolves rapidly when dispersed in water. To explain the further crystallization of such solutions when again ultracentrifuged, it is necessary to postulate incomplete fractionation or subsequent inactivation leading to crystallizable material.

Loss of infectivity during the course of purification is not a necessary preliminary to crystallization. In the winter months, when conditions are such that frozen leaf extracts contain little sedimentable material except virus, crystalline pellets are often obtained at the first centrifugation, and if these are suspended in their own supernatant fluids they are as infective as the original extracts. Thus if infective virus does

not itself crystallize, then it seems that freshly-expressed extracts from infected plants must already contain much non-infective virus.

A third interpretation of our results is that the virus is only a minor component of our purified preparations, and that our crystalline material is merely a specific disease product. A similar suggestion could be made for all the other purified virus preparations, but it necessitates the introduction of extra hypotheses with no obvious advantage. With the Rothamsted culture it would also be necessary to postulate that the rate of solution of the specific disease product alters as the virus loses its infectivity. The fewest hypotheses are needed if we assume that the virus readily changes into a non-infective derivative without altering any of its other properties that have been tested except rate of solution. On this assumption, this plant virus seems to have smaller particles than any other previously studied. The X-ray crystallographic measurements suggest that the particles are spherical and have a minimum weight equivalent to a molecular weight of 1,600,000 (Crowfoot and Schmidt, 1945), which agrees well with the figure of 1,850,000 calculated from the sedimentation constant, assuming that the particles are spherical, unsolvated and obey Stokes' law.

SUMMARY.

A nucleoprotein that is not present in the leaves of healthy plants has been isolated from bean and tobacco leaves infected with the Rothamsted culture of tobacco necrosis virus. This has not crystallized when precipitated with salt, but it crystallizes slowly from concentrated salt-free solutions or during sedimentation by ultra-centrifugation. It has a sedimentation constant of 49S, smaller than that of other preparations of plant viruses previously studied. The Rothamsted culture of tobacco necrosis virus readily loses infectivity, and the relationship between the crystallizable protein and the virus is uncertain. It is most likely that much of the protein is a non-infective derivative of the virus having many physical, chemical and serological properties in common with it.

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