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A PRELIMINARY DESCRIPTION OF PREPARATIONS OF SOME OF THE VIRUSES CAUSING TOBACCO NECROSIS.

F. C. BAWDEN AND N. W. PIRIE.

From Rothamsted Experimental Station, Harpenden, Herts.

WITH AN ADDENDUM ON

EXAMINATION IN THE ULTRACENTRIFUGE.

A. G. OGSTON.

From the Department of Biochemistry, Oxford.

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TOBACCO necrosis was named and described by Smith and Bald (1935); they showed that it differed in two main ways from any previously recognized plant virus disease. Firstly, no host was found in which systemic infection occurred, and secondly, the symptoms on naturally infected tobacco plants differed from those on plants inoculated experimentally. Symptoms appeared only on the lowest leaves of naturally infected plants and consisted of a necrosis spreading from and along the veins, while the upper leaves looked healthy and were virus-free. Nevertheless, these leaves were not immune, for when rubbed with infective sap they developed lesions. The lesions were restricted to the leaves actually rubbed and consisted of discrete necrotic rings or spots. Plants other than tobacco, such as cowpea (*Vigna sinensis*) and French bean (*Phaseolus vulgaris*), were also found to be susceptible and to give necrotic local lesions when inoculated. Smith (1937) found that the roots of tobacco and of some other plants were often infected, although neither the roots nor the leaves showed any symptoms; extracts of the roots of such apparently healthy plants produced the typical necrotic lesions when rubbed on to leaves of bean or tobacco.

Pirie *et al.* (1938) purified Smith's culture of tobacco necrosis virus with results different from any obtained with other viruses. Their end-product invariably consisted of two fractions, one of which crystallized in the form of thin plates, whereas the other was amorphous. The crystalline and amorphous fractions gave different sedimentation constants. Bawden (1941) showed that the symptoms typical of tobacco necrosis could be caused by serologically unrelated viruses. He purified two cultures of serologically unrelated tobacco necrosis viruses and got no crystals, but showed that the culture used by Pirie *et al.* (1938) contained both of these, and suggested that the crystalline

fraction of their products was yet another virus serologically unrelated to the amorphous ones. We have now confirmed this suggestion. We have purified tobacco necrosis viruses from other sources and obtained crystals, some in the form of flat plates and others in forms previously undescribed. This paper consists largely of a description of the preparation of these crystals and of the serological reactions of the different viruses.

MATERIALS AND METHODS.

Tobacco necrosis viruses from four different sources have been used. The first was derived from a single local lesion on a tobacco leaf, produced by rubbing with an extract from the roots of an apparently healthy potato plant. The second came from the roots of an apparently healthy tobacco plant. The third source was a single local lesion on bean, produced by using as inoculum a dried, necrotic bean leaf supplied by Dr. W. C. Price, of Princeton, U.S.A. The fourth was a direct descendant by bulk culture from that previously described as the Rothamsted culture (Bawden, 1941). These four cultures will be referred to as the potato, tobacco, Princeton and Rothamsted cultures respectively.

The first and third were derived from single lesions ; it is therefore reasonable, in the absence of any evidence to the contrary, to assume that they consist of only one strain of virus.

It became obvious, after several separate preparations had been carried through, that the second culture was a mixture. Bean leaves were therefore rubbed with diluted infective sap and six discrete lesions were isolated and used as inocula on six separate bean leaves. Sap from these leaves gave enough inoculum for preparations from tobacco plants. These isolates are called tobacco I to VI. The Rothamsted culture, being a bulk culture, may well be a mixture, but we have made no attempt to prove this. This set of names has arisen purely as a matter of convenience during the investigation, and each name refers only to the origin of the culture and has no systematic connotation.

Six antisera have been used. Two were prepared by Dr. E. T. C. Spooner in 1938 ; one of these was the serum used in the work of Pirie *et al.* (1938), and the other was prepared against the crystalline fraction of their product, but had not been tested before. The other four were prepared by Dr. A. Kleczkowski against the Rothamsted and potato cultures and against two of the single lesion isolates from the tobacco culture. All the virus preparations were good antigens, and antisera precipitating at dilutions greater than 1 in 200 were made by two injections of 0.5 mg. of purified virus into rabbits. The tests were made as previously described (Bawden, 1941).

We have tried many methods of purifying these tobacco necrosis viruses, but have found none that is uniformly satisfactory. Except for the Rothamsted culture, which is much less resistant to the purification processes than the others, the following method has given the best results, although sometimes only one-quarter of the virus present in the original sap is recovered in the final product. No doubt better methods can be devised using a quantity high-speed centrifuge, but we are not yet in a position to use this method.

Leaves are picked from 6-8 days after inoculation, when the lesions are

well developed, put through a domestic meat mincer, and the mince frozen solid. This freezing is not essential, but it tends to increase the yield and gives a product that is easy to handle. After thawing, the sap is expressed by hand through thin, closely woven cloth, such as madapollam, and the leaf residue is mixed with a 4 per cent. solution of Na_2HPO_4 . If tobacco leaves are used 250 ml. are added for each litre of sap, but with bean leaves it is well to use 360 ml. Phosphate precipitates much of the normal leaf protein and calcium ions, and the partial removal of these at this stage lessens the loss of virus by adsorption that their removal at a later stage entails. The success of the later alcohol precipitation does, however, depend on the presence of some calcium, for no virus can be separated by this method from sap to which an excess of potassium oxalate has been added. The precipitate of calcium phosphate has also the virtue of adsorbing some coloured contaminants that are otherwise difficult to remove from the preparation. The residue is again minced, the liquid is expressed, mixed with the sap, and half a volume of 90 per cent. alcohol is added with continuous, thorough stirring. The green coagulum is centrifuged off and discarded. Approximately 2 N HCl is added to the fluid to bring the pH to 4.5 or 4, and this protein precipitate is also centrifuged off and discarded. Two litres of 95 per cent. alcohol are added to each litre of centrifugate and the mixture is stirred occasionally for an hour; after lying for another hour, as much of the fluid as possible is removed by decantation. The precipitate contains the virus and is packed tightly by centrifuging at 3500 r.p.m. for half an hour. It is extracted with a 3 per cent. solution of ammonium sulphate in 0.5 per cent. acetic acid; the calcium salts, of which the precipitate is largely composed, are converted into hydrated calcium sulphate and the virus goes into solution. Three extractions are generally adequate if the mixture is left for about an hour during each extraction before centrifuging, and if the combined extracts have about one-tenth the volume of the original sap. Saturated ammonium sulphate solution is added to the clear brown extract at the rate of 30 to 60 ml. per 100 ml. The smaller volume is sufficient for tobacco VI virus, but the large volume is safer with the others. In general there is no immediate precipitate, for most of the normal leaf protein has been removed in the earlier stages. Even after 5 hours the precipitate may be barely visible; after 5 to 24 hours at room temperature the fluid is centrifuged for at least 45 minutes at 3500 r.p.m. If the removal of protein at the earlier stages has been satisfactory, a litre of sap will give 1 to 2 ml. of clear and nearly colourless precipitate. This is extracted two or three times with water so that the final volume of extract is about 1/100 of that of the sap. The combined extract is colourless and faintly opalescent, and should show no anisotropy of flow when shaken between crossed polarizers. The insoluble residue is either colourless and of a heavy, coarsely crystalline texture (presumably calcium sulphate) or is darkly coloured. If it is pale and settles out of solution slowly after stirring, it probably still contains virus. This can be recovered by extracting for 4-5 hours at 0°C. with 2 per cent. sodium chloride solution. The slowness with which crystalline virus precipitates sometimes dissolve will be commented on later.

With all the cultures this method usually leads to a loss of virus on the discarded precipitates. If the products from the potato, tobacco and Princeton

cultures are diluted to give the same serological titre as the original sap, however, they are as infective as the sap and often more so. Price and Wyckoff (1939) have also commented on a similar increase in infectivity with their preparations made by ultra-centrifugation. The reason for this increase is not clear, but it is most likely that purification removes material that acts as an inhibitor of infectivity. Such phenomena make it impossible to be certain what proportion of the initial virus is isolated in a fully active form.

The Rothamsted culture gives very variable results with this method. Occasionally it yields an infective product, but most often the product is serologically active, but almost non-infective. As a rule the infectivity is lost during the precipitation with alcohol, but it may survive this and still be lost in later operations. Using the method described by Bawden (1941) we have sometimes got reasonably active preparations of this culture, but even this usually leads to a product that is much less infective than that from the other cultures (Table I), and less infective than the virus in freshly-expressed sap. This method gives satisfactory products with the other strains and, when a large volume of sap is being handled, is to be recommended; on a small scale, however, the method described here is both more convenient and more reliable.

The virus from all four cultures is more soluble in dilute ammonium sulphate solution at 0° C. than at room temperature, and this property can be used for fractionating the colourless products further and for their crystallization. Saturated ammonium sulphate solution is added drop by drop to the partially purified preparation at 20° C. until the first signs of opalescence appear, when it is cooled to 0° C. and left for a few hours. If a precipitate separates it is centrifuged off at 0° C., using the method of lagging a centrifuge tube that we have described (Bawden and Pirie, 1942). This precipitate is generally free from virus and can be discarded; the supernatant fluid is kept at 0° C. for crystallization to proceed. Crystallization may begin in a few hours, but occasionally, for reasons that we do not understand, it is delayed for several weeks. If there is no crystallization in a few days a drop of saturated ammonium sulphate solution should be added for each ml. of fluid, but if more than a quarter of a volume of ammonium sulphate solution is added the precipitate that separates is likely to be amorphous.

Preparations of all cultures have been made from both beans and tobacco, and the host has not been found to influence the end-product. Purification of preparations from infected beans is generally the more difficult, for a larger amount of normal leaf protein remains in a water-soluble state in the fluid from which the virus is being crystallized. Unlike the viruses, this protein is more soluble at room temperature than at 0° C., and is largely removed when the preparation is centrifuged at 0° C. before crystallization. Further traces of normal protein can also be separated from dialysed preparations at 0° C. The somewhat erratic behaviour of these viruses on dialysis is described later in this paper.

YIELDS.

No definite figures can be given for the yields of virus, for these vary with the numbers of lesions on the leaves used for the preparation. In general,

however, the yield is higher from tobacco than from bean, and is higher when plants are grown under poor light conditions during the winter than in the summer. Purification is also considerably easier in the winter, for then the plants contain smaller quantities of other soluble proteins that interfere with the separation of the virus. Constant differences are obtained between the yields from different cultures. With tobacco I and II the yield from plants with large numbers of lesions varies from 1 to 6 mg. per litre, whereas with other cultures it varies from 15 to 100 mg. per litre. This difference is reflected in the infectivity and the serological titres of sap taken from leaves infected with the different cultures. Often sap with tobacco I and II gives no precipitate with antiserum, and the highest dilution ever to react was 1 in 4. By contrast, sap from plants rubbed with the potato, tobacco VI, Princeton and Rothamsted cultures precipitates with antiserum at dilutions of from 1 in 8 to 1 in 64; it is also 10 or more times as infective as sap from plants infected with tobacco I or II.

PROPERTIES.

Crystal Forms of the Different Viruses and Virus Strains.

All the viruses resemble Bushy Stunt in that they dissolve more slowly when in the crystalline state than when amorphous, and a useful fractionation can often be made by washing the crystals quickly with water. After crystallization it is often difficult to get the viruses completely into solution. They give opalescent suspensions that flocculate on warming and can give confusing results in serological tests. When left for some hours at 0° C., however, these suspensions become nearly clear and then remain stable on warming. This effect is probably responsible for at least part of the increase in clarity that was noticed by Pirie *et al.* (1938) as a result of freezing a mixture of tobacco necrosis viruses.

Potato culture.—This culture has behaved quite systematically, and the eighteen preparations that have been made have all crystallized completely, after lying at 0° for a few days, in the form of thin, lozenge-shaped plates (Fig. 1). The smaller pair of angles made by the edges of these plates is 79°; this is the same as the angle in the crystalline material made by Pirie *et al.* (1938), and there seems to be no reason to think that the potato culture is not identical with the crystalline fraction of that preparation. Solutions rendered opalescent by the addition of ammonium sulphate have an intense sheen and show anisotropy of flow when shaken between crossed polarizers. The crystals are birefringent when viewed edgewise. Crystallization seems to proceed by the regular piling of these plates on one another as in a pack of cards, for preparations that are examined under the microscope after the minimum disturbance contain thicker plates than usual, and show but little anisotropy of flow when shaken lightly. On more intense shaking the plates separate and the usual amount of anisotropy of flow appears. Further evidence that this is the original manner of crystallization is given by the frequency with which an identical nick appears on many of the malformed crystals in a preparation. Slotta and Fraenkel-Conrat have described (1938) a somewhat similar manner of crystallization with the protein from rattlesnake venom.

Tobacco culture.—Preparations from this culture have always given crystals, but the crystal form has varied widely in successive preparations. Dodecahedra, bipyramids, irregular laminae and other forms were all encountered, and sometimes more than one form was present in the same preparation (Fig. 3). It seemed likely that these variations resulted from the fact that the culture contained a mixture of viruses. Attempts to avoid this difficulty were made by isolating six local lesions from this culture and using each as a separate inoculum, in the hope that a single local lesion would contain only one virus. Only three of the isolates have been studied in any detail; these will be referred to as tobacco I, II and VI. The preparations of VI have behaved quite consistently, whereas those of I and II have varied, although we have no reason to believe that they are mixed cultures.

The crystals separated from the first few preparations of I and II were dissimilar, so that it was thought that the cultures were probably different, but, after six separate preparations of each had been made, each had given all the different crystal forms. For these and other reasons, it now seems likely that I and II are very similar if not the same. Four modes of crystallization have been recognized. From solutions more acid than pH 5 the virus separates as irregular laminae; when these form on the walls of the vessel they have the more or less circular outline shown in Fig. 6 and they are generally slightly puckered. In the bulk of the fluid, pieces of irregularly crumpled sheet are to be seen. Between pH 5 and pH 7 the usual forms are bipyramids, dodecahedra and several types of twinned crystal. The bipyramids may be mistaken for regular octahedra when they are small, but when large crystals separate their birefringence is apparent and they tend to be elongated as shown in Figs. 9 and 10. The rhombic dodecahedra appear to be regular and even the largest have shown no birefringence. A variety of many-pointed stars has been found, but the most common type is shown in Fig. 4. It appears to be a cube on to each of the six faces of which a square pyramid has been built; the angle at the apex of the pyramid is, however, more acute than that which would have resulted in a rhombic dodecahedron, and a 24-faced solid is formed. These crystals often seem to have a laminated structure. Except that the irregular laminae are formed in acid solution, the conditions leading to the different manners of crystallizations are not understood. After clarifying the infective sap, we have sometimes split it into two and worked up the two parts separately, and from one half we have obtained rhombic dodecahedra and from the other birefringent bipyramids. Similarly, we have dissolved dodecahedra, re-crystallized and obtained bipyramids. Preparations at the stage for crystallization have been split, and part seeded with dodecahedra and part with bipyramids, but this has introduced no more consistency into the crystallization. Soon after the addition of ammonium sulphate solution all preparations of I and II show a definite sheen, but this disappears in a day or two when crystallization is complete.

Tobacco VI has always crystallized in the form of hexagonal prisms, with pointed ends. The only variation is that sometimes lateral growth proceeds so far that the points are replaced by hexagonal faces; this is illustrated in Figs. 5 and 7.

These prisms are birefringent when viewed from the side, but not when

viewed end on (Fig. 8). Crystallization is slower with this virus than with any of the others, and is often only complete after a fortnight. The difference in the solubility of this virus at 0° C. and 20° C. seems greater than that of the others, for the addition of sufficient salt to produce an opalescence at 20° C. is often insufficient to produce crystals when the solution is kept at 0° C.

Princeton culture.—After a short time at 0° C. in ammonium sulphate solution, purified preparations of this culture develop an intense sheen and, if shaken between crossed polarizers, they show strong anisotropy of flow. When examined microscopically the precipitate is seen to consist of small uniform particles, resembling bacteria about one-and-a-half times as long as they are wide. This elongation would seem insufficient to account for the pronounced optical phenomena and it is possible that the particles are plate-like. We have attempted to increase their size by slow precipitation methods, but have obtained nothing that can certainly be considered a crystal.

Rothamsted culture.—From a large number of preparations of this culture we have never obtained any crystalline material. Occasionally the preparations have given a sheen when precipitated with ammonium sulphate, but otherwise the precipitates have been amorphous. It is, of course, possible that the loss of infectivity suffered during purification is responsible for this, and that purified, fully-active preparations would crystallize. With other viruses, such as bushy stunt and the potato culture of tobacco necrosis, loss of infectivity without loss of serological activity has no effect on the ability to form crystals. Unless the changes undergone by the Rothamsted culture on purification are different from those rendering these other viruses non-infective, this explanation is unlikely to be true.

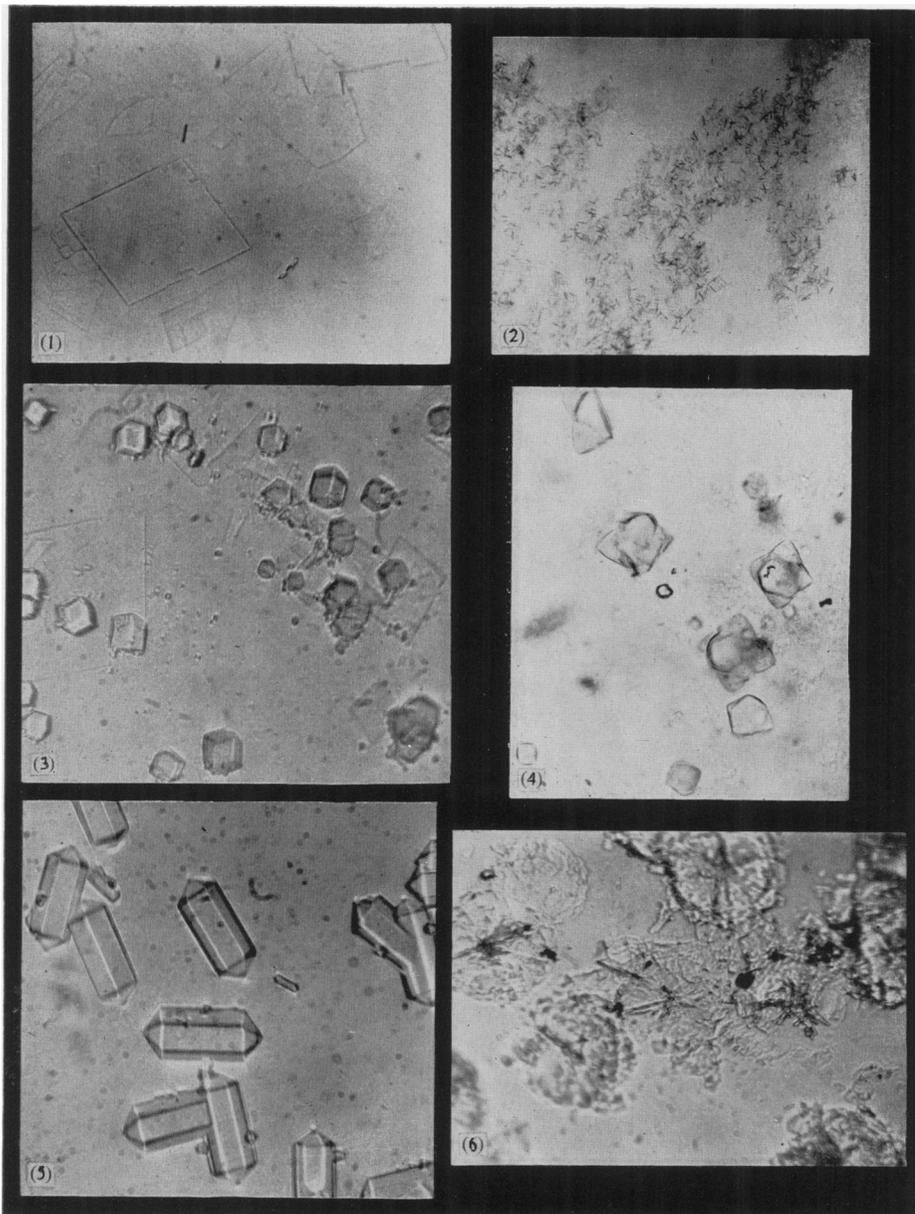
ACTIVITY AND SEROLOGICAL RELATIONSHIPS.

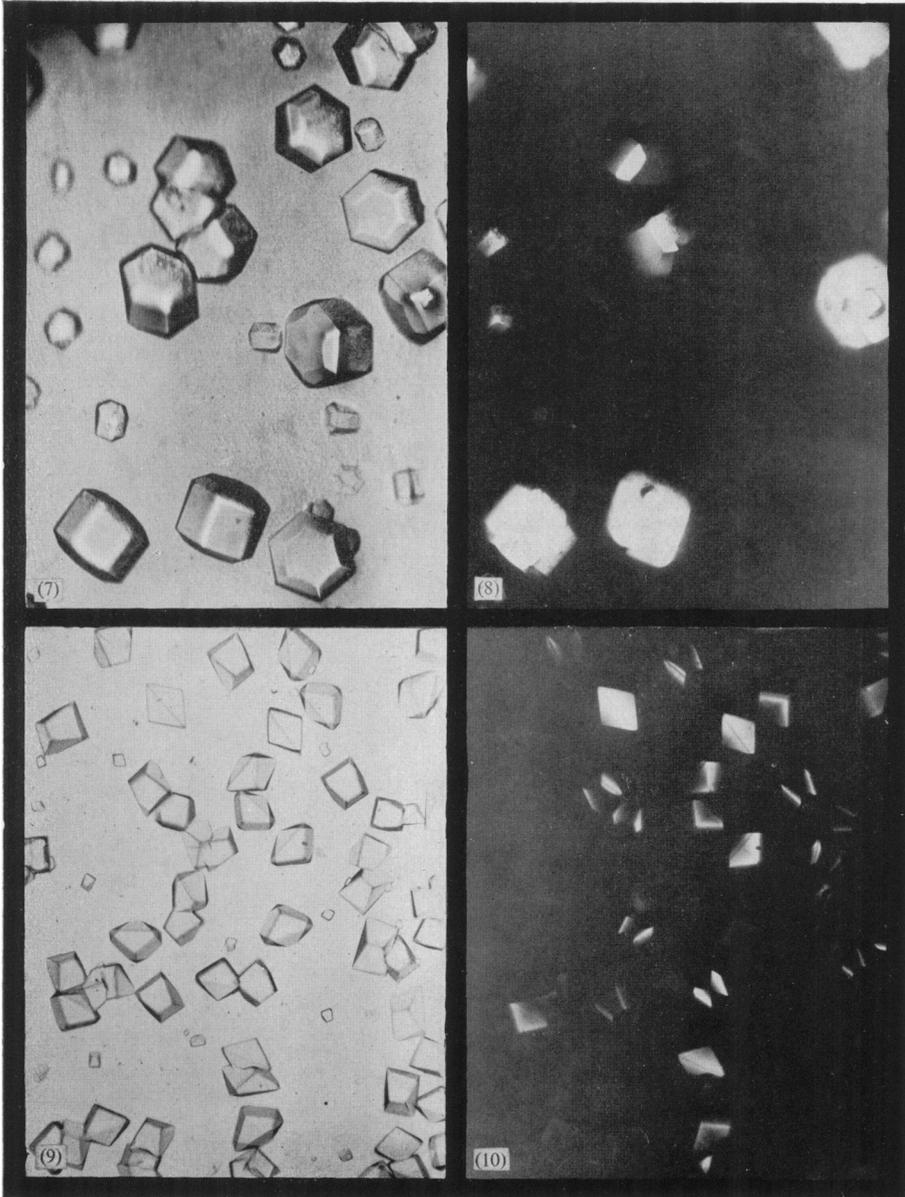
Table I shows the activity of a purified preparation of each culture. These tests were done at different times, so that exact comparisons of the infectivity of the different cultures cannot be made. The reduced infectivity of the purified preparation of the Rothamsted culture, however, is obvious, and often the effect is greater than the one shown. The higher serological titre is a further constant feature of preparations of this culture. This is probably

EXPLANATION OF FIGURES.

Crystalline forms of Tobacco Necrosis Viruses.

- 1.—Lozenge-shaped crystals from the potato culture. × 200.
- 2.—Crystals of the potato culture, some seen edgewise, photographed while still attached to the wall of the tube in which the preparation was crystallized. × 70.
- 3.—Crystals from the original tobacco culture before single lesion isolates were made. Lozenge-shaped crystals and dodecahedra occur together. × 70.
- 4.—Twinned crystals from a culture of tobacco I. × 200.
- 5.—Crystals in the form of hexagonal prisms with pointed ends, produced by rapid crystallization of tobacco VI. × 200.
- 6.—Crystals of tobacco I. Round laminae separating on the walls of the tube from a preparation at pH 4.5. × 200.
- 7.—Tobacco VI crystallized slowly. × 70.
- 8.—The same field as fig. 7, photographed between crossed Nicol prisms. × 70.
- 9.—Tobacco II in the form of bipyramids. × 70.
- 10.—The same field as fig. 9, photographed between crossed Nicol prisms. × 70.





another intrinsic difference between the virus (or viruses) in this culture and in the others, for the least rigorous purification methods are used with this culture ; it is therefore the most likely to contain impurities and its serological titre may be minimized in our tests.

TABLE I.—*Activity of Purified Preparations of Different Cultures of Tobacco Necrosis Viruses.*

Culture.	Serological titre.	Infectivity.			
		Average number of lesions per leaf at—			
		10 ⁻⁵ .	10 ⁻⁶ .	10 ⁻⁷ .	10 ⁻⁸ .
Potato	1/400,000	350	115	23	3
Tobacco I	1/300,000	270	72	26	5
Tobacco VI	1/400,000	300	80	13	2.5
Princeton	1/400,000	325	120	20	2
Rothamsted	1/600,000	86	9	1	0

When sap from plants infected with any of the cultures is dried and resuspended, its infectivity and serological activity are not greatly affected. Drying after purification, on the other hand, reduces the serological titre to about one-half and the infectivity to less than one-tenth of the original. For example, the preparation of tobacco I in Table I gave, after drying, a titre of 1/150,000 and at 10⁻⁵ and 10⁻⁶ gave an average of 41 and 5 lesions per leaf, and the preparation of the Rothamsted culture after drying gave a titre of 1/400,000 and no lesions at 10⁻⁵. The preparations were dried unfrozen and the material dissolved readily in saline.

The serological relationships of these different cultures of tobacco necrosis viruses are summarized in Table II.

TABLE II.—*Serological Relationships of Different Cultures of Tobacco Necrosis Viruses.*

Culture.	Antiserum					
	Potato.	Tobacco I.	Tobacco II.	Rothamsted.	C.1.	C.2.
Potato	+	-	-	-	+	+
Tobacco I	-	+	+	-	-	-
Tobacco II	-	+	+	-	-	-
Tobacco VI	+	-	-	-	+	+
Princeton	+	-	-	-	+	+
Rothamsted	-	-	-	+	+	-

C. 1 is the serum used in the work of Pirie *et al.* (1938) and C. 2 the serum prepared against their crystalline product.

A + indicates that the particular culture has precipitated with that antiserum and a - that it has not, even though tests have been made over a wide range of antigen/antibody ratios in both constant antigen and constant antibody tests. It will be seen that the potato, tobacco VI and Princeton cultures are serologically related and all react with the same sera. Tobacco I and II are related to each other, but to no others, and are the only cultures not sero-

logically related to any of the constituents of the virus mixture investigated by Pirie *et al.* (1938). The Rothamsted culture is also serologically distinct. This reacts with the serum made against the bulk culture of Pirie *et al.* (1938), but not with the serum made against their crystalline material, confirming the suggestion made previously (Bawden, 1941) that this was a distinct virus from the amorphous material. It is likely that their crystalline product is very similar to, if not identical with, the culture here referred to as potato.

The rhombic dodecahedron is one of the more common forms in which proteins crystallize, e.g. bushy stunt virus, lysozyme and some of the haemoglobins and haemocyanins have all been found to form crystals of this type. Their occurrence in preparations of viruses causing tobacco necrosis, therefore, clearly cannot be taken as evidence of relationship between these and bushy stunt virus. However, to test this possibility, and to eliminate the chance of contamination, all preparations of tobacco cultures I and II which contained dodecahedra were tested against antiserum to bushy stunt virus. None ever reacted. Similarly, tests with the other viruses causing tobacco necrosis have failed to show any serological relationships between these and bushy stunt virus.

SEDIMENTATION CONSTANTS.

The only evidence conflicting with the view that the crystalline virus isolated by Pirie *et al.* (1938) is the same as our potato culture is an apparent difference in the sedimentation constant. Dr. A. S. McFarlane, quoted by Pirie *et al.* (1938), found that this material was homogeneous with $S_w^{20^\circ} = 130 \times 10^{-13}$, whereas Dr. Ogston finds an $S_w^{20^\circ} = 116 \times 10^{-13}$ for our potato culture. Similar differences occur in the measurements made on bushy stunt virus (Bawden and Pirie, 1942) by Dr. McFarlane and Dr. Ogston in their centrifuges, and it is now agreed (*cf.* addendum to Bawden and Pirie, 1942) that the older values obtained by McFarlane were too high because of an uncertainty in the temperature of the rotor. Dr. Ogston also finds an $S_w^{20^\circ} = 116 \times 10^{-13}$ for our tobacco VI and Princeton cultures, which are serologically related to the potato culture. This is in reasonable agreement with the value of 112 given by Price and Wyckoff (1939) for the culture from which our Princeton one was derived.

The amorphous fraction of the preparations made by Pirie *et al.* (1938) was found by Dr. A. S. MacFarlane to have several components, the chief of which gave $S_w^{20^\circ} = 58$ and 220×10^{-13} . Similarly, the constants found by Dr. Ogston for our amorphous preparations of the Rothamsted culture differ from those for the other viruses. Except for one preparation, which contained a small amount of material with $S_w^{20^\circ} = 235 \times 10^{-13}$, these have been homogeneous and they have given $S_w^{20^\circ} = 49 \times 10^{-13}$. All preparations have had equal serological activity, and the preparation containing the heavy component was no more infective than the others. There is, therefore, no evidence that the heavy component is residual infective virus, but there is also no certainty that 49×10^{-13} is the sedimentation constant of fully active virus rather than of a non-infective derivative. With the other viruses that we have studied, loss of infectivity without loss of serological activity has not affected the sedimentation constant, but it cannot be assumed that this is also true of this

virus. If the value should turn out to be that associated with the active material, this will be the smallest plant virus on which centrifugal measurements have been made.

The yields of tobacco I and II cultures are so small that we have at no time had sufficient fresh material for centrifugation. Dr. Ogston's results on the other four cultures are set out in the Addendum.

CARBOHYDRATE AND PHOSPHORUS CONTENTS.

These viruses have not as yet been available in sufficient quantity for any detailed analytical work, but a fairly extensive series of carbohydrate (Pirie, 1936) and phosphorus (Kuttner and Lichtenstein, 1932) determinations have been made. No consistent differences between the different strains have been noticed and, with highly purified products, all values have fallen in the ranges—carbohydrate 7·0 to 8·5 per cent., phosphorus 1·7 to 2 per cent. The rates of colour development with the orcin reagent used in the carbohydrate estimation is the same with each strain, and the rate is compatible with the hypothesis that, as in the other plant viruses, the carbohydrate occurs in the form of a nucleic acid of the ribose type. The contaminants, other than inactive materials derived from the viruses, that are most frequently encountered in these preparations have a higher carbohydrate but a lower phosphorus content than the viruses. This gives these determinations some value as an index to the probable purity of a virus preparation.

SOLUBILITY.

Tobacco VI culture.—Purified preparations of this virus have never precipitated from solution in distilled water or dilute salt solution at any pH value between 3 and 8, even on prolonged standing.

When, on the other hand, crystals that have separated from ammonium sulphate solution are suspended in water or dialysed at pHs in the neighbourhood of 4·5 little of the virus goes into solution, and even after some days the fluid contains only 1 to 2 g. of virus per litre. The material which dissolves under these conditions has the same infectivity and serological activity as that which remains insoluble. At pH 8 a crystal suspension in saline dissolves immediately, and at pH 6·7 it dissolves in a few minutes. The rather critical pH range in which solution is quick is illustrated in Table III. The supernatant fluids were tested serologically and, under these conditions, a serum precipitation end-point of 1 : 128 represents substantially complete solution. At pH 5 the crystals dissolve on heating to 70° or on the addition of urea, but these treatments cause partial inactivation.

TABLE III.—*Solubility of Crystals of Tobacco VI at Different pHs.*

pH of crystal suspension.	Serological titre of the supernatant after 150 minutes at 0°.
6·4	1 : 128
6·1	1 : 64
5·7	1 : 32
5·3	1 : 16

The suspension contained 0.4 mg. of virus in the form of crystals in $M/7$ NaCl containing $M/20$ phosphate buffer at the pH stated.

Potato culture.—Solutions of this virus in distilled water or dilute salt solution do not precipitate at first at any pH between 3 and 8. Occasionally however, slightly acid solutions, in the absence of salt, have crystallized in the usual form after lying either at 0° or at room temperature for some months. This crystallization has always been incomplete and the crystals dissolve on the addition of a little salt, especially at about pH 8. There is no significant difference between the activities of the crystalline and soluble fractions separated in this way. The crystals that separate from ammonium sulphate solution will dissolve slowly after washing with distilled water or after dialysis. Solution seems to be most rapid at pH 4.5, although it occurs over a wide range, and it is favoured by cooling. It is noteworthy that a suspension of crystals at pH 4.5 will dissolve after a few days and then crystallize again after lying undisturbed for some months. We have no explanation for this phenomenon. The increase in clarity and stability in solutions of crystals when they are left at 0° for some hours has already been mentioned in the section on serology. It will be more fully investigated when more material is available.

Princeton culture.—We have studied the virus from this culture less fully than the others to which it is serologically related, but like them it has not precipitated from water or dilute salt solutions at any pH in the normal working range. We have failed to get indubitable crystals from this virus, but the precipitates produced by ammonium sulphate show a phenomenon comparable with the slow solution of the crystals from the tobacco VI and potato cultures. When the precipitates are taken up in water nothing can be sedimented by centrifuging at 4000 r.p.m., but the clarity of the suspensions increases progressively during the first few hours.

Tobacco cultures I and II.—Between pH 4 and 5 and in the absence of salts, precipitates invariably separate from solutions more concentrated than 0.2 per cent. and often separate from more dilute solutions. The precipitates redissolve readily on the addition of salt or on neutralization. Most of the activity of a preparation is found in the precipitate, suggesting that the virus itself has a low solubility, or is insoluble, at pH 4.5 in the absence of salts. However, several factors complicate this interpretation. Of the normal plant proteins still present most can be removed by dialysis at pH 6 followed by centrifugation at 0° C., and the precipitate characteristic of the preparations from these two cultures does not occur with the others, but the yields with tobacco I and II are so much smaller than with the others that the importance of contaminants is increased. The serological titres of freshly made preparations are sufficiently high to suggest that there is no gross contamination.

Rothamsted culture.—There is considerable uncertainty about the solubility of the virus from this culture. Our tests have all been made on preparations that have lost most of their infectivity, and no attempt has been made to ensure that the culture contains only one virus or virus strain. Also, the method of purification used is less rigorous. Precipitates usually separate on dialysis at pH 6–7, but these consist of normal leaf protein, have low phosphorus and carbohydrate contents and carry little or no activity. After

preparations have been freed from normal proteins by this treatment, they give a further precipitate when dialysed at about pH 4.5 ; as with preparations of tobacco I and II, the precipitate carries part of the activity with it. Precipitation is not complete, and most of the serological activity remains in the supernatant. The infectivity is usually fairly evenly divided, but occasionally we have had preparations in which almost all of the residual infectivity has appeared in the precipitate, although the serological activity has been predominantly in the supernatant. The precipitate dissolves on the addition of salt, or when brought to pH 7, and it has the same phosphorus and carbohydrate contents as the material that is soluble at pH 4.5.

BEHAVIOUR ON HEATING.

No significant difference has been found between the thermal inactivation point of the different cultures. In different tests the temperature at which ten minutes' heating has destroyed infectivity has varied between 75° C. and 85° C., the variations apparently depending more on the virus content of the preparation and the sensitivity of the test plants than on the virus culture. The results of one test using infective sap clarified by freezing and centrifuging are given in Table IV. It will be seen that heating at temperatures well below

TABLE IV.—*Effect of Heat on Tobacco Necrosis Viruses.*

Average number of lesions per leaf using infective sap diluted 1/1 and 1/50. Culture.

Temperature.	Potato.		Tobacco I.		Princeton.		Rothamsted.	
	1/1.	1/50.	1/1.	1/50.	1/1.	1/50.	1/1.	1/50.
Unheated	147	125	113	96	155	152	190	157
50°	135	23	97	23	195	44	215	35
60°	24	3.5	26	2	29	3	19	1.5
70°	4	0	3	0	7	0.5	3.5	0
80°	1.5	0	0	0	1.5	0	0	0

the thermal inactivation point causes great loss of infectivity with all cultures. This loss is not accompanied by any corresponding fall in the serological titres, and by longer heating at these temperatures preparations can be rendered completely non-infective without any apparent change in their serological activity. None of these cultures has ever given any lesions after heating for ten minutes at or above 85° C., although the Princeton culture was derived from one for which Price (1938) found the thermal inactivation point to be 92° C. This is the temperature at which the viruses denature and lose their serological activity, and it is likely that with more concentrated inocula or more sensitive test plants some infectivity would be detected in preparations heated to this extent. Differences of this kind rather than differences in the heat stability of the viruses most probably account for these discrepancies (Table IV).

DISCUSSION.

The fact that similar symptoms are often caused by different viruses, while different symptoms are caused by related ones, has been one of the main reasons for the confusion that has arisen in the nomenclature of plant viruses.

It has also been an important factor in preventing the development of any system of classification, for it was early recognized (Johnson, 1926) that symptomatology alone provided no basis for this. Although there are no generally accepted criteria for classifying viruses, in recent years two tests have been widely used as methods for determining whether viruses causing different symptoms should be regarded as related strains. The first is the demonstration that a plant fully infected with one is protected against infection with the other, and the second is the demonstration that the two are serologically related. The first has been the more widely used, for serology as yet has had only limited applications, but wherever the two have been applied to the same viruses in critical tests, they have given the same results. In other words, wherever viruses have failed to protect plants against each other they have also failed to react with each other's antisera.

The plant protection method cannot be used as a test of relationships with these tobacco necrosis viruses, because they all produce identical symptoms and no host is known in which any becomes systemic. Therefore, serological tests remain the only standard method of indicating close relationships. These show that some of the cultures with which we have worked have antigens in common, whereas others are serologically distinct. Provided that conditions are such that a positive test is obtained with homologous sera, we regard a negative result with a heterologous serum as of greater significance than a positive result, for a positive test can only be taken as evidence of relationship if it is known that the antigen used to prepare the test serum was homogeneous. For example, if all our tests had been made using only the serum prepared by Pirie *et al.* (1938), the Rothamsted and potato cultures would appear to share antigens, for the immunizing antigen used to prepare this serum contained viruses from each culture. The antigen preparation used to prepare the serum to our potato culture was derived from a single local lesion, and we have no reason to believe that it contained more than one type of virus. Therefore the fact that tobacco VI and Princeton cultures react with it we take as evidence that they are serologically related, and we regard these and the potato culture as related strains of the same virus. Tobacco I and II and the Rothamsted culture, which do not react with this serum or with each other's sera, we regard as distinct viruses.

The grouping indicated by serological methods is supported by the other properties of the various cultures, for those that do not share antigens also differ more in other ways than those that do share antigens. The serologically related potato, tobacco VI, and Princeton cultures have been found to differ only in their behaviour when precipitated with ammonium sulphate, whereas, in addition to differences of this type, the serologically unrelated Rothamsted culture is less stable and tobacco I and II multiply less readily in infected plants. Tobacco I and II and the Rothamsted culture also differ from the others in their behaviour at pH 4.5 in the absence of salts. Further work may invalidate the relationships now indicated by serological methods, but at the moment it seems simplest, in view of the current usage of the terms, to regard tobacco necrosis as a disease that can be caused by distinct viruses, each of which may exist in a number of strains. In this respect, the disease resembles acroncrosis of the potato more than any other, but the different viruses and

virus strains that cause this disease can be distinguished by their reactions on a series of differential potato varieties, as well as by serological methods and plant protection tests (Bawden, 1936). In the same way it may be that the viruses and virus strains causing tobacco necrosis could be distinguished by studying their reactions on a wide range of hosts, for we have only worked with them on tobacco, *Nicotiana glutinosa*, and bean. What the relationships are between the various serologically unrelated viruses is as difficult to decide as with any other viruses that are serologically unrelated. It is obvious that all those with which we have worked do share some properties, but most of these are also shared by tomato bushy stunt virus, which also produces somewhat similar necrotic local lesions in bean and tobacco. We would therefore hesitate to suggest at this stage of our knowledge that the Rothamsted and tobacco I cultures are more closely related to each other, or to the potato culture, than they are to tomato bushy stunt virus, but it is obvious that all these do fall naturally into one group clearly distinct in most of their properties from other plant viruses that have been studied in any detail.

SUMMARY.

The purification of six separate cultures of viruses causing tobacco necrosis is described; these cultures have been labelled Potato, Princeton, Tobacco VI, Rothamsted, Tobacco I and Tobacco II. The last two are probably identical, but the remainder differ in their properties, although they produce identical symptoms in tobacco and bean. The first three share antigens but are serologically unrelated to the others, suggesting that the disease can be caused by different viruses, each of which may occur in a number of strains. On precipitation with ammonium sulphate the products from four cultures have behaved systematically: Rothamsted gives an amorphous precipitate; Princeton shows anisotropy of flow but gives no recognizable crystals; potato crystallizes as thin lozenge-shaped plates; and tobacco VI as hexagonal prisms. Tobacco I and II, for unknown reasons, crystallize in a variety of different forms. The two commonest are dodecahedra and bipyramids, but thin round laminae and elaborately twinned structures also occur. The materials isolated from all the cultures of tobacco necrosis seem to be essentially nucleoproteins.

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