THE PURIFICATION OF INSECT-TRANSMITTED PLANT VIRUSES.

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IN the past few years specific nucleoproteins have been isolated from plants infected separately with a number of different viruses. These nucleoproteins have been obtained in apparently pure forms, and there is no evidence conflicting with the view that they are the viruses themselves. All the viruses that have yet been isolated, namely, tobacco mosaic, potato " X ", cucumber 3, tomato bushy stunt, and tobacco necrosis, have been found to have closely similar analytical compositions, differing significantly only in the quantity of nucleic acid they contain (Bawden and Pirie, 1939). These have widely different properties, both *in vivo* and *in vitro*, but they are all among the more stable viruses, and the list contains no virus known to be transmitted by insects. Therefore, although it is reasonable to expect that some other plant viruses are also nucleoproteins, it would be premature to assume from the results obtained with the five named viruses that all plant viruses are chemically similar.

The viruses that are known to be insect-transmitted can be divided conveniently into two types on the basis of their relationships with their insect vectors. Viruses of the first type are not transmitted by their vectors immediately after a short feeding period on infected plants. A so-called incubation period is necessary before the insects become infective, but once they are able to transmit they continue to do so for long periods, sometimes for the remainder of their lives. Viruses of the second type have no significant incubation period in their vectors, and a few hours after ceasing to feed on infected plants these lose their power of infecting healthy plants. That this difference is determined by the virus and not the vector is shown by the fact that some insects transmit both types of viruses.

In this paper attempts to purify two insect-transmitted viruses of the second type are described. Most of the work has been done with potato virus " Y ", but many preparations of Hyoscyamus virus 3 have also been made. Watson (1936, 1938) has shown that these two viruses are transmitted by *Myzus persicæ* Sulz. and some other aphids in essentially the same manner. One curious feature in their transmission is that the efficiency of the vectors is much reduced by continuous feeding. The optimum conditions for transmission are obtained by using aphids that have been starved for at least 1 hour, allowing these to feed for periods of only a few minutes on the infected plants and transferring them immediately to healthy plants.

These two viruses occur in infective sap in much smaller concentrations than those previously described. As they are also less stable and their properties in many ways approximate more closely to those of some of the normal plant proteins, their purification is more difficult, and it is impossible to make confident statements about either the purity or properties of the preparations. Sufficient evidence has been gained, however, to indicate that these two insect-transmitted viruses do not differ from those previously isolated in the general plan of their chemical constitution.

Preparation of Potato Virus " Y ".

Preparations of potato virus " Y " have all been made from tobacco plants, variety White Burley. The work was done mainly in the winter months, for the yield of virus " Y " is then greater than in the summer ; furthermore, plants then contain smaller amounts of troublesome constituents that interfere with the purification. The plants were cut down about a month after infection, when showing definite dark-green vein-banding symptoms, minced, and the sap expressed through muslin.

Na_2HPO_4 is added to the sap until a copious precipitate is produced, and this is removed by a few minutes' centrifugation at 3500 r.p.m. 300 g. of ammonium sulphate is added to each litre of the clarified sap, together with some NH_4OH to keep the mixture neutral, because much of the virus is lost if the pH falls sufficiently to coagulate the normal plant proteins. After standing for an hour to allow complete flocculation of the virus, the fluid is centrifuged for about half-an-hour, the brown supernatant fluid being discarded. The precipitate is suspended in a measured volume of water (about one-fifth of the volume of the original sap) and centrifuged. One-quarter of a volume of saturated ammonium sulphate solution is added to the supernatant fluid and, after standing for an hour, the mixture is centrifuged. The bulky, greyish-green precipitate is discarded, and the supernatant fluid is two-fifths saturated with ammonium sulphate. After standing for an hour, the fluid is centrifuged and the yellow supernatant fluid discarded.

The precipitate at this stage is a soft brown mass that will not pack tightly in the centrifuge tube. It is suspended in one-fifth of its volume of phosphate buffer at pH 8 and dialysed against running tap-water for from 6 to 8 hours. The dialysed fluid usually has a volume of about one-twentieth of the original sap and a solid content of from 1–2 per cent. If this solution is treated with different amounts of ammonium sulphate between one- and two-fifths of saturation, it can be separated into a number of fractions. Although these have different physical properties, each contains virus, and the process does not lead to any useful fractionation of the virus. Some virus can be prepared directly by high-speed centrifugation of the concentrated dialysed solution, but as it contains many other proteins that also sediment readily, it is more convenient to destroy some of these by incubation with trypsin. Incubation for 6 hours at 37° C. and pH 8 in the presence of 0·4 per cent. " Pangestin " (Difco) removes most of the contaminating proteins without seriously reducing the virus content. Unless the solution is highly buffered

it will be necessary to readjust the pH by the addition of alkali during the incubation. After such incubation, one-quarter of a volume of saturated ammonium sulphate solution is added and, after standing for 2–3 hours at 0° C., the mixture is centrifuged. Two-fifths of a volume of saturated ammonium sulphate solution is added to the supernatant fluid, and after standing for an hour the mixture is centrifuged for an hour at 3500 r.p.m. to pack the precipitate tightly. The precipitate is suspended in a volume of water equal to one-hundredth of that of the original sap, the pH adjusted to pH 7·5, and the fluid centrifuged for a few minutes to get rid of insoluble materials. The final supernatant fluid is then centrifuged at 15,000 r.p.m.

For unknown reasons it has never proved possible, at this stage, to sediment all the virus from a solution by centrifuging at 15,000 r.p.m. in a rotor of 8 cm. radius. About 80 per cent. is sedimented in from 3 to 4 hours and some of the remainder can be sedimented by a further period of centrifugation, but some always remains in the supernatant fluid. The pellets resulting from the high-speed centrifugation are usually opaque, showing only the faintest trace of birefringence at their edges. Occasionally, however, they are pale green and almost clear. The material constituting the final virus preparation is merely a minor constituent of these pellets. The nature of the major constituent varies somewhat in different preparations. Most often it is either a tough brown material that is suspended in water with difficulty, or a pale greenish-grey material that is suspended readily. Some of the properties of the second material, which sediments from neutral solutions much more readily than the virus, will be described later. The pellets are suspended in a volume of $M/20$ NaCl solution equal to about one-tenth of that from which they were sedimented, and then centrifuged for an hour at 10,000 r.p.m. Many of the contaminants sediment with this treatment, whereas most of the virus remains in the supernatant fluid, which now, for the first time, shows definite anisotropy of flow if shaken between crossed polarizing screens.

The process of differential centrifugation, that is, 3 to 4 hours at 15,000 r.p.m., followed by shorter periods at 10,000 r.p.m. on the resuspended pellets, is continued until a 0·3 per cent. solution is colourless, and the pellet obtained by centrifuging at 10,000 r.p.m. is small, homogeneous and birefringent. All the pellets separating at the lower speed contain some virus. They are therefore mixed, extracted with $M/20$ NaCl solution at pH 7·5, and the process of differential centrifugation repeated to recover the virus. By this method about one-half of the virus, estimated by serological titres, that was present in a preparation before incubation with trypsin can be obtained in a colourless state showing strong anisotropy of flow. The yields obtained have varied between 0·5 mg. and 1·5 mg. per litre of infective sap. This contrasts widely with the 2 g. or more virus that can be isolated from a litre of sap from plants infected with tobacco mosaic virus. As tobacco mosaic virus is the commonest contaminant in tobacco plants, all the anisotropic preparations of potato virus "Y" were tested for its presence, both serologically and by inoculation to *Nicotiana glutinosa*. This was necessary, for if one plant out of the 200 or so used for each preparation became infected, even before symptoms of tobacco mosaic became obvious on it, the material isolated would consist largely of tobacco mosaic virus.

Properties.

In describing preparations of other viruses that we have made it was possible to show by various tests that they appeared to be homogeneous. No similar claim can be made for the preparations of potato virus " Y ", for too little material has been available for rigorous testing. In their general physical properties, however, the preparations of this virus resemble those of the other anisotropic viruses, especially those of potato virus " X ", although they are more readily denatured by heat, acid and ageing. The intensity of anisotropy of flow that they show is similar to that shown by preparations of potato virus " X ", *i. e.* it is clearly visible when a 0·02 per cent. solution in a tube of 1 cm. diameter is shaken in a polarizing box. The liquid crystalline pellets prepared by the high-speed centrifugation of solutions of viruses " X " and " Y " also resemble one another closely, and the two viruses precipitate in a similar manner with acid and ammonium sulphate. When completely precipitated the material is difficult to see and appears amorphous under the microscope. But with insufficient acid or salt (about pH 5 or one-tenth saturation with ammonium sulphate) for complete precipitation the fluids develop a slight sheen and show increased anisotropy of flow. The precipitates produced from highly purified preparations of virus " Y " with acid and salt dissolve with difficulty, although with cruder preparations the precipitation is readily reversible. Potato virus " Y " sediments from neutral solution rather more slowly than potato virus " X ". No differences have been found between the two to suggest why one should be so readily transmitted by aphids and the other not.

The activity of purified preparations of virus " Y " is considerably less than that of preparations of the other anisotropic viruses previously isolated, and it seems probable that the purification processes inactivate much of the virus. Activity has been tested both by serological and infectivity tests. Attempts to produce antisera in rabbits by the intraperitoneal injection of crude infective sap failed, but good precipitating antisera were obtained by two intravenous injections of ·2 mg. of a partially purified preparation. The virus-antiserum precipitate has a fluffy, open structure, similar to that given by the other anisotropic viruses and by bacterial flagellar antigens (Bawden and Pirie, 1938*b*). The serological titres of the purified preparations, *i. e.* the greatest dilution at which 1 c.c. gives a visible precipitate when mixed with1 c.c. of diluted antiserum, have varied between $1 : 5 \times 10^5$ to $1 : 10^6$. These titres are approximately one-sixth of those obtained with carefully purified preparations of potato virus " X ". Some of this difference results from the different method used for testing virus " Y ". It is rapidly inactivated at temperatures around 55° C., and the tests could not, therefore, be carried out for 12 hours at 50° C. Instead, the tubes containing the virus-antiserum mixtures were placed in the water-bath at 50° C. for a few minutes at hourly intervals to ensure complete mixing by convection currents, and were kept for the remainder of the time at 1° C. Readings were made after 24 hours. With preparations of potato virus " X " this method was found to give titres about half as great as those obtained by the method previously used. Even when an allowance

is made for methods of testing, therefore, the serological activity of the virus " Y " preparations is only about one-third of that of virus " X " preparations.

The weight of a purified preparation required to cause infection when rubbed on to tobacco plants has varied from 10^{-5} to 10^{-7} g., that is, the infection end-point is little or no greater than the precipitation end-point. In the clarified untouched sap this is not so, for the precipitation end-point of this varies with different samples from about 1/4 to 1/12, whereas the infection end-point is usually greater than 1/10,000. The ratio of infectivity to serological activity, therefore, is greatly reduced by the purification methods. As the virus does not give countable local lesions, accurate quantitative infection tests could not be made, but the reduction in the ratio seems too great to be explained by aggregation as suggested for tobacco mosaic virus (Bawden and Pirie, 1937). It seems likely that during the isolation many of the virus particles undergo slight changes that render them non-infective but do not destroy their serological activity. Loring (1938) has suggested that salts do this to potato virus " X ", and it has been found possible to bring about changes of this type in all the other viruses that have been studied by treatment with agents like formaldehyde, nitrous acid, hydrogen peroxide, ultra-violet light and X-rays.

Occasionally the whole, or the greater part, of a preparation has appeared finally as a serologically inactive and non-infective product that shows strong anisotropy of flow but is insoluble, that is to say, it sediments in an hour when centrifuged at 2500 r.p.m.. Insufficient material of this type has been available for a detailed study, but its content of phosphorus and carbohydrate is similar to that of the soluble preparations. An explanation of this cannot be given, but the insoluble material may be analogous to the anisotropic precipitates given by tobacco mosaic virus and potato virus " X " with clupein and related materials ; or it may be simply an extreme case of the aggregation that is characteristic of the anisotropic viruses that have been salted out.

Provided that the purified preparations of virus " Y " are kept cold they remain active and show anisotropy of flow for some weeks, but at room temperature they become inactive and lose their optical properties in a few days. If crude sap is acidified to below pH 5 the virus is lost. By contrast, exposure for 3 hours to pH 5 at 15° C. had no effect on the activity of a purified preparation, and after 3 hours at pH 4 the activity was only reduced by one-half. After 3 hours at pH 9·2 a preparation was non-infective but still retained one-half of its serological activity. After this time at pH 10·3 both infectivity and serological activity were destroyed.

Purified preparations of virus " Y " are not inactivated by freezing and thawing. Thus they differ from those of tomato bushy stunt virus, but resemble those of the other anisotropic viruses that have been purified. As many of the normal plant proteins are irreversibly coagulated by this treatment, it might be of use in the purification. However, it has been found that this virus is readily absorbed on to surfaces, and procedures separating the virus as a precipitate, *e. g.* centrifugation, lead to much smaller losses than those in which contaminants are precipitated from fluids containing the virus. During the course of preparation the whole of the virus has occasionally disappeared,

a loss attributed to the absorption of virus either on to the precipitate separating with one-fifth saturation with ammonium sulphate, or on the precipitate produced by the ageing of the normal sap constituents. This is in agreement with the observation that virus " Y " is inactivated at greater pH values in crude preparations than when purified, for normal sap contains constituents precipitated at about pH 5. Virus " Y " is inactivated by absorption on to charcoal, but not by kieselguhr, talc or kaolin. Attempts have been made to use these materials in the purification methods. They have not proved as useful as the methods described above, but it is possible to separate the virus by centrifuging suspensions of kieselguhr in partially fractionated preparations for a few hours in the closed rotor of a Sharples centrifuge. The greater part of the virus is held by the thin layer of sedimented kieselguhr while the rotor is being emptied, from which it can easily be extracted in a small volume of water.

The purified preparations of potato virus " Y " contain from 14–16 per cent. of nitrogen—a value inside the range usually found for proteins. The most usual impurity is a complex containing both lipoid and carbohydrate. This contains from 6–10 per cent. of nitrogen, and its presence, therefore, leads to low nitrogen figures. Total carbohydrate estimations have been carried out by the orcin method (Pirie, 1936) used for other viruses, but with virus " Y " the results are often less certain because of the turbidity of the reaction mixture. This turbidity probably results largely from the presence of the contaminating lipo-protein, for this alone gives an intense turbidity. The virus preparations most active serologically have shown the least turbidity, and have given a colour corresponding to 3 per cent. of carbohydrate, estimated as glucose. As with potato virus " X ", it is often difficult to reduce the carbohydrate content to below 5 per cent. The phosphorus content is from 0·3–0·5 per cent. and is a fairly reliable criterion of virus activity, for the other proteins present in partially purified preparations contain no phosphorus and the contaminating lipoid-protein contains little. Too little material has been available for attempts to isolate a nucleic acid from this virus, but from its general behaviour it seems probable that this preparation consists essentially of a nucleoprotein more similar to tobacco mosaic virus and potato virus " X " in its content of nucleic acid than it is to tomato bushy stunt or tobacco necrosis viruses.

Absorption spectra.

It is difficult to get optically-clear solutions even at dilutions as low as 0·02 per cent., for during the necessary centrifugation there is a serious loss of virus. The interpretation of the absorption spectrum, therefore, is rather more difficult than with the viruses previously studied. Tobacco mosaic, tomato bushy stunt and tobacco necrosis viruses all have spectra of the same type, *i. e.* there is a maximum at 260 mμ., followed by a minimum at 245 mμ., and at shorter wave-lengths the absorption becomes intense. With potato virus " Y ", by contrast, although the absorption at 260 mμ. (the region associated with absorption by nucleic acid and other purine-containing substances) is as strong as that with solutions of tobacco mosaic virus of the same concentration,

there is no minimum at 245 mμ. This effect may result from the summation of an absorption curve similar to that found for the other viruses with another curve produced partly by another constituent with an absorption maximum at about 245 mμ., and partly by the light scattered by the small amount of suspended material. If, as in other systems, the scattering is proportional to 1/(wave-length)^4, scattering alone could not explain the phenomenon. A similar effect has also been obtained with both strains S and L of potato virus " X ". Preparations of these were made by the methods previously described (Bawden and Pirie, 1938*a*). They were as clear as protein solutions ever are, yet they showed a similar anomalous absorption at 245 mμ. An attempt to explain this phenomenon will be made later when more material is available.

The lipoid-containing contaminant.

In preparations made during the spring and summer the most troublesome contaminant has been a grey, or sometimes greenish material that is not precipitated with one-fifth saturated ammonium sulphate solution but is precipitated at two-fifths of saturation. It can be readily differentiated from the virus, as it is much more easily sedimented from neutral solutions by high-speed centrifugation and is apparently unaffected by boiling. Solutions of this material do not show anisotropy of flow when agitated in polarized light, but they are opalescent and often show a pronounced shimmer in ordinary light, similar to that shown by suspensions of blood-corpuscles. The material does not react with virus-antiserum.

When solutions of the contaminant are dried frozen they give a material with a light, open structure, from which 20 per cent. to 40 per cent. by weight can be extracted with lipoid solvents such as benzene. The extracted lipoid is partly crystalline when solutions in benzene or alcohol are evaporated, and it usually contains 0·1–0·2 per cent. of phosphorus; as would be expected, the phosphorus can be concentrated into the acetone-insoluble fraction. After removal of the fat, the residue contains all the nitrogen and carbohydrate of the original material; its nitrogen content is from 9–14 per cent., and the carbohydrate is much more variable but is usually between 10 and 20 per cent. of the defatted residue.

The nature of this material is unknown. It may be a specific product of virus " Y " activity, for no such material has been found either in healthy plants or in plants infected with other viruses. The material is reversibly precipitated with acid and ammonium sulphate and is heat-stable, so that none of the treatments used in preparing the other viruses would be expected to destroy it. However, extracts of plants infected with other viruses have not been re-investigated since this material has been recognized in preparations of potato virus " Y ". If present in them it is certainly removed from the viruses with greater ease than from virus " Y ", and this removal is accompanied by less serious losses of virus. But the quantitative differences in virus-content of sap from plants infected with virus " Y " and with the viruses previously investigated are so great that this contaminant, if present in other infective

extracts, would be only a minor constituent of a virus preparation and so might remain unnoticed.

Preparation of Hyoscyamus Virus 3.

The work on Hyoscyamus virus 3 was done mainly in the summer, for infected plants are so crippled in the winter that they yield little sap. Using similar methods to those for potato virus " Y ", material showing anisotropy of flow has been isolated with properties essentially similar to those of virus " Y ". The yields have been about 1–3 mg. per litre of sap, but the yield per plant has been less than with virus " Y " because of the greater reduction in growth caused. Although these two viruses have such similar properties and are transmitted in the same way, they are not related strains, for antisera prepared against one does not react with the other.

SUMMARY.

Liquid crystalline preparations of nucleoproteins have been made from the sap of tobacco plants infected with the two insect-transmitted viruses, potato virus " Y " and Hyoscyamus virus 3. The yields are from 0·5 to 3·0 mg. per litre of infective sap. These proteins give specific precipitates, of the flagellar type, with antisera when diluted to $1 : 10^6$. They are infective, but the methods used in the purification processes seem to destroy the infectivity of much of the virus without destroying its serological activity. The chemical and physical properties of preparations of these viruses closely resemble those of potato virus " X ". Some properties of a lipoid-containing contaminant are described.

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