LIQUID CRYSTALLINE PREPARATIONS OF POTATO VIRUS "X".

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IN earlier papers we have presented evidence suggesting that potato virus "X" either contains or is intimately associated with protein. Plants infected with various strains of this virus contain antigens not present in healthy plants, and evidence has been accumulated that these antigens are either the viruses themselves or parts of them (Bawden, 1935; Spooner and Bawden, 1935). Also, potato virus "X" is susceptible to attack by certain proteolytic enzymes (Bawden and Pirie, 1936). Then there was no reason to believe that our purified virus preparations were homogeneous, but now we have isolated the antigens specific to the infected plants in the form of nucleoproteins. Although these nucleoproteins have some properties in common with those isolated from plants infected with strains of tobacco mosaic virus (Bawden and Pirie, 1937*a*), and with cucumber viruses 3 and 4 (Bawden and Pirie, 1937*b*), they differ from them in all respects in which the viruses are known to differ, and the evidence suggests that they are the viruses themselves.

Recently, Stanley and Wyckoff (1937) and Loring and Wyckoff (1937) claim to have isolated a protein of high molecular weight with the properties of potato virus "X" (latent potato mosaic virus) by the high-speed centrifugation of clarified infective sap. As yet, however, they have presented little evidence in support of the claim, for their published results merely show that the sediments contain both virus and protein. No analyses of their products have been given; the statement that their material is a protein being based on the fact that it gives the usual protein colour reactions, and only centrifugal data have been given to support the view that the products are homogeneous.

The potato virus "X" nucleoproteins are much more difficult to obtain in a relatively pure state than are those of the tobacco mosaic virus type. It is necessary to handle the potato virus more carefully, for it is easily destroyed by acids, heating, ageing and proteolytic enzymes. Furthermore, certain physical properties of the potato virus, for example, its higher viscosity and the greater readiness with which it adsorbs impurities, also render its isolation more difficult and more uncertain.

Virus Strains Used.

Salaman (1933) described three strains of potato virus " X ", named S, G and L respectively, and showed that plants infected with one acquire an immunity to further infection with the other strains. The three strains are closely related serologically, possess similar general properties *in vitro* (Spooner and Bawden, 1935 ; Bawden, 1935), and are differentiated because they cause different symptoms in certain host-plants. Chester (1936*b*), using filtered infective sap, showed by means of cross adsorption experiments that each strain contains specific as well as common antigens.

The virulent strain, S, is the only one which produces local lesions, and is therefore the only one with which quantitative infection experiments can conveniently be made. Most of the work described in this paper has been done with this strain. In tobacco (var. White Burley) and in *Nicotiana glutinosa* the S strain produces white necrotic rings at the site of inoculation ; these are followed by systemic symptoms of a ringspot type, all the young actively growing leaves being covered with white, concentric necrotic rings. In tomato (var. Kondine red) it produces an interveinal mottle and black necrotic spots and rings.

When plants are infected with both tobacco mosaic virus and potato virus " X " they show characteristic symptoms, distinct from those produced by either virus alone. Tomatoes infected with both show the well-known experimental or glasshouse streak, and tobaccos show a severe general necrosis, the plants are stunted and the leaves wilt and sometimes collapse.

We have also used the mild, G strain. This produces no recognizable symptoms in infected tomato plants, and its effects on tobacco and *N. glutinosa* are slight. At first the infected plants show no definite symptoms, but after a few weeks they usually develop a faint interveinal mottle.

No significant differences have been found between the nucleoproteins isolated from plants infected with the different strains, except that each produces its characteristic effects when plants are inoculated with it. We have isolated the two virus strains from each of the three hosts, and have found that the nucleoprotein obtained is independent of the species of the host-plant.

Preparation.

Plants are inoculated when small and the leaves picked about a month after infection, that is, with strain S, about three weeks after systemic symptoms appear. The yield of virus per volume of sap is greatest at this time and the isolation is easier, for sap from old plants contains much pigment which is difficult to remove ; also, if old tobacco plants are inoculated with potato virus " X " the infection tends to be localized and the virus does not spread readily out of the inoculated leaves. The leaves are minced and the sap expressed through muslin, heated to 60° C., and then rapidly cooled. This causes a green, flocculent coagulum that is easily thrown down by a few minutes centrifuging at 3000 r.p.m. The heating must be done carefully and with continuous stirring to prevent local overheating, for the virus is rapidly inactivated at temperatures around 66° C.

The brown opalescent supernatant fluid is now either one-quarter saturated

with ammonium sulphate (185 g. per litre) or brought to pH 4·5 by the addition of sulphuric acid. These treatments produce a brown precipitate containing all the virus. After centrifuging, the precipitate from 1 litre of sap is suspended in 100 c.c. of water, neutralized with dilute NaOH, and centrifuged to free from insoluble materials. If the resulting solution is shaken between crossed Nicol prisms it now shows anisotropy of flow. We have been unable to find any materials in healthy plant sap which show anisotropy of flow, and examination of the fluids during the course of preparation in polarized light is the most convenient way of testing for the presence of the virus.

The virus is again precipitated by the addition of from one-third to one-half of a volume of saturated ammonium sulphate solution, centrifuged, and the coloured supernatant fluid discarded. The precipitate is suspended in water and the precipitations with ammonium sulphate repeated until the supernatent fluid is no longer brown. About six repetitions of this treatment are necessary, and a few drops of dilute NaOH solution must be added each time to keep the solution neutral. The precipitates obtained from a given volume of infective sap are smaller with potato virus " X " than they are with strains of tobacco mosaic virus ; they also differ in appearance. The dense white, apparently crystalline precipitates with the intense sheen, so characteristic of tobacco mosaic virus preparations, are not obtained at any stage in the preparations of potato virus " X ". In contrast, these precipitates are light, flocculent and amorphous, and, after centrifugation, form soft, brown, translucent masses. If, however, insufficient ammonium sulphate is added to potato virus " X " preparations to cause precipitation, the fluids do show a sheen, but the sheen is less pronounced than that shown by solutions of tobacco mosaic virus in the same circumstances.

When no further colour can be removed by precipitations with ammonium sulphate, the precipitate is again dissolved at pH 7 and centrifuged until free from insoluble material. The material deposited is dark brown and rich in carbohydrate. It also contains some virus, part of which can generally be recovered in a soluble form after incubation with trypsin. The turbid, brown supernatant fluid is adjusted to about pH 4·5 with acetic acid, and the flocculent precipitate produced is centrifuged down. Generally the whole and always the greater part of the virus precipitates at this pH, but sometimes after prolonged centrifugation at 3,000 r.p.m. the supernatant fluid still shows anisotropy of flow, a part of the virus being soluble at pH 4·5. The virus in solution at pH 4·5 can usually be precipitated by adjusting the pH of the fluid either to 5 or to 4. The acid precipitate is freed from ammonium sulphate by repeatedly suspending in water, centrifuging and discarding the supernatant fluid. As the washings decrease the salt content a larger proportion of the virus becomes soluble at pH 4·5. The actual ratio of the virus soluble to that insoluble at pH 4·5 varies with different preparations, but a part is invariably in each state. No differences have been noticed between the two strains, and both preparations at this stage contain virus soluble and insoluble at pH 4·5. If the preparations at pH 4·5 are frozen solid and allowed to thaw before being centrifuged, most of the virus can be removed at 3000 r.p.m. After this treatment the virus does not dissolve on further washing, and for a given amount of centrifuging it packs more tightly than does the unfrozen

virus. After the precipitate has been washed once or twice, it is suspended in water, frozen solid, thawed, centrifuged, and the washing with water continued. Alternatively, the ammonium sulphate can be removed by dialysis, but this is not to be recommended. When large quantities of virus are handled it is slow, and virus is often lost on an insoluble precipitate which separates during dialysis. The actual loss depends on the quantity of impurities present, and highly purified preparations can be dialysed without losing activity or forming precipitates.

The well-washed, virus-containing, precipitate at pH 4·5 is dissolved by the addition of sufficient N/20 NaOH to raise the pH to 7, and the solution is centrifuged until clear. Tobacco mosaic virus preparations made by precipitation with acid and ammonium sulphate from young infected plants are often colourless, and, if sufficiently concentrated, deposit a liquid crystalline layer. Preparations of the two strains of potato virus " X " made as described from tobacco or *N. glutinosa* plants are clear but brown, and they are never spontaneously birefringent. Preparations from tomato plants are usually less coloured, but otherwise similar. Preparations at this stage are often as infective and as serologically active as those which have been subjected to further fractionations, but these methods are unreliable as tests for purity, for the preparations are still contaminated with sufficient impurities to affect the analytical figures, especially the carbohydrate content, and to prevent the formation of spontaneously birefringent solutions.

Further Purification and the Liquid Crystalline State.

Impure preparations of tobacco mosaic viruses can be fractionated by incubation with trypsin and by sedimenting the viruses in a high-speed centrifuge. Incubation with trypsin is the more satisfactory method, for only in preparations treated thus could no normal plant protein be detected by the sensitive anaphylactic test (Chester, 1936*a* ; Bawden and Pirie, 1937*c*). Both of these methods have been used successfully in the further purification of potato virus " X ".

We have published data (Bawden and Pirie, 1936) on the rate at which trypsin inactivates potato virus " X ". We now find that the activity of partially purified preparations, containing about 0·4 p.c. of solids, is reduced by one-third by incubation with 0·05 p.c. pancreatin (Merk) for 90 minutes at 38° C. and pH 7·4. During incubation there is a loss of anisotropy of flow corresponding to the fall in activity. After six hours in these conditions the preparations show no anisotropy of flow, and the activity is reduced to one-eighth. Before testing the infectivity of the incubated mixtures, the virus must be freed from trypsin by precipitations with ammonium sulphate and acid, for the presence of trypsin in the inoculum greatly reduces the infectivity.

The coloured virus preparations containing about 0·4 p.c. of solids are incubated with 0·05 p.c. pancreatin for only 90 minutes in the conditions stated. Brown material can now be removed by centrifuging, and the virus is precipitated by the addition of one-third of a volume of saturated ammonium sulphate solution. The precipitation is repeated until the supernatant fluid is no longer coloured.

Although the virus is not precipitated until the concentration of ammonium sulphate is greater than one-sixth of saturation, the precipitated virus will not dissolve until the concentration of ammonium sulphate is reduced to about one-twentieth of saturation. One incubation with trypsin is usually sufficient to give colourless preparations, but sometimes, especially with material from older plants, it is necessary to repeat the treatment. The ammonium sulphate is removed by precipitating the virus at pH 4·5, and washing with water. The virus is dissolved in sufficient N/10 NaOH to bring the pH to 7. Before the last centrifugation at pH 4·5 the preparation should be frozen and thawed, otherwise the neutral solution will be too dilute to give a liquid crystalline layer. Even after incubation with trypsin it is difficult to obtain clear, homogeneous preparations of potato virus " X " without using a high-speed centrifuge. With experience it is possible to obtain colourless solutions, which, if more concentrated than about 3 p.c., will separate on standing into two layers. The lower layer is liquid crystalline, has the greater solid content and is clear, while the upper layer is turbid and shows anisotropy of flow.

By the use of trypsin it is possible to purify the coloured virus preparations without losing more than one-third of the virus. High-speed centrifugation is a quicker and more certain method of purification, but less of the virus is recovered in the purified state. When the partially purified preparations, containing from 1 to 2 p.c. of solids, are centrifuged for three hours at 14,000 r.p.m. (16,000 times gravity), from a fifth to a third of the virus is sedimented in the form of turbid birefringent pellets, containing from 15–20 p.c. of solids. When dissolved in from 5 to 10 volumes of water, the pellets give opalescent solutions, from which brown precipitates, rich in carbohydrate, can be removed by light centrifugation. The remaining supernatant fluid is now almost colourless and is spontaneously birefringent. If the solutions are now diluted until they are no longer spontaneously birefringent, and again centrifuged for three hours at 16,000 times gravity, one-half or more of the virus sediments. The birefringent pellets again contain small amounts of insoluble material, but are now colourless. The insoluble material is removed by dissolving the pellets in water, and lightly centrifuging. More virus can be recovered from the supernatant fluids from the high-speed centrifugations either by a further period of centrifugation, or by the use of trypsin.

Prepared in this manner 2 p.c. solutions of both strains of the virus are colourless and only faintly opalescent. At room temperature they are liquid crystalline, and they may keep this property until diluted to about 1·5 p.c. The intensity of anisotropy of flow shown by dilute solutions is of the same order as that shown by solutions of tobacco mosaic virus. Solutions of potato virus " X " are more viscous, and the separation of originally homogeneous solutions into layers takes place more slowly. With a 2·5 p.c. solution some separation is noticeable after a day at 0° C., but the interface between the layers is not sharp until the solution has stood undisturbed for a week. With tobacco mosaic virus the layering phenomenon could be used in the further purification, but with virus " X " it cannot, for we have been unable to get a preparation of this virus to settle into layers until the purification has been carried so far that the differences in properties and concentration of the materials in the two layers are extremely small.

More care must be taken over the storage of solutions of virus " X " than of tobacco mosaic virus, for they are more susceptible to contamination with bacteria, and they lose their birefringence fairly rapidly at room temperature. Preparations of virus " X " kept for some weeks decompose, even when stored in the cold, and become viscous. A 0·5 p.c. solution of old virus may be too gelatinous to flow at 0° C. ; in this condition the preparations are sometimes feebly birefringent, and they are highly thixotropic. When shaken or warmed they will again flow and then show anisotropy of flow, but this is less intense than that shown by a solution of freshly prepared virus of the same concentration.

Although the optical properties of neutral solutions of freshly prepared potato virus " X " and those of tobacco mosaic virus are similar, they are distinct when the viruses are precipitated with acid or with ammonium sulphate. Potato virus " X " precipitates from highly purified preparations are amorphous and difficult to see, although they are thrown down by centrifuging at 3000 r.p.m., and they are detected by the increased readiness of the fluid to hold bubbles. The anisotropy of flow shown by tobacco mosaic virus preparations is greatly increased by precipitating the virus by either of the methods ; neutral solutions of tobacco mosaic virus diluted beyond the point at which they show anisotropy of flow will show the phenomenon if the virus is precipitated with either acid or with ammonium sulphate. In contrast, neutral solutions of potato virus " X ", at concentrations at which they show definite anisotropy of flow, lose this property when the virus is completely precipitated with acid or ammonium sulphate. When acid or ammonium sulphate is added to solutions of potato virus " X " in quantities insufficient to cause precipitation, their behaviour more nearly resembles that of tobacco mosaic virus solutions. At about pH 5, or in about 10 p.c. saturated ammonium sulphate, the potato virus preparations develop a slight sheen and show increased anisotropy of flow.

We have shown that the tobacco mosaic type viruses give paracrystalline precipitates with clupein, resembling those obtained with acid or ammonium sulphate. When clupein is added to neutral solutions of potato virus " X ", precipitates are formed, which also resemble those produced by the addition of acid or ammonium sulphate. The precipitates are amorphous and difficult to see, but are readily separated by centrifuging at 3000 r.p.m. Thus, although we have obtained potato virus " X " in the form of birefringent solutions and birefringent jellies, closely resembling in optical properties those of tobacco mosaic virus, we have been unable to obtain any solid preparations of this virus which could be described as " crystals ". Similarly, there is a difference in the kinds of inclusion bodies formed by the two viruses, for whereas plants infected with tobacco mosaic virus contain both amorphous " X "-bodies and hexagonal crystals, plants infected with virus " X " contain only amorphous " X "-bodies.

Analyses.

The physical properties of potato virus " X " are greatly affected by processes of purification which have no measurable effect on the activity or the analytical figures. The effects with tobacco mosaic virus were much

smaller, and more confident statements could be made about properties. The properties of potato virus " X " described in this paper are those characteristic of preparations believed to be the purest, but it is impossible to make positive statements until some better criteria of purity are available.

Landsteiner and Heidelberger (1923) and Northrop (1930) have pointed out the importance of solubility measurements for determining the purity of protein preparations, and they suggest that when the solubility is independent of the amount of solid protein present it is probable that the preparation is pure. We have been unable to make similar measurements on the solubilities of any of the viruses we have used. No solid phase separates from solutions as they are concentrated, but the fluids become more viscous and turn to gels. If experiments are carried out in conditions in which a solid phase will separate, *e.g.* at pH values or at ammonium sulphate concentrations insufficient for complete precipitation of the viruses, the amount remaining in the supernatant fluid (*i.e.* the solubility) depends upon the intensity of centrifugation.

As the maximum solid content of solutions showing only anisotropy of flow (top layer) depends upon the purity, the formation of a liquid crystalline layer is valuable as an indication of purity. When the two layers are in equilibrium, the difference in concentration between them becomes less as the purity increases. Therefore it is possible that with a perfectly pure virus preparation the two layers could not occur together, for a small addition of water or of virus, or a rise or fall in temperature, would change the whole system from one state to the other.

Preparations of potato virus " X " with reasonably constant carbon, hydrogen, nitrogen and ash contents, are easily made by precipitation methods, for the probable impurities have compositions similar to the virus. The carbohydrate content of a preparation, measured by an orcinol method (Pirie, 1936), is a more reliable index of the purity. The coloured, impure preparations described in this paper have carbohydrate contents of from 5 p.c. to 10 p.c. When these preparations are centrifuged at 14,000 r.p.m., the pellet of virus contains brownish flakes, which do not dissolve when the pellet is suspended in water. A second centrifugation of the redissolved virus, however, usually gives more of these insoluble flakes. Analysis on the supernatant fluids from the high-speed centrifugation, on the insoluble flakes, and on the soluble birefringent sediment, show that the first two are rich in carbohydrate and that the last is not. By repeating the process of centrifugal fractionation a few times, preparations can be obtained with a carbohydrate content of from 2·5 p.c. to 3 p.c. The extra carbohydrate is not an essential constituent of the virus, for reducing the carbohydrate content to this level does not decrease the activity of the virus preparation. We have been unable to reduce the carbohydrate content to below 2·5 p.c. by centrifugation. Removal of this carbohydrate results in inactivation of the virus.

It is well known that proteins readily adsorb other materials and this presumably accounts for the sedimentation of pigmented material, rich in carbohydrate, together with the virus during high-speed centrifugation, and for the fact that the impurity is only gradually removed by repeated centrifugation. Because of this property of proteins it is impossible to assume that material sedimenting in intense gravitational fields is necessarily all virus, for

unless there are other reasons for considering the material to be homogeneous, the sedimentation rate measured may merely be that of an adsorption complex containing virus.

Highly purified, liquid crystalline preparations of potato virus " X " have given the following analyses, and no differences have been detected between the analytical figures of the two strains used.

Carbon . . .	47·7 to 49·5 p.c.
Hydrogen . .	7·1 ,, 7·7 ,,
Nitrogen . .	15·7 ,, 17·0 ,,
Phosphorus . .	0·4 ,, 0·5 ,,
Carbohydrate . .	2·5 ,, 3·0 ,,
Ash . . .	2·0 ,, 2·5 ,,

Within the limits of experimental error, therefore, virus " X " has the same analytical composition as the strains of tobacco mosaic virus and cucumber viruses we have already described. As a nucleic acid can be isolated from inactivated preparations of potato virus " X ", it seems that this, like the other viruses, is also a nucleoprotein.

Activity.

The yields of potato virus " X " obtained have averaged about 100 mg. per litre of infective tobacco sap, but they have varied with the number and type of treatments used to purify the sample. The final yield represents only a part of the virus present in infective sap, for the treatments used destroy much of the virus ; from the serological titres of clarified, infective saps it seems probable that the virus content can be as great as 500 mg. per litre.

The purified virus preparations were tested for their infectivity and their serological activity. Infectivity tests with the S strain were made by the local lesion method, the hosts used being tobacco and *N. glutinosa*. Tobacco proved much the more sensitive, and virus solutions giving some hundreds of lesions on a tobacco leaf often gave only a few on a leaf of *N. glutinosa*. The first-formed true leaves are most sensitive, the young upper leaves giving few lesions. For this reason the first, second and third leaves were used, and 1 c.c. of a solution containing a known weight of the purified virus preparations was carefully rubbed over the surfaces of six leaves. Bald (1937) has shown that the presence of electrolytes reduces the numbers of lesions produced by potato virus " X ", and the dilutions were therefore made in water.

Serological titres were measured by the methods previously described. Antisera were prepared both by a series of intraperitoneal injections of crude infective sap, and by intravenous injections of the purified viruses. The titres of the virus preparations were found to be independent of the method of preparing the antiserum, and to be the same whether the individual strains were titrated against their homologous or their heterologous antisera. Changes in the antiserum affected only the optimal precipitating points of virus preparations and not their precipitating end-points.

The results of infectivity and serological tests with six purified preparations of strain S are given in Table I. The infectivity of preparations of strain G was estimated by inoculating plants with 1 c.c. of solutions containing known

amounts of protein, and finding the smallest amount required to produce systemic infection. Inoculated plants which showed no symptoms were reinoculated with the S strain. If susceptible to infection with the severe strain they were taken as uninfected. The amount of protein required to produce systemic infection varied in different tests from 10^{-7} to 10^{-9} g. The serological titres of the purified preparations of the G strain have also varied from $\frac{1}{3 \times 10^6}$ to $\frac{1}{6 \times 10^6}$. The higher serological titres have usually been obtained with preparations from plants grown in the summer and the lower from plants grown in the winter.

TABLE I.—*Activity of Purified Preparations of the S Strain of Potato Virus "X".*

Serological titre.	Infectivity on tobacco (var. White Burley). Average number of lesions per leaf at various dilutions.					
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
$\frac{1}{4 \times 10^6}$	775	214	35	3	0·6	0
$\frac{1}{6 \times 10^6}$	550	160	40	6	2	0·3
$\frac{1}{4 \times 10^6}$	95	15	2	0·3	0	0
$\frac{1}{3 \times 10^6}$	450	252	31	6	1	0·3
$\frac{1}{6 \times 10^6}$	196	38	9	2	0·3	0
$\frac{1}{5 \times 10^6}$	660	220	42	7	1	0

The serological titre is expressed as the highest dilution, in grams per c.c., at which there was a visible precipitate after 24 hours when 1 c.c. of virus solution was mixed with 1 c.c. of antiserum at 1 in 50. The dilutions in the infectivity tests are given as grams per c.c. Six leaves were rubbed with 1 c.c. The tests were made at different times with different preparations, and the large differences in the infectivities are due to differences in the susceptibilities of the host-plants. The results of serological tests are constant and reproducible, but the results of infectivity tests vary greatly with the host-plants.

Potato virus " X " does not precipitate with tobacco mosaic virus antiserum, and all our purified preparations were tested against this antiserum to ensure that they were not contaminated with tobacco mosaic virus. This was necessary for, although the two viruses have such different physical properties, none of the purification methods described in this paper will separate them completely one from the other. As tobacco mosaic virus is more stable, pure preparations of this can easily be obtained from plants infected with both viruses by exposing the extracted sap to conditions which inactivate potato virus " X " but not tobacco mosaic virus, but as yet it has proved impossible to isolate from such a mixture a preparation of potato virus " X " free from tobacco mosaic virus. All the plants used in this work were also carefully examined for the symptoms characteristic of a mixed infection.

The decrease in numbers of local lesions accompanying dilution is not directly proportional to the dilution, but more are obtained at high dilutions than would be expected. With tobacco mosaic virus this deviation from the expected is much more pronounced, and sometimes dilution may actually result in an increased number of lesions. Bald and Briggs (1937), from a mathematical interpretation of the dilution curve of purified preparations of tobacco mosaic virus, state that the deviation from the expected agrees well with the suggestion that the virus particles are aggregated in concentrated solutions, and that dilution breaks up the aggregates. Although this explanation may account for the behaviour of purified preparations when diluted, it is unlikely that it can account for the deviations in all dilution curves. For example, the dilution curve of crude tobacco mosaic sap resembles that of purified virus preparations. As we have shown that aggregation into rods is largely an effect of purification, it is improbable that the results obtained by diluting crude sap can also be explained solely by the breaking up of the virus aggregates. It is perhaps more probable that the deviations in the dilution curve of crude sap are caused by relatively inactive complexes of virus-host material breaking up when diluted. Some support for this comes from the fact that the addition of healthy tobacco sap to infective sap causes a reduction in the infectivity, but when the mixtures are highly diluted with water they regain most of their activity.

As solutions of the purified virus " X " show anisotropy of flow, and in some conditions show a sheen, the particles in them are presumably rod-shaped. These rods also appear to be formed by the linear aggregation of smaller particles. The fact that the fall in infectivity with dilution is more proportional to the dilution with purified preparations of potato virus " X ", suggests that the aggregated rods of this virus are more stable than those of tobacco mosaic virus, or, alternatively, if they do break up on dilution, that they are built up of fewer components.

Evidence that small particles of virus " X " aggregate to form rods when precipitated, comes from experiments comparing the filterability, activity and optical properties of crude infective sap with those of the purified preparations. These experiments are similar in type to those described in more detail with tobacco mosaic virus (Bawden and Pirie, 1937*a*). Smith and Doncaster (1936) found that potato virus " X " in untreated, clarified sap passed through collodion membranes with an average pore size of 150 mμ., but after partial purification it would not pass through a membrane with an average pore size of 240 mμ., and they suggested that the virus aggregated. Filtration experiments with out purified preparations have given similar results ; the virus in untreated sap has passed through membranes with an average pore size of 150 mμ., but with solutions of the purified virus we have been unable to obtain infectious filtrates through membranes with an average pore size of 400 mμ. The virus need not be carefully purified to show this reduction in filterability, for after one precipitation with acid or ammonium sulphate it will no longer pass through a membrane of pore size 150 mμ.

Similarly treated potato virus " X " preparations with the same serological activity are equally infective, but preparations treated differently can have the same serological activity but different infectivities (Bawden, 1935). Crude

sap always produces more lesions than a solution of the purified virus with the same serological titre. Any type of aggregation of the virus particles would result in a reduction of the filterability, and in a reduction of the ratio of infectivity to serological activity. We suggest that the particles aggregate to form rods because of the optical properties of the purified preparations, for only an aggregation which results in a definite departure from the spherical shape could increase the degree of anisotropy of flow shown by a given amount of virus. Direct comparisons of the optical properties of the virus before and after treatment cannot often be made, because the virus content of clarified sap is too small for anisotropy of flow to be seen. After precipitation with ammonium sulphate and resolution in a volume of water equal to that of the original sap, a few samples have shown anisotropy of flow, and when dissolved in a tenth of a volume of water they all show the phenomenon.

Inactivation of Potato Virus " X ".

We have shown previously that potato virus " X " preparations can be inactivated in two ways. Some treatments cause loss of both serological activity and infectivity, whereas others, *e.g.* nitrous acid and formaldehyde, destroy infectivity without in any way affecting the serological activity (Bawden 1935 ; Bawden and Pirie, 1936 ; Bawden, Pirie and Spooner, 1936). With tobacco mosaic virus similar results were obtained, and treatments of the second type were found to have no effect on the ability of the virus preparations to show anisotropy of flow and to form liquid crystalline solutions (Bawden and Pirie, 1937*a*). With the purified preparations of virus " X " we now find that treatments denaturing the protein cause loss of infectivity, serological activity, and the ability to show anisotropy of flow, whereas treatments causing loss of infectivity without denaturing the protein leave the serological activity and the ability to show anisotropy of flow unaffected.

Heating.—Purified preparations of tobacco mosaic virus are unaffected by long periods of heating at 70° C., but when heated to above 90° C. they break down to give an insoluble coagulated protein and a soluble free nucleic acid. Potato virus " X " is much more sensitive to heating, and the effect of heat seems to be different. Heating neutral salt-free solutions for some time at 60° C. has no apparent effect on their activity or on their ability to show anisotropy of flow. A few minutes at temperatures above 66° C. is sufficient to inactivate ; the solutions become more opalescent, and lose their infectivity, serological activity and anisotropy of flow, but no precipitate is formed. Further heating has no apparent effect, and such solutions can be boiled without the separation of a protein coagulum. The addition of a little neutral salt to the boiled solution causes the precipitation of some denatured protein, which is free from phosphorus. If the fluid is now acidified gradually, a succession of protein fractions is obtained, and the precipitation of protein is complete when the pH of the fluid is about 4. These fractions contain phosphorus, the amount increasing with the increase in acidity required to cause precipitation. The addition of a strong acid to the remaining supernatant fluid causes the separation of a gummy precipitate of crude nucleic acid, apparently similar to that isolated from inactivated tobacco mosaic virus.

If the virus preparation used for such an experiment has a carbohydrate content of 3·5 p.c. or higher, the first precipitates formed are rich in carbohydrate, although they may contain less than 0·1 p.c. of phorphorus. By incubating this precipitated protein with trypsin a brownish precipitate can be made ; this contains a pentosan similar to that which can be removed from the preparation by repeated high-speed centrifugation, and it accounts for most of the carbohydrate of the first denatured protein. Good preparations of the virus, containing only 2·5 p.c. of carbohydrate, give no precipitate of this type, and this is confirmatory evidence for the view that virus preparations containing more than this amount of carbohydrate can be still further purified.

Drying.—If leaves, or expressed sap, from plants infected with potato virus " X " are completely dried they lose their infectivity (Smith, 1933). Highly purified preparations of the virus can be dried and still be active, but the activity and physical state of the dried virus are so greatly affected by the salt content of the solution, and by the method used for drying, that reproducible results are not easily obtained. Alternate freezing and thawing has no effect on the virus solutions, and the virus solutions were dried frozen in a vacuum desiccator.

With incomplete drying, that is, using sodium sulphate or ammonium sulphate as the desiccating agent, the virus film is birefringent, and when taken up in water shows little loss of activity. When completely dried over phosphorus pentoxide, the virus film is not birefringent, and on resolution there is always reduction in the infectivity and serological activity. The loss depends on the pH value of the solutions dried : in pH 6 buffer the activity is reduced to about one half of the original, while at pH 4 and pH 8 more than 90 p.c. of the activity may be lost, and much of the material is rendered insoluble. After complete drying at from pH 6 to pH 7, about one-quarter of the material is not soluble in water, and the soluble portion has lost to a considerable extent its ability to show anisotropy of flow. The soluble part can be fractionated by both high-speed centrifugation and by incubation with trypsin, and fully active virus recovered showing the usual amount of anisotropy of flow. It seems, therefore, that the disintegration products produced by drying potato virus " X ", like those from tobacco mosaic virus, impede the arrangement of the particles that is necessary for anisotropy of flow to be seen. The insoluble part of the dried virus has a slightly higher nucleic acid content than the original virus, and when extracted with 80 p.c. acetic acid it readily gives a residue of crude nucleic acid.

Acid.—The virus precipitates optimally from pure preparation at about pH 4·5, but it can be completely sedimented by long periods of centrifugation at low speeds over a considerable range on both sides of this point. At room temperature and pH 4 denaturation of the protein is slow, but at pH 3 a few hours are sufficient to denature and inactivate.

When treated with 80 p.c. acetic acid, both strains of potato virus " X " break down to give proteins and nucleic acid, but it is more difficult to separate the two components than it is with similarly treated viruses of the tobacco mosaic type. The most promising results have been obtained by adding four volumes of acetic acid to the tightly-packed precipitates formed by centrifuging the virus preparations at pH 4·5. The proteins rapidly dissolve and leave

a residue of crude nucleic acid, or nuclein, containing about 4 p.c. of phosphorus. The nucleic acids obtained from the strains of virus " X " have not yet been investigated in any detail.

Alcohol.—The addition of an equal volume of 80 p.c. alcohol to infective sap precipitates virus " X " completely, and the sediment must be washed several times in water before all the virus will go into solution. In the purified salt-free preparations, precipitates form only slowly in 50 p.c. alcohol; with 60 p.c. or more alcohol the precipitation is rapid, and the sedimented virus readily redissolves in water. Denaturation does not proceed at all rapidly until the concentration of alcohol reaches 85 p.c. In the presence of calcium chloride the purified virus is immediately precipitated by 40 p.c. alcohol, and the resulting sediment is insoluble in water. All the virus, however, is not inactivated, for some lesions are obtained when plants are rubbed with the insoluble precipitate. Janssen (1937) has described a similar effect with the virus causing foot-and-mouth disease of cattle.

Sodium dodecyl sulphate.—Pancreas contains, in addition to trypsin, a substance soluble in petrol ether which inactivates potato virus " X " (Bawden and Pirie, 1936). This substance also inactivates some animal viruses (A. Pirie, 1935), and the type of inactivation is presumably analogous to that obtained with soap, bile and other related surface-active substances, which are well known as inactivators of certain viruses. A. Pirie (1938) has also studied the inactivation of several animal viruses by surface-active substances used as commercial wetting and spreading agents (long chain sulphonic acids, and the sulphuric half esters of long chain alcohols). We find that these agents rapidly inactivate potato virus " X ", and of those that we have tried sodium dodecyl sulphate is the most effective.

TABLE II.—*Effect of Incubating 1 p.c. Solutions of Potato Virus "X" with 0·33 p.c. Sodium Dodecyl Sulphate.*

Time of incubation.	Serological titre.	Infectivity. Average number of lesions per leaf. Inoculum virus content 10^{-4} gm. per c.c.
Mixed, but not incubated	$\frac{1}{2 \times 10^6}$	420
40 mins.	$\frac{1}{10^5}$	45
150 mins.	$\frac{1}{10^4}$	3·6
300 mins.	No. precipitate at $\frac{1}{2 \times 10^3}$	0

Table II shows the results of one inactivation experiment. 1 c.c. of fluid containing 10 mgm. of purified virus and 3·3 mgm. of sodium dodecyl sulphate was incubated at 38° C. At different intervals 0·2 c.c. of the mixture were removed and immediately diluted with 1·8 c.c. of water. Dilution to this extent almost stops the action of the wetting agent, for the virus is but little

affected by 0·03 p.c. sodium dodecyl sulphate, although the more concentrated virus solution is rapidly inactivated by 0·3 p.c. The inactivated virus shows no anisotropy of flow, and the anisotropy of flow diminishes during incubation at approximately the same rate as the activity. Nothing can be sedimented from the completely inactivated virus preparations by centrifuging for 3 hours at 16,000 times gravity, but the protein can still be precipitated with acid or with ammonium sulphate.

Irradiation.—Solutions of potato virus " X " exposed to either X-rays or to ultra-violet light lose their infectivity, the loss being proportional to the time of irradiation. The inactivated preparations are unchanged in appearance, and they still show anisotropy of flow and react specifically with virus anti-serum.

TABLE III.—*The Effect of X-rays and Ultra-violet Radiation on Solutions of Potato Virus "X".*

Radiation.	Time.	Serological titre.	Infectivity. Average number of lesions per leaf. Concentration of inoculum 10^{-4} gm. per c.c.
X-rays	100 mins.	$\frac{1}{2 \times 10^6}$	22
	360 ,,	$\frac{1}{2 \times 10^6}$	0
Ultra-violet	3·3 mins.	$\frac{1}{3 \times 10^6}$	40
	10 mins.	$\frac{1}{3 \times 10^6}$	1
	30 ,,	$\frac{1}{2 \times 10^6}$	0
Control	Not irradiated	$\frac{1}{3 \times 10^6}$	342

The results of one experiment are given in Table III. 0·2 p.c. solutions of purified virus were used. In the ultra-violet test a layer 1 mm. thick, under a quartz plate, was irradiated at a distance of 8 cm. from a 2 amp. lamp. In the X-ray test, the solution was irradiated at a distance of 8 cm. from a copper anticathode of an X-ray tube run at 30 k.v. and 20 m.a.

Hydrogen peroxide.—Stanley (1936) has shown that hydrogen peroxide inactivates tobacco mosaic virus without affecting its specific reaction with antiserum. We have confirmed this, and also obtained similar results with potato virus " X ". Virus " X ", however, is more sensitive to oxidation, and the treatment must be carried out more cautiously than with tobacco mosaic virus. Treatment with concentrated H_2O_2 denatures the protein, and causes loss of all characteristic properties, while treatment with dilute solutions destroys infectivity without affecting the serological reactions or the anisotropy of flow. At room temperature, solutions of potato virus " X " are rapidly inactivated by H_2O_2 at concentrations between 0·2 p.c. and 1 p.c. without the serological activity being affected, but concentrations above 1 p.c. denature the virus.

DISCUSSION.

The isolation of tobacco mosaic virus as a nucleoprotein was little reason for thinking that other viruses were necessarily chemically similar, for it is an exceptionally stable virus and its properties differ greatly from those of other viruses. Potato virus " X " is more stable *in vitro* than many viruses, but its properties are more typical of the virus group as a whole, and the recognition of this virus as a nucleoprotein does suggest that other viruses may also be found to be proteins. It is interesting to note that nucleoprotein has been found in all the purified preparations of viruses and bacteriophages that have been tested for it, and in this respect the viruses seem to be similar to all known cells. The plant viruses which we have purified are apparently solid masses of nucleoprotein, for the preparations contain no appreciable amounts of water or of diffusible substances, and the X-ray measurements show that the virus particles have a perfect internal regularity of the type found in some large protein molecules. Recent work on coli phage (Schlesinger, 1936) and staphylococcus phage (Northrop, 1937) suggests that these are also nucleoproteins. It is perhaps improbable that this will be found to be a general feature of viruses, and it is more likely that some viruses, especially the larger ones, will be found to be more highly organized and to contain substances other than nucleoprotein. With vaccinia elementary bodies there is already some evidence in support of this view (Hughes, Parker and Rivers, 1935; Salaman, 1937).

In spite of the chemical relationship which exists between the known purified viruses, there are considerable differences in their properties. For example, potato virus " X " and tobacco mosaic virus break down differently when heated, and the former is susceptible to tryptic digestion while the latter is not. Also, nothing analogous to the linear aggregation, so characteristic of the purified preparations of these two virus types, has been noticed with the animal viruses or phages, but that these may be affected by purification processes is suggested by the observation of Schlesinger and Galloway (1937) that foot-and-mouth virus is more readily adsorbed on surfaces when partially purified than it is when in the crude state. The power of linear aggregation, however, does not seem to be a general property of plant viruses, for we have been unable to obtain anisotropic preparations of some plant viruses, although highly active, protein-containing sediments can readily be obtained by high-speed centrifugation.

The method by which these viruses increase in the infected plants is unknown, but the fact that the presence of strain G prevents the multiplication of strain S suggests that the two behave identically. Healthy plants do not seem to contain any proteins similar to those isolated from the infected plants. Healthy plant sap heated to 60° C. gives a coagulum similar to that obtained by heating infective sap, but a smaller precipitate separates when the remaining supernatant fluid is one-quarter saturated with ammonium sulphate. This precipitated material shows no anisotropy of flow when redissolved; it is also less stable than that obtained from infected plants, and the quantity becomes much reduced by repeated precipitations with ammonium sulphate. Nothing can be sedimented from solutions by 3 hours centrifugation at 16,000 times gravity.

Although the 2 strains used cause such different symptoms, we have found no significant differences between them, either in the yields obtained or in the physical or chemical properties of the isolates. No doubt there are small differences of the type found between the various strains of tobacco mosaic virus, and Chester's (1936*b*) experiments show that the strains possess specific as well as common antigens. As plants infected with the different strains do not contain greatly different amounts of virus, it is improbable that the symptoms are a direct result of virus multiplication in weakening the host. It is more probable that the symptoms are the result of a secondary effect of the virus on the host, produced by active side groups, and that the different strains cause their characteristic symptoms because they possess specific side groups. The fact that each strain contains specific antigens is some support for the view that they possess such specific groups.

SUMMARY.

Methods are described for the isolation of nucleoproteins from *N. tabacum*, *N. glutinosa* and *Lycopersicum esculentum*, infected with the S and G strains of potato virus " X ". These have not been isolated from healthy plants, and the available evidence suggests that they are the viruses themselves. Infections were obtained with 10^{-9} gm., and specific serological reactions with $\frac{1}{6 \times 10^6}$ gm. Concentrated solutions are spontaneously bi-refringent and dilute solutions show anisotropy of flow; when sedimented by high-speed centrifugation the nucleoproteins form birefringent jellies, but when precipitated with acid or ammonium sulphate the material appears amorphous under the microscope.

The filterability of the virus after purification is less than that of the virus in untreated sap, and purification appears to cause the virus particles to aggregate into rods.

Two types of inactivation are described: one leads to a loss of infectivity without changing the optical properties or serological reactions, whereas the other denatures the protein and destroys all three. The effects of heating, drying, acid, alcohol, sodium dodecyl sulphate, irradiation with X-rays and ultra-violet light, and hydrogen peroxide are described. The properties of virus " X " are compared with those of tobacco mosaic virus, and the results discussed.

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THE ISOLATION OF ANTIGENIC SUBSTANCES FROM STRAINS OF *BACT. TYPHOSUM*.

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IN a recent paper one of us (W. T. J. M., 1937) described a method for the isolation of a specific antigenic substance from *Bact. dysenteriæ* (Shiga). This substance is apparently free from protein and as far as it has been determined, it induces in the rabbit an antibacterial response indistinguishable from that produced by the untreated bacterial cells.

We have extended the use of this method to the study of *Bact. typhosum*; this communication deals with the extraction of two of its antigenic components and with some of their immunological and chemical properties.

Methods.

The strains of Bact. typhosum *examined.*—These are representative of the O agglutinable or O forms, the O inagglutinable or O + Vi forms and the so-called rough Vi forms believed to contain only Vi antigen; they have been described by Felix and Pitt (1934*a* and *b*, 1935). We are indebted to our colleague Dr. Felix for these strains and also for valuable information concerning their maintenance.

The maintenance of the cultures.—The strains were kept at 37° C. on a trypsin-digest agar; they were passaged daily and plated on this medium at least once a week. The antigenic condition of each strain was regularly