SUMMARY.

In this preliminary paper the action of fibrinolysin in producing a change in the antitoxic pseudoglobulin molecule, so that it becomes disaggregated into protein components having different physical and chemical properties, is reported. By taking advantage of this property of the enzyme, a method of critical differential heat denaturation has been evolved ás a method for the further purification of antitoxins.

The specific action is not limited to fibrinolysin, but appears to be a property of all the proteolytic enzymes provided they are used under the correct conditions. To be of use for this method the action of the enzymes must be so limited that no hydrolysis or digestion in its generally accepted sense takes place ; if this occurs differential denaturation fails, because obviously it cannot affect non-coagulable protein fragments.

Based on the methods outlined, a process for the large scale purification of antitoxins has been evolved, and will form the subject of other papers. As a point of interest it may be stated that by using these methods, antitoxin of such purity that all the protein present can be specifically precipitated by diphtheria toxin has been prepared experimentally.

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CRYSTALLINE PREPARATIONS OF TOMATO BUSHY STUNT VIRUS.

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

From the Rothamsted Experimental Station, Harpenden, and the Biochemical Laboratory, Cambridge.

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IT has been shown that proteins, with characteristic properties and infective at high dilutions, can be isolated from plants infected separately with each of three strains of tobacco mosaic virus, two strains of potato virus " X ", and cucumber viruses 3 and 4 (Bawden and Pirie, 1937*a*, *b* ; 1938*a*). The proteins have not been found in healthy plants, but have been isolated from different species of infected plants. The particular protein isolated is determined solely by the infecting virus, is independent of the species of host plant, and has the stability typical of that virus. It is highly probable that the proteins are the viruses themselves—a view now becoming more widely accepted (Laidlaw, 1938)—but proof of this can be obtained only by a conclusive demonstration

that the protein preparations are pure. This, however, is impossible in practice, but experiments of a number of different kinds have failed to show any appreciable heterogeneity.

Although these viruses have different physical and chemical properties they are all nucleoproteins with similar analytical compositions, and they all have rod-shaped particles. Because of their shape they are readily orientated and the purified preparations have some properties characteristic of crystalline materials, but no true crystals, *i. e.* visible solids in which the particles are arranged with a three-dimensional regularity, have yet been produced *in vitro*. Concentrated solutions are liquid crystalline, dilute solutions show the phenomenon of anisotropy of flow, and birefringent jellies can be prepared. The solids formed by precipitating potato virus " X " with acids or salts are amorphous, while the precipitates of tobacco mosaic type viruses consist of needle-shaped paracrystals or micro-tactoids. These have been described as true crystals (Stanley, 1937 ; Wyckoff and Corey, 1936), but they are one of the forms of the liquid crystalline state, for the constituent particles are regularly arranged in two dimensions only (Bernal and Fankuchen, 1937).

We have now isolated from plants infected with Bushy stunt virus (Smith, 1935 ; Ainsworth, 1936 ; Bawden and Pirie, 1938*b*) a protein with the characteristic properties of this virus, and which has not been found in healthy plants. Like those previously isolated it is a nucleoprotein, but it differs from them in its analytical composition and in many other respects. Its particles are probably spherical, or at least not appreciably elongated, and the purified preparations, both liquid and solid, are isotropic. When precipitated with salts this virus forms true crystals, mainly rhombic dodecahedra ; these belong to the cubic system and, as would be expected, are isotropic. There is no conclusive evidence that our preparations of Bushy stunt virus are pure, but as they are fully crystalline, and show a high degree of homogeneity in different tests, a stronger claim can be made for their purity than for the liquid crystalline preparations of the other viruses, and in this paper the protein will be called virus.

METHODS.

The original source of the virus used in this work was an infected tomato plant supplied by Dr. Kenneth M. Smith. Bushy stunt virus is more difficult to transmit than the viruses we have previously investigated and the reactions of infected plants are more variable. It produces necrotic, local lesions on a number of species, including tobacco and *Nicotiana glutinosa*, but systemic infection has been obtained only in tomato and *Datura stramonium*. Most of our isolations have been made from tomatoes, but the same protein has been isolated from infected *Datura*.

The reasons for the variations in symptoms in tomato are not fully understood, but the reactions depend greatly upon the age and condition of growth of the inoculated plants. The first symptoms are local necrotic rings, and often no further effect is obtained. Systemic infection is first shown by a curling downwards and inwards of the petiole of the inoculated leaf, and by the check in growth of the main stem. Young seedlings then develop a severe

general bright yellow mottle with necroses, and are sometimes killed. Older plants react less severely, usually developing only scattered yellow blotches and necroses. Plants infected for a period of some weeks may produce numerous side shoots, giving the plants a characteristic bushy appearance from which the disease takes its name.

The yields of virus isolated have averaged about 50 mg. per litre of expressed sap, but they have varied widely, and are directly correlated with the severity of the symptoms shown by the plants. Sap from seedlings showing the severe general mottle may contain as much as 0·5 mg. of virus per c.c., while that from systemically infected plants showing only scattered blotches may contain less than 0·02 mg. per c.c.

Young plants are therefore used and cut down about three weeks after infection when they are showing the most definite symptoms, minced in a meat mincer and the sap expressed through bolting silk. The sap is heated to 60° C., cooled, and the bulky green coagulum produced is removed by a few minutes' centrifugation at 3000 r.p.m. The clear brown supernatant fluid is rather more than one-quarter saturated with ammonium sulphate by the addition of 200 gm. per litre. A small fawn-coloured precipitate separates, which readily sediments and packs tightly after a short period of centrifugation at 3000 r.p.m. The coloured supernatant fluid is discarded, and the precipitate is suspended in a volume of water equal to about one-tenth of the volume of original sap. The fluid is centrifuged until clear, and the preparation is further concentrated by a second precipitation with quarter-saturated ammonium sulphate solution. The virus-containing precipitate now dissolves in water to give a clear but darkly coloured solution, from which a small amount of insoluble material can be removed by centrifugation.

The type of precipitate of Bushy stunt virus produced by ammonium sulphate depends largely on the conditions of precipitation. Rapid precipitation in the presence of much salt gives amorphous material, while slow precipitation gives crystals. The virus has different solubilities in the two states, the amorphous material being considerably more soluble in dilute ammonium sulphate solution at 0° C. than at room temperature, whereas the crystals are not. By applying these two properties of the virus it can be freed completely from coloured contaminants, and obtained in a fully crystalline state with careful precipitations with ammonium sulphate. In this respect Bushy stunt virus differs sharply from tobacco mosaic virus and potato virus " X ", for they adsorb impurities much more readily, and it is usually impossible to obtain colourless, homogeneous preparations of them merely by repeated precipitations with acids and salts.

A saturated solution of ammonium sulphate is added slowly to the coloured virus preparations at room temperature with constant stirring until there is a trace of opalescence not dissipated by stirring. If kept at room temperature the opalescence soon increases and precipitation is complete in about an hour, but the material which separates is amorphous and darkly coloured, and better fractionation is obtained at 0° C. Unless too much ammonium sulphate has been added, the amorphous precipitate dissolves and the fluid becomes clear on cooling to 0° C., but after some time, the exact time depending upon the pH value and the virus content of the preparation, a crystalline deposit separates,

appearing first on the side walls and later as a precipitate at the bottom of the vessel. The process of crystallization at 0° C. is allowed to proceed for about 48 hours, when the fluid is centrifuged for a few minutes in a chilled centrifuge. The supernatant fluids are decanted, and as they warm up to room temperature a coloured amorphous precipitate separates, from which more virus can be recovered.

Bushy stunt virus differs greatly from the other viruses we have purified in that there is apparently no pH value at which it is insoluble in water or dilute salt solutions. At about pH 5, however, the crystals that separate from ammonium sulphate solutions dissolve only slowly in water. In contrast, precipitated amorphous material dissolves rapidly. The crystals are therefore washed by being suspended in about three times their volume of water and then centrifuged off again immediately. The rapid washing dissolves any amorphous pigmented materials, and these are removed in the supernatant fluid. The crystalline deposits are again suspended in water and left for an hour with frequent stirring; the crystals dissolve and a small amount of insoluble material can be removed by centrifugation.

Preparations of the virus at this stage are faintly opalescent but almost colourless. They will usually crystallize completely if the process described above is repeated. The initial turbidity at room temperature is produced by the addition of from one-sixth to one-fifth of a volume of saturated ammonium sulphate solution, and crystallization at 0° C. is complete in 2 days. The crystals are centrifuged off at or near 0° C., rapidly washed in water, and then dissolved in water. The solution is brought to about pH 3 by the addition of HCl and thoroughly dialysed against distilled water. Dialysis at pH 3 often gives a further fractionation not obtained at pH 7, for a colourless or fawn precipitate, consisting of a mixture of carbohydrate and denatured virus, separates and is centrifuged off when dialysis is complete.

The crystallization mother liquors and the water used for washing the crystals contain some virus. This can be recovered, after further concentrating the solutions by precipitation with ammonium sulphate, by repeating the process of slow crystallization at 0° C. Alternatively, it can be recovered by sedimenting the virus in a high-speed centrifuge or by incubating the preparations with pancreatin, which destroys contaminating proteins but not the virus, and then crystallizing the virus with ammonium sulphate. Incubation with pancreatin is the more convenient method, for Bushy stunt virus sediments more slowly in a high-speed centrifuge than viruses of the tobacco mosaic type. When a 1 per cent. solution of the virus is centrifuged for 2 hours at 14,000 r.p.m. in a rotor of 8 cm. radius only about one-quarter of the virus sediments. The pellets of sedimented virus are clear, and differ from those of tobacco mosaic virus and potato virus " X " in being isotropic. The material finally prepared as crystals has identical chemical, physical and serological properties regardless of the method used for purification.

The preparations can be crystallized completely so that microscopic examination of the ammonium sulphate precipitates shows only crystals in the form of rhombic dodecahedra. In order to obtain homogeneous preparations of this type it is essential to do the crystallization in dilute ammonium sulphate solution at 0° C., for preparations wholly crystalline in these

conditions may give amorphous precipitates if much ammonium sulphate is added at room temperature. It is noticeable that larger crystals are formed from slightly impure preparations than from those which have been repeatedly crystallized. This effect is illustrated in Fig. 1. The crystals were photographed on the wall of a vessel in which the same preparation was twice crystallized. The larger crystals were produced by the first crystallization and the smaller ones by the second. In addition to the rhombic dodecahedra, occasionally a few icositetrahedra are formed when slightly impure preparations are allowed to crystallize slowly, and at the top of the fluid there can usually be found crystals which appear to be flat hexagons. Such crystals have been isolated by Dr. F. M. L. Sheffield and inoculated to plants. They caused typical Bushy stunt symptoms, and it is therefore probable that they are merely malformed crystals of the same protein, produced by unequal crystal growth.

Preparations of Bushy stunt virus can also be crystallized with sodium sulphate and magnesium sulphate, but these offer no apparent advantage over ammonium sulphate. The crystal form is the same, and the precipitated material at room temperature is again largely amorphous and more soluble at 0° C. than at room temperature.

Tobacco mosaic virus and potato virus " X " form with clupein sulphate insoluble complexes having microscopic appearances similar to the precipitates of these viruses produced with acid and ammonium sulphate. The addition of clupein sulphate to salt-free solutions of Bushy stunt virus produces an amorphous precipitate, which resembles the ammonium sulphate precipitate in being more soluble at 0° C. than at room temperature. Also, after standing for some time at 0° C. a part of the preparation turns into rather misshapen dodecahedra. The clupein complexes of all three viruses have similar properties ; they contain 5 per cent. or less of clupein sulphate and dissolve in salt solutions more concentrated than M/10, but the minimum value necessary for solution depends on the salt used, the pH value, and the amount of clupein sulphate present.

Nothing whatever can be isolated from healthy tomato plants by the purification method described. This is largely the result of two of the processes used ; heating the sap to 60° C. denatures most of the normal plant proteins precipitated by one-quarter saturated ammonium sulphate solution, and the residual protein is no more soluble in ammonium sulphate solution at 0° C. than at room temperature. The behaviour of some of the constituents of normal plant sap is described in the accompanying note.

The solid content of the purified virus preparations is determined by freezing a sample and drying it *in vacuo* over P_2O_5 while frozen. The dried material is highly hygroscopic, and rapidly adsorbs from 5 to 8 per cent. of water on exposure to the air. The dried preparations differ from those of tobacco mosaic virus for they are completely insoluble in water or in dilute salt solutions. Solutions of the virus have been dried at a number of different pH values, in the absence of salts and in various buffer solutions, frozen and unfrozen, but all were insoluble and quite inactive. Freezing and thawing was found to have no effect on the viruses with which we have previously worked, but it denatures Bushy stunt virus. Precipitates develop in solutions frozen and thawed and the virus is inactivated.

RESULTS.

Serological Reactions and Infectivity.

Bushy stunt virus is antigenic, and sera giving specific precipitates at dilutions of over 1/1000 were prepared by giving rabbits a single intravenous injection of 2 mg. of purified virus. The serological titres of the virus preparations have largely been used to determine activity, for they are much more constant than infectivity tests, which vary widely with individual plants. The titres were determined by adding 1 c.c. of antiserum at a constant dilution to a series of tubes each holding 1 c.c. of solution containing a known weight of antigen, and they are expressed as the smallest weight in grammes per c.c. of antigen solution to give a precipitate visible to the naked eye. The tubes were kept for 12 hours with the fluid columns half immersed in a water-bath at 50° C., and the readings were made after a further 12 hours at room temperature.

The serological titres of the crystalline Bushy stunt virus preparations have been consistently lower than those of the liquid crystalline preparations of tobacco mosaic virus and potato virus " X ". This difference probably has its origin in the different shapes of the viruses. The precipitates of tobacco mosaic virus and virus " X " with their antisera are of the same type, but differ greatly from those of Bushy stunt virus. They form rapidly, are flocculent, have an open structure, and after settling take up a large volume, *i. e.* they closely resemble the precipitates obtained with bacterial flagellar (" H ") antigens. On the other hand, the precipitates of Bushy stunt virus with its antiserum are of the type obtained with bacterial somatic (" O ") antigens. They form more slowly, are granular, and settle out into compact masses at the bottom of the tubes. The differences are illustrated in Fig. 2. The central tube contains saline. The three left-hand tubes contain 1 c.c. of Bushy stunt antiserum at 1/50 and 0·1, 0·05 and 0·025 mg. of Bushy stunt virus in 1 c.c. respectively. The three right-hand tubes contain 1 c.c. of tobacco mosaic virus antiserum at 1/50 and 0·1, 0·05 and 0·025 mg. of tobacco mosaic virus in 1 c.c respectively. After adding the antisera to the virus solutions, the tubes were immediately placed in the water-bath at 50° C. A precipitate was at once obvious in the most concentrated solution of tobacco mosaic virus, and within two minutes there was a bulky precipitate in this and precipitation was also apparent in the more dilute solutions. The first signs of precipitation were not seen in the most concentrated solution of Bushy stunt virus until the tubes had been in the bath for 6 minutes, and it was 30 minutes before the most dilute solution precipitated. After 12 hours in the water-bath the tubes were left at room temperature, and they were photographed after standing undisturbed for a further 12 hours.

The particles of tobacco mosaic virus and of potato virus " X " can be readily orientated by streaming and this, and other allied phenomena, show that they are greatly elongated. Structurally, therefore, the particles of these viruses will resemble flagella. It is to be expected that when these rods flocculate and come together they will become entangled and support one another, giving a fluffy open structure. The particles of Bushy stunt virus cannot be orientated by streaming, and there is no evidence to suggest that

they are not spherical. Spheres, coming together at random, will pack more tightly than rods, and a precipitate composed of spheres will probably be more compact than one made up of rods. Thus, for a given weight of antiserum precipitate, it is to be expected that either potato virus " X " or tobacco mosaic virus will produce a more voluminous and easily seen precipitate than Bushy stunt virus, and, if the viruses precipitate similar amounts of serum protein at the antigen dilution end-point, it is to be expected that the rod-shaped viruses will give visible precipitates at higher end-points than Bushy stunt virus.

There are no measurements of the ratio of antigen to antibody in these virus-antiserum precipitates. However, Boyd and Hooker (1934 ; 1936) have pointed out that the amount of antibody combining with a given amount of antigen corresponds closely with the amount necessary to form a monomolecular layer of antibody particles over the surface of the antigen particles, and with the existing knowledge on the sizes of the virus particles this ratio can be calculated roughly. If the tobacco mosaic virus particles are long prisms the ends can be neglected and it is unnecessary to know their length, for the ratio of antigen to antibody will be approximately the ratio of the cross-sectional area of the virus to the area of a ring of antibody particles on the surface of the virus particle. The cross-sectional area is 20,100 sq. A (Bawden and others, 1936) and, within the degree of accuracy needed for this argument, we may regard the rod as a cylinder of about 8 mμ. radius, although it is highly probable that the rods do not have a strictly circular section. Kabat and Pedersen (1938) give the molecular weight of rabbit pneumococcus antibody as 157,000, and presumably the antibodies to the viruses will have a similar value. Such a particle, if spherical, would have a radius of about 3·7 mμ. As a first approximation, therefore, the ratio of virus to antibody in the serum precipitates of tobacco mosaic virus should be the ratio of the area of a circle of radius 8 mμ. to the area of the ring between circles of 8 mμ. and 11·7 mμ. radius, *i. e.* 1 : 1·1.

As Bushy stunt virus appears to have a spherical particle, its molecular weight can be legitimately calculated from its sedimentation constant. McFarlane and Kekwick (1938) find the sedimentation constant to be $S_{20} = 146 \times 10^{-13}$ and the density to be 1·353, and give the molecular weight as 8,800,000 and the radius as 13·7 mμ. With this virus, therefore, the ratio of antigen to antibody in the precipitates will be approximately the ratio of a volume of a sphere of 13·7 mμ. radius to the volume of a shell of 13·7 mμ. internal radius and 17·4 mμ. external radius, *i. e.* 1 : 1·0. The only large particles of size comparable with the viruses for which such ratios have been measured are the hæmocyanins (Boyd and Hooker, 1936), and the values of from 0·6 to 1·4 agree sufficiently well with our calculated ratios to suggest that they are of the right order. It seems reasonable to assume, therefore, that tobacco mosaic virus and Bushy stunt virus precipitate comparable amounts of antibody, and that this quantity is approximately equal to the weight of virus in the system at optimal proportions, and not greatly in excess of it in the region of antibody excess. When particles of the size of these viruses give precipitation end-points of 10^6, the precipitate in 2 c.c. final volume of fluid will be made up of 0·001 mg. of virus and not much more than 0·001 mg.

of antibody, *i. e.* the precipitate has a weight of about 0·002 mg. Other more or less spherical antigens whose serological titres have been determined are mainly those with molecular weights of under 100,000. These will have a greater surface to weight ratio than the larger antigens, and the antibody to antigen ratio would be expected to be correspondingly greater. The figures collected by Marrack (1938) range from 10 to 21 in the region of antibody excess, and with such antigens a titre of 10^6 corresponds to from 0·01 mg. to 0·02 mg. of precipitate. It is to be expected, therefore, that large spherical antigens, such as Bushy stunt virus, will give lower serological titres than smaller spherical antigens, or than greatly elongated antigens giving precipitates of an especially easily visible type.

The serological titres of Bushy stunt virus preparations are more dependent on the concentration of antiserum used than are those of tobacco mosaic virus, for inhibition of precipitation by excess of antibodies is more definite. The effect of varying the concentration of antiserum on the precipitation of a Bushy stunt virus preparation is shown in Table I. Because of this effect the titres were determined with antiserum diluted at least 1/200 with 0·85 per cent. NaCl solution.

TABLE I.—*Effect of Antiserum Concentration on Precipitation of Bushy Stunt Virus.*

Antiserum.	Time.	Antigen in mg. per c.c. 0·2.	0·1.	0·02.	0·01.	0·005.	0·0025.	0·002.	0·0016.	0·0012
1/5	2 min.	++	−	−	−	−	−	−	−	−
	30 ,,	+++	++	−	−	−	−	−	−	−
	90 ,,	++++	+++	±	−	−	−	−	−	−
	24 hr.	++++	++++	+++	+	±	−	−	−	−
1/50	2 min.	++	−	−	−	−	−	−	−	−
	30 ,,	+++	+++	±	−	−	−	−	−	−
	90 ,,	++++	+++	++	+	−	−	−	−	−
	24 hr.	++++	++++	+++	++	+	+	±	−	−
1/500	30 min.	−	−	±	−	−	−	−	−	−
	90 ,,	−	−	++	+	±	−	−	−	−
	24 hr.	−	−	+++	++	+±	+	+	+	±

1 c.c. of antiserum at the stated concentration added to 1 c.c. of antigen solution containing given weight of antigen. Tubes were immediately placed in the water-bath at 50° C.
+ signs indicate the degree of precipitation.

Infectivity tests were made by the local lesion method, *Nicotiana glutinosa* being used as the host. The lesions resemble those produced by tobacco mosaic virus, but if equal weights of the two viruses are inoculated to opposite halves of the same leaves the tobacco mosaic virus produces many more lesions. The results of precipitation end-point determinations and infectivity tests on five different crystalline preparations of Bushy stunt virus are shown in Table II. The precipitation end-points of a large number of preparations have all been similar, with antiserum at a dilution of 1/200, varying only between 1 : 600,000 and 1 : 800,000. With antiserum at 1/800 titres of

TABLE II.—*Activity of Crystalline Preparations of Bushy Stunt Virus.*

Serological titre.	Infectivity on *N. glutinosa*. Average number of lesions per leaf at various dilutions.					
	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	10^{-8}.
1 : 800,000	84	31	11	1	0·4	0
1 : 800,000	..	37	20	2·5	0·5	0
1 : 600,000	48	28	11	1	0	0
1 : 600,000	81	20	7	1·5	0·5	0
1 : 800,000	74	53	11	4	0·4	0·2

Serological titre is expressed as the smallest amount, in gm. per c.c., of antigen giving a visible precipitate with 1 c.c. of antiserum at 1 in 200. Dilutions in infectivity tests given as gm. per c.c., and 1 c.c. is rubbed over from 5 to 6 leaves.

$1/10^6$ have been obtained. The activity of a fully crystalline preparation is unaffected by recrystallizations or by repeated sedimentations in a high-speed centrifuge, but the activity of partially purified preparations is increased by these treatments. Further evidence that the virus activity is in the crystals was obtained by inoculating isolated large crystals, after washing in one-quarter saturated ammonium sulphate solution and dissolving in water, to *N. glutinosa* and tomato, when typical symptoms were obtained.

Analyses and Isolation of Nucleic Acid.

Analytical figures have been determined on a number of fully dried preparations and have fallen in the following ranges :

Carbon	47 to 50 per cent.	Hydrogen	7·2 to 8·2 per cent.
Nitrogen	15·8 ,, 16·4 ,,	Sulphur	0·4 ,, 0·8 ,,
Phosphorus	1·3 ,, 1·5 ,,	Carbohydrate	5 ,, 6 ,,
Ash	1·7 ,, 5 ,,		

Phosphorus was determined by the method of Kuttner and Lichenstein (1932). The carbohydrate estimations were made by an orcin method of Pirie (1936) with glucose as a standard and the figures given are referred to glucose. As the sugar of the virus is probably ribose, to refer the figures to ribose they should be multiplied by 0·84. The other constituents were determined by the usual Pregl methods.

Unless the virus has been carefully prepared the carbohydrate content may be 8 per cent. The extra 2 to 3 per cent. of carbohydrate can be removed, without inactivating the virus, either by recrystallizing and thoroughly washing the crystals with distilled water, or by repeatedly sedimenting the virus in a high-speed centrifuge. This carbohydrate differs from that found contaminating partially purified preparations of potato virus " X " (Bawden and Pirie, 1938*a*), for that was insoluble in water and always associated with coloured material. The presence of the extraneous carbohydrate in preparations of Bushy stunt virus can be demonstrated by extracting from 5 to 10 mg.

of the dried virus with 1 c.c. of $N/20$ ammonium hydroxide for a few hours at room temperature. This treatment extracts nothing from preparations with a carbohydrate content of from 5 to 6 per cent., for the protein is insoluble and the nucleic acid is not extracted unless the mixture is heated, but any contaminating carbohydrate dissolves and can be estimated in the usual way. Alternatively, it can be isolated from preparations inactivated by freezing and thawing ; the insoluble virus can be removed from the fluid by centrifugation, but the excess carbohydrate remains in the clear supernatant fluid. This method is the most convenient for the routine examination of the virus preparations, and detects from 0·5 to 1·0 per cent. of contaminating carbohydrate.

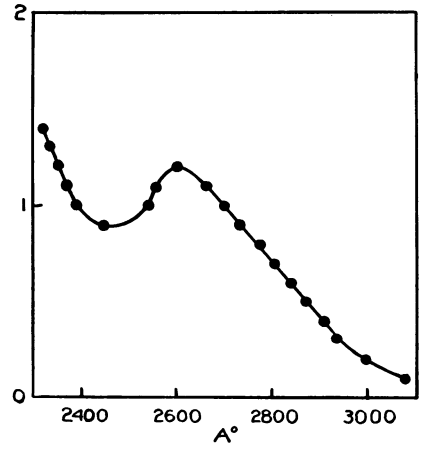


FIG. 4.—Ultra-violet absorption curve of a solution of Bushy stunt virus.

The carbon, hydrogen and nitrogen figures are very similar to those obtained with tobacco mosaic virus and potato virus " X ", and to those of proteins in general, but the phosphorus and carbohydrate figures are more than twice as great, suggesting that Bushy stunt virus has a higher nucleic acid content. This suggestion is reinforced by a study of the ultra-violet absorption spectra of the viruses. The tobacco mosaic group of viruses give general absorption beginning at about 2500 A and also a definite absorption maximum at 2600 A (Bawden and Pirie, 1937*a*), the region generally associated with absorption by nucleic acid. Solutions of Bushy stunt virus absorb ultra-violet light even more strongly at 2600 A ; with tobacco mosaic virus $\log_{\cdot 10} I_0/I$ is 1·2 at 2600 A with a 2 cm. column of a 0·02 per cent. solution, whereas, under the same conditions, this value is given by a 0·012 per cent. solution of Bushy stunt virus. Fig. 4 is the absorption curve of a solution of Bushy stunt virus.

In Bushy stunt virus the nucleic acid is more firmly bound than in the plant viruses we have previously studied. A few minutes' heating of salt-free solutions at 90° C. inactivates the virus, but even when boiled such solutions

remain clear. On the addition of a little salt to the boiled solutions a protein coagulum separates, but carries with it all the nucleic acid. Extraction of the virus inactivated by either heating or drying with boiling $N/80$ ammonia solution dissolves most of the nucleic acid and the residual protein has a low phosphorus content. The addition of acid causes the fluid to become opalescent, and in a few hours a precipitate forms with the typical resinous character of nucleic acid. The precipitate is ground up with a little water and washed by centrifugation, and the residue dissolves completely when sufficient alkali is added to make the preparation neutral. After reprecipitation with acid, the sodium nucleate is dried in the frozen state and gives a colourless, easily soluble, feathery mass containing 6 to 7 per cent. of phosphorus and from 27 to 31 per cent. of carbohydrate. It gives neither a Schiff reaction for desoxypentose nor Dische's reaction with diphenylamine (1930) after hydrolysis with acid, but it gives as much colour as the same weight of yeast nucleic acid by McCance's (1926) method of pentose estimation. There is not sufficient of the nucleic acid available for a rigorous identification of the carbohydrate, but it seems reasonable to conclude that the nucleic acid is of the ribose type similar to that in yeast and tobacco mosaic virus (Bawden and Pirie, 1937*a*). The value of the specific rotation, $[\alpha]_D^{20^\circ} = 58^\circ \pm 4^\circ$, supports this conclusion.

Material, apparently identical with that obtained by extracting the virus with boiling alkali, can be prepared by incubating the coagulum produced by heating the virus in the presence of a little salt or acid with trypsin free from nuclease.* After the coagulum has been digested the nucleic acid can be precipitated with acid. Bushy stunt virus is destroyed by warm acetic acid and by wetting and spreading agents such as sodium dodecyl sulphate (Bawden and Pirie, 1938*a*). A phosphorus-free protein can be precipitated with ammonium sulphate at neutrality from preparations inactivated by these agents, and from the supernatant fluids the nucleic acid can be isolated by precipitation with acid.

The amount of nucleic acid isolated is from 15 to 20 per cent. of the weight of virus taken. In view of the practical difficulties in isolating substances of this kind on a small scale, the yield accounts satisfactorily for so much of the phosphorus and carbohydrate of the virus preparations that there is no reason to suspect the presence of any phosphorus or sugar containing components other than the nucleic acid.

The ash content of preparations recrystallized several times with ammonium sulphate and dialysed for many days against distilled water at from pH 3 to pH 5 does not usually fall below 3 per cent. Some of this tightly bound ash can be removed by electrodialysis against distilled water, but an ash-free preparation has not yet been obtained.

Effect of Acids and Alkalis.

Although so sensitive to dehydration, Bushy stunt virus is less affected by changes of pH than the other viruses we have studied. It does not precipitate, and undergoes no apparent change during exposure of some hours at room

* The trypsin preparation was kindly supplied by Dr. J. H. Northrop.

temperature to pH values of between 1·5 and 9·5. At pH 1 the virus solutions rapidly lose their original faint opalescence and become water-clear. Nothing can be sedimented from such solutions by high-speed centrifugation, but on neutralization the protein precipitates and the virus is inactivated. Exposure to pH 10 or higher for a few hours at room temperature destroys the infectivity. No precipitate develops in these solutions on neutralization unless they are kept for about 48 hours, when a part of the material separates. If this is removed by centrifugation the remaining solution is found to be still capable of reacting with antiserum, although it is non-infective. The effect of exposure to different pH values is shown in Table III.

TABLE III.—*Effect of Acid and Alkali on Bushy Stunt Virus.*

pH value.	Serological titre.	Infectivity at 10^{-4}. Average number of lesions per leaf.
1	..	0
1·5	1 : 600,000	41
7	1 : 600,000	38
9·5	1 : 600,000	51
11	1 : 200,000	0

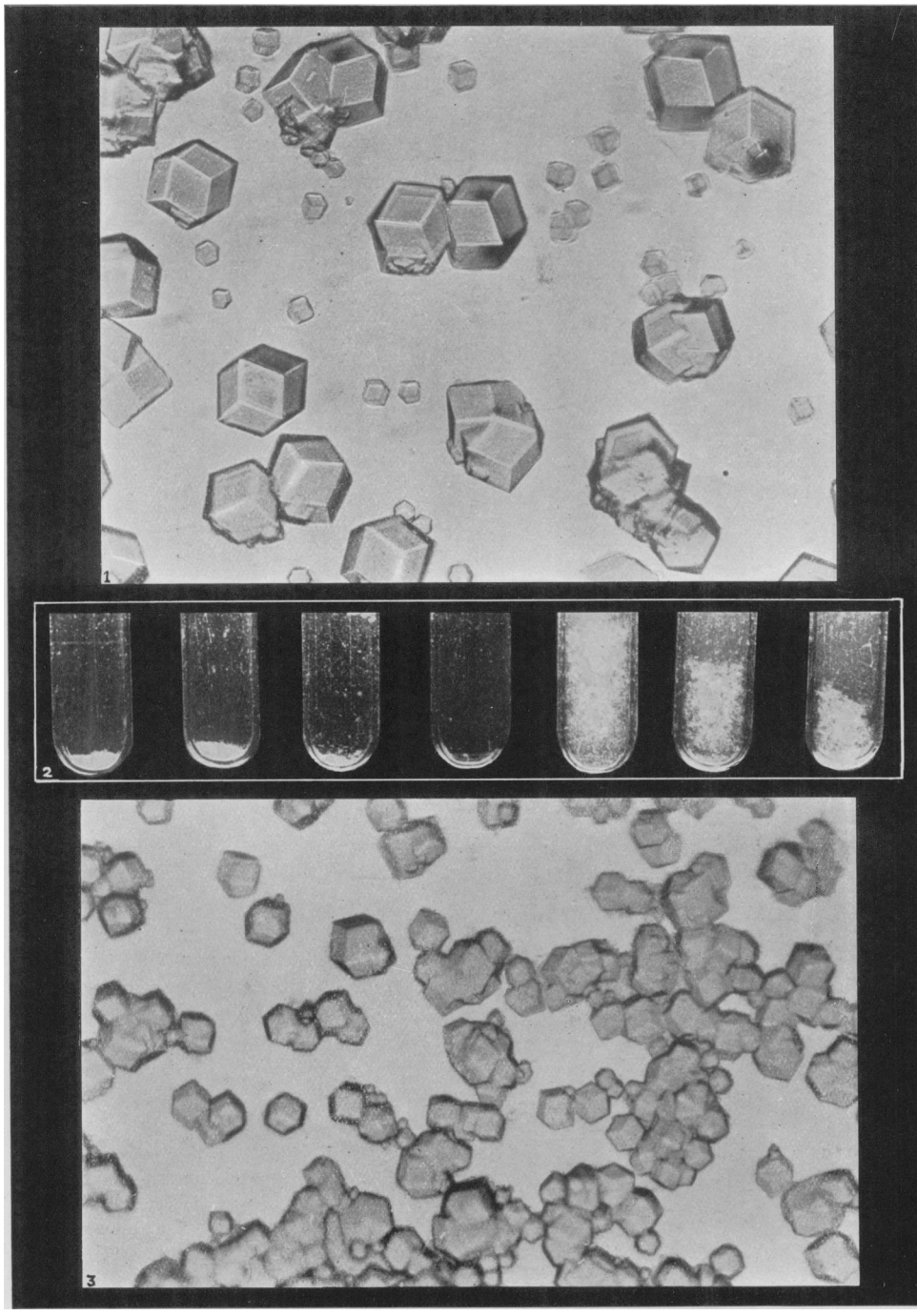
Mixtures left for 2 hours at 18° C. at the pH stated.

Inactivation with Nitrous Acid and Ultra-violet Light.

Tobacco mosaic virus and potato virus "X" can be inactivated with nitrous acid and ultra-violet light without being denatured, and without losing their serological properties or their characteristic optical properties (Bawden and Pirie, 1937*a*; 1938*a*). Bushy stunt virus is also inactivated by these treatments without appreciably modifying its serological or physical properties.

Provided the solution is kept cold, no denaturation takes place when solutions are irradiated with ultra-violet light under a quartz plate. A 1·5 per cent. solution irradiated for 40 minutes at 8 cm. from a 2 amp. mercury vapour lamp was found to be non-infective, but gave a precipitation end-point of 1 : 600,000. On the addition of ammonium sulphate it crystallized well. The crystals were again dodecahedra, but showed a greater tendency to form crystal aggregates than the fully active virus (Fig. 3). The inactive preparations can be recrystallized, and after dialysis dissolve in the same manner as the active virus.

Exposure for 10 hours at 0° C. and at pH 3·4 in the presence of 1·2 per cent. sodium nitrite destroyed the infectivity of a 0·68 per cent. solution of the virus without affecting its precipitation end-point with antiserum. The protein precipitated in the usual way on the addition of ammonium sulphate, and when recrystallized formed dodecahedra showing all the properties associated with the active virus. Treatment with formaldehyde and hydrogen peroxide also inactivates Bushy stunt preparations without destroying their crystallinity or serological reactions.



Bawden and Pirie.

SUMMARY.

The isolation of a protein, probably the virus itself, is described from plants infected with tomato Bushy stunt virus. This protein not only differs from the normal plant proteins, but it also differs more from the other purified plant viruses than these differ from one another. It is fully crystalline instead of liquid crystalline. It has a higher nucleic acid content than tobacco mosaic virus or potato virus " X ", and is more stable towards pH changes, but less stable towards dehydrating agents. Its particles are not elongated, and liquid and solid preparations are isotropic. 1 c.c. of solution containing 10^{-7} gm. produces infections when rubbed on to *N. glutinosa*, and 1 c.c. containing 10^{-6} gm. gives a specific precipitate with antiserum. Precipitates of the rod-shaped viruses with their antisera resemble those obtained with bacterial flagellar (" H ") antigens, but those of Bushy stunt virus resemble those with somatic (" O ") antigens. When irradiated with ultra-violet light or treated with nitrous acid the virus loses its infectivity, but it can still be crystallized and still retains its serological activity.

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FIG. 1.—Dodecahedral crystals of Bushy stunt virus. The larger crystals were produced by a first crystallization with ammonium sulphate and the smaller by a second. × 250.

FIG. 2.—Precipitates of Bushy stunt virus and tobacco mosaic virus with their homologous antisera. Central tube saline control. Three left-hand tubes, 0·1, 0·05 and 0·025 mg. of Bushy stunt virus; three right-hand tubes, same weights of tobacco mosaic virus. Note compact precipitate of Bushy stunt and bulky fluffy precipitate of tobacco mosaic virus.

FIG. 3.—Crystals of Bushy stunt virus inactivated by irradiation with ultra-violet light. Material is still serologically active. × 300.