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# THE SEPARATION AND PROPERTIES OF TOBACCO MOSAIC VIRUS IN DIFFERENT STATES OF AGGREGATION.

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THE problem of the size and shape of the particles of tobacco mosaic virus has received much attention for many years, and the conclusions reached have been about as numerous and varied as the workers engaged and the methods employed. For long it was tacitly assumed that the particles were incompressible spheres and calculations of size were made on this basis, although Takahashi and Rawlins suggested in 1933 that they might be rod-shaped. Studies of purified virus preparations within the last nine years have proved that the virus can occur in the form of greatly elongated particles. We contributed some of the evidence for this, but we also suggested that these particles may be artifacts, produced during the course of purification by the linear aggregation of smaller particles, for the purified virus filtered less readily, was less infectious, and showed more anisotropy of flow than virus in clarified sap (Bawden, Bernal and Fankuchen, 1936; Bawden and Pirie, 1937*a*). Infective sap contained some rod-like particles, for it showed some anisotropy of flow, but there was no evidence to show whether all the virus present was of a uniform size and shape. We have urged the need for caution in translating physical data on tobacco mosaic virus into "molecular" weights and sizes, for the physical properties are so anomalous that conclusions based on them are suspect. Even if the underlying theory were unequivocal, in a system that aggregates so readily there is a danger that the size calculated might be neither that of the smallest particle capable of causing infection nor that of the most common particle in untreated material.

Loring, Lauffer and Stanley (1938) agreed that virus in sap aggregates linearly when precipitated with acid, salts or alcohol, but claimed that homogeneous, unaggregated preparations could be made by ultra-centrifugation of infective sap. At different times various weights and sizes have been assigned to the particles in such preparations, but since 1941 Stanley and Lauffer have postulated a basic particle or molecule about 15m $\mu$ . thick and 280m $\mu$ . long. They consider that aggregation is restricted to the end-to-end joining together of two or three such fundamental particles.

Evidence from a number of different sources suggests that particles of approximately this size are common in preparations of tobacco mosaic virus, but it seems unjustifiable to assume that these are the equivalent of virus "molecules" when there is nothing to show that they are not simply one of the more stable aggregates. Indeed there are suggestions that smaller particles exist. For example, Frampton (1942) measured the approximate lengths of 159 particles shown in electron micrographs of tobacco mosaic virus published by Stanley and Anderson (1941), and pointed out that many were shorter than the postulated molecules, some being as short as 37m $\mu$ . However, this is not conclusive, for drying inactivates the virus and reduces the anisotropy of flow (Bawden and Pirie, 1937*a*); it may therefore be causing breaks in the rods. There is no published evidence to show whether these small fragments are formed by a disruption of the fundamental particle during the drying involved in the

preparation of the electron microscope specimen, or whether they are the true units from which the larger particles are built by aggregation.

In the present paper we describe further experiments showing how readily the properties of tobacco mosaic virus can be altered. Preparations made by the method we have described recently show much less anisotropy of flow than our first preparations (Bawden and Pirie, 1943, 1937*a*). They need to be more concentrated to give a liquid crystalline layer and they give lower serological titres, although they behave qualitatively in precipitin tests in much the same manner. Heating the sap to 70° C. and incubation with trypsin were steps in our first method that were omitted later; the differences arise from these omissions, and by restoring either of these treatments preparations are obtained showing the extreme orientation phenomena. The differences between these preparations made by different methods are insignificant compared with those between such preparations and those that can be made by using carefully chosen starting material. From such material we have prepared virus solutions with physical properties so different from those of our earlier preparations that it now seems probable that the particles responsible for the anisotropy of flow in infective sap are themselves aggregates.

Virus preparations with different physical properties also behave very differently in serological tests. As ordinarily prepared, tobacco mosaic virus gives with its anti-serum an open, fluffy type of precipitate, resembling that given by bacterial flagellar antigens. We attributed this to elongation of the particles, for other anisometric viruses behave similarly, whereas viruses with spherical particles give dense, granular precipitates, similar to those given by bacterial somatic antigens (Bawden and Pirie, 1938). We now have further evidence for the view that serological behaviour depends on the shape of the antigen, for preparations of tobacco mosaic virus that show little or no anisotropy of flow resemble somatic rather than flagellar antigens in precipitin tests. Such preparations are readily transformed into ones showing anisotropy of flow and they then behave like flagellar antigens.

#### MATERIALS AND METHODS.

Most of this work was done with a mild strain of the common tobacco mosaic virus propagated in tobacco, var. White Burley, but essentially similar results were obtained with this and with tomato aucuba mosaic virus propagated in tomato, var. Kondine Red.

In previous work on tobacco mosaic virus the sap that is expressed from leaves after maceration with a pestle in a mortar or by passage through a meat mincer has been used. We have tried many different kinds of leaf extract in experiments preliminary to those now described, and these have shown that tobacco mosaic virus aggregates as a result of short exposures to conditions obtaining in sap from minced whole leaves. The virus isolated from sap may, therefore, be in a different state from that in which it occurs in infected cells. That this aggregation proceeds in expressed sap but not in intact cells need occasion little surprise, for the sap from minced leaves probably has little or nothing in common with any fluid that exists in intact cells. For simplicity, only one procedure for extracting virus in its least aggregated form will be described.

#### *Preparation of Virus-containing Extracts.*

Leaves between 2 and 5 in. long are picked about a month after the tobacco plants have been infected, the midribs are cut out and discarded, and the blades are frozen for 5–20 hours at –10°. The frozen block of leaves is put into 30 times its weight

of water at room temperature and stirred gently till thawed ; as much fluid as possible is pressed out by hand without rubbing or shearing. This brown fluid contains some virus and its properties will be described later. The mass of leaf blades is carefully unravelled, suspended again in 30 times its weight of water and pressed out ; this washing is repeated. Enough of the leaf components that normally lead to aggregation in sap have now been removed for relatively stable extracts of virus to be made. The leaf blades are passed through a domestic meat mincer, the fibre is mixed with a little water, squeezed in a cloth and the residue minced and re-extracted. The weight of the combined extracts is about three-quarters of the original weight of the leaf blades, i.e. the volume of extract is about equal to the volume of sap that would have been got by direct mincing. The extract is at pH 6.0-6.5 and, after centrifuging for half an hour at 3000 r.p.m., it is clear and pale brown. It will be called " mincer extract." Residual free virus in the mince can be removed by repeatedly suspending the fibre in water and pressing. This washing is generally combined with neutralization for the pH of the mince falls, as in the comparable experiments on the liberation of tomato bushy stunt virus (Bawden and Pirie, 1944), because of the demethylation of pectin (Holden, 1945). Four washes with 60 ml. of water to 10 g. of fibre are sufficient to reduce the virus content of the last wash to one thousandth of that of the first extract after mincing.

The washed fibre is now moistened to get a suitable consistency and passed through the triple-roller mill which we have described previously (Bawden and Pirie, 1944). The smooth paste is suspended in about 5 parts of water ; after a few minutes the fluid is separated either by squeezing through madapollam or, more conveniently if a small quantity is being handled, by centrifuging through a cloth bag held in the mouth of a centrifuge tube by a rubber band. The fluid is then frozen for 10-12 hours at  $-10^{\circ}$  C. On thawing, a heavy green precipitate separates, leaving a clear yellowish-green supernatant whose volume is about equal to that of the mincer extract already made. This fluid will be called " mill extract." The preliminary washing of the minced fibre is not essential, but it enhances the differences between mincer and mill extract.

#### *Separation of Virus Preparations from the Extracts.*

By centrifugal fractionation of such extracts, preparations of virus with widely different properties can be made. For this purpose we have used an air-driven centrifuge of the type described by Masket (1941), run at either 40,000 r.p.m. (R.C.F. 80,000) or 24,000 r.p.m. (R.C.F. 30,000). The ten tubes each containing 7.5 ml. of fluid are inclined at only  $10^{\circ}$  to the axis of rotation, so that the centrifugal field is relatively uniform throughout the tube. The virus particles sediment towards the side of the tube, but, from purified virus preparations, the material always slides down the wall to form a pear-shaped pellet. When fractionations are attempted the fluids should be free from fatty materials, such as leaf chromoprotein, for these stick to the walls of the tube in the form of a streak in which virus remains entangled during centrifugation ; when the centrifuge stops this partly redissolves in the supernatant.

Centrifuging for 30 minutes at 40,000 r.p.m. sediments all the virus, and concentrates it partly as a colourless, birefringent, coherent pellet, and partly as a brown uncompact dense but mobile fluid, which we call uncompact sediment.

The supernatant fluids are removed carefully with a syphon so as to leave the layer of uncompact sediment undisturbed ; the supernatants are discarded and the uncompact layers combined. The pellets are mixed, suspended in water and centrifuged for 10 minutes at 10,000 r.p.m. after dilution to one-third of the original volume. The brownish, slightly opalescent fluid is again ultracentrifuged for 30 minutes

at 40,000 r.p.m., and the uncompact sediments collected as before and mixed with the earlier samples. The pellets are dissolved, diluted to one-quarter of the original volume, and separated from any insoluble material by centrifugation at 10,000 r.p.m.

There are now two products, all the uncompact sediments and the pellets that have been compact twice. The latter contains less than 0.5 g. per litre of material uncompactable at 40,000 r.p.m. and is ready for further fractionation, but the former needs a further sedimentation to reach this condition. It is therefore centrifuged at 40,000 r.p.m., the supernatant and the small pellet are discarded, the layer of uncompact sediment is kept. Both products are now fractionated by centrifugation for 30 minutes at 24,000 r.p.m. The uncompact sediment gives another tiny pellet (B), and a supernatant which merges into an uncompact layer at the bottom of the tube (A). The pellets give three fractions which are separated, a supernatant which is little but water, an uncompact sediment (C) and a pellet (D). If D shows any anisotropy of flow in 1 per cent solution it can be fractionated further into a pellet and supernatant by centrifuging for 30 minutes at 17,000 r.p.m.; only the latter is used.

The course of a fractionation from starting material to end-products is illustrated in Table I; the letters assigned to the end-products will be used for fractions whose

TABLE I.—*Scheme of the Differential Ultracentrifugations used in the Separation of Virus Preparations in Different States of Aggregation. Intensity of Centrifugation in R.C.F.*

	First 80,000 g.	Second 80,000 g.	Third 80,000 g.	Fourth 30,000 g.	Final fraction.
Original extract .	Supernatant				
	Uncompact sediment	Supernatant	Supernatant	Supernatant	A
			Uncompact sediment		
	Pellet	Uncompact sediment	Supernatant	Supernatant	C
			Pellet		

properties are described later, so that there can be unambiguous reference to their history. All the centrifugations are done within as short a time as possible, with solutions containing less than 8 g. of solids per litre and the pH is kept between 6.5 and 7. In this type of centrifuge the surface of pellets always lies vertically, and because of their density they disperse rapidly as soon as the effective direction of gravitational acceleration changes from horizontal to vertical as the centrifuge stops. The centrifuge is therefore decelerated from 10,000 r.p.m. in 5 to 10 minutes and the tubes removed and the fractions separated as rapidly as possible.

Comparable products can be made from mincer or mill extracts, but there are consistent differences in the ratios in which the different fractions occur in the two starting materials. Table II gives figures for eight fractionations and shows that,

although there are wide variations in the ratio of compacted to uncompacted material in different batches of leaves, there is consistently more uncompacted sediment in the mincer than in the mill extract. Thus the first column of ratios covers a wide range, but the second column of ratios, which relates the mincer extract ratios to the mill extract ratios, is more uniform and all the numbers in it are greater than 1. There is a more or less continuous gradation between fractions of types A and D, and our division into four distinct fractions is arbitrary. From some leaf extracts, for example those giving the fractions whose sedimentation constants are set out in the following paper (Johnston and Ogston, 1945), the material in fraction C is larger than that in fraction B. No correction for this has been made in Table II, where the fractions are divided broadly into two groups; any such correction, however, would be small, because fractions B and C usually contain about equal amounts of material.

TABLE II.—*Yields of Pellet and Uncompacted Sediment from Mincer and Mill Extracts.*

Source.	Yield in grammes per litre in the form of—		Ratios.	
	Pellet (Fractions B + D).	Uncompacted sediment (Fractions A + C).	Pellet Uncompacted.	Mincer ratio. Mill ratio.
Mincer ext. . . . .	1.95	0.45	4.33	} 3.2
Mill ext. . . . .	3.7	2.7	1.37	
Mincer ext. . . . .	0.49	0.41	1.19	} 1.8
Mill ext. . . . .	0.68	1.0	0.68	
Mincer ext. . . . .	1.20	0.54	2.22	} 1.3
Mill ext. . . . .	1.57	0.88	1.78	
Mincer ext. . . . .	0.91	0.65	1.40	} 1.6
Mill ext. . . . .	1.87	2.14	0.87	
Mincer ext. . . . .	0.41	0.85	0.48	} 1.4
Mill ext. . . . .	0.56	1.64	0.34	
Mincer ext. . . . .	0.34	0.15	2.27	} 1.6
Mill ext. . . . .	0.57	0.40	1.42	
Mincer ext. . . . .	1.0	0.29	3.45	} 1.7
Mill ext. . . . .	1.46	0.73	2.00	
Mincer ext. . . . .	1.62	0.29	5.56	} 1.5
Mill ext. . . . .	3.76	1.03	3.65	

The mincer and mill extracts that form a pair were made by the method described in the text from the same batch of leaves. Batches were worked up at different dates throughout the year.

In the method of preparation described, the frozen leaves are put directly into a large volume of water so as to ensure the rapid removal of crystalloids from the flaccid leaf cells. There is a little virus in this fluid and, judging by its serological behaviour, it is in the aggregated state, but it is too dilute for isolation to be feasible. A few preparations have been made by ultracentrifuging the juice that runs out of the frozen leaves when they are pressed gently after being allowed to thaw. About one-tenth of the total virus that can be isolated from the leaf is in this fraction; it is more highly aggregated than any of the other fractions, although the aggregation can be carried still further by the methods described later. No sedimentable but uncompacted material has been found in this juice.

### *Methods of Testing.*

Infectivity tests were made by the local lesion method, using a Latin square design on *Nicotiana glutinosa*. Precipitin tests were made by adding 1 ml. of diluted antiserum to each of a series of tubes containing 1 ml. of antigen at various dilutions. The tubes were immediately placed in a water bath at 50° C., with their fluid columns half immersed to ensure continuous mixing by convection, as previously described (Bawden and Pirie, 1937*a*, 1943). In earlier work attention was given largely to the precipitation end point obtained, for this gave a reasonably accurate measure of the quantity of virus in the antigen under test. In the present work, however, differences in end-point were obtained with preparations known to contain the same amount of virus, and different fractions behaved qualitatively very differently in precipitin tests. These differences are most simply demonstrated by comparing the behaviour of a mill extract of infected leaves before and after incubation with trypsin (Table III). Before incubation the extract showed no anisotropy of flow, and with antiserum precipitated only slowly and then over only a small range of antigen/antibody ratios. After incubation the extract showed anisotropy of flow strongly, and it then precipitated with antiserum almost immediately and over a wide range of antigen/antibody ratios. After incubation the precipitate was also of a different type, bulky and flocculent (typical "H"-type) instead of small and rather granular. When comparing different preparations, therefore, in addition to recording the precipitin titre, records were also made of the time of the first appearance of precipitation, the antigen concentration in the optimal tubes, the zone of precipitation and the character of the precipitate.

Our observations of anisotropy of flow were made simply by examining the fluids in polarized light as they are tipped from end to end of half-filled test tubes 0.5 cm. in diameter. Under these conditions the phenomenon is detectable in solutions containing as little as 0.1 g. of aggregated virus per litre. We prefer to use this rough method of comparing anisotropy of flow rather than to make measurements of the amount of birefringence built up by a given rate of shear (Lauffer and Stanley, 1938), for, in our first experiments on anisotropy of flow, we found that preparations varied greatly in the rate of shear needed to elicit a given amount of birefringence, and that if high rates of shear were used, very significant differences were missed. Furthermore, the intensity of anisotropy of flow was greatly influenced by other components in the system. It seemed, therefore, that the precision apparently lent to a set of observations by a measurement of the birefringence was likely to be spurious. This view is confirmed by the critique that Robinson (1939) has published of all measurements on anomalously flowing liquids made in capillary apparatus. In our opinion only a series of measurements made at a number of different rates of shear would give as much information about differences between two preparations as is given by a simple observation of them shaken side by side.

#### PROPERTIES OF CENTRIFUGALLY FRACTIONATED VIRUS PREPARATIONS.

##### *Infectivity and Serological Activity.*

Table IV shows the results of precipitation and infectivity tests made on the four fractions obtained by differential centrifugation from the mincer and mill extracts of one sample of infected leaves. These are typical of many such tests that have been made. In all, the fraction compacting at 24,000 r.p.m. (D) and that uncompacting at 40,000 r.p.m. (A) differ strikingly in their serological behaviour, and the former is highly infective, whereas the latter is almost non-infective. The fractions intermediate

TABLE III.—Effect of Aggregation by Trypsin on the Serological Behaviour of Tobacco Mosaic Virus.

Test antigen.	Antigen dilution.									
	1:1.	1:2.	1:4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	
Freshly prepared mill extract	+	+	+	+	+	+	+	+	+	+
Mill extract after incubation with 0.1% trypsin at pH 7 for 8 hours.	+	+	+	+	+	+	+	+	+	+

Antiserum used at 1:400.

TABLE IV.—Serological Behaviour and Infectivity of Centrifugally Isolated Fractions from Mincer and Mill Extracts.

Fractions from mincer extract.	Time for first appearance of floccules.	Dilution of antigen in first tube to precipitate.	Zone of precipitate after 3 hours.	Infectivity.	
				Average number of lesions per leaf at—	
A	3 hours	1:64	1:64	0	0
B	1 hour	1:32	1:16-1:128	3	0
C	15 minutes	1:16	1:1-1:256	54	10
D	5 "	1:2	1:1-1:512	116	34
Fractions from mill extract.					
A	3 hours	1:64	1:64	0	0
B	1 hour	1:32	1:8-1:128	1	0
C	15 minutes	1:16	1:4-1:256	24	5
D	5 "	1:2	1:1-1:512	85	14

Antiserum used at 1:400. Antigen dilution 1:1 = 1 mg. per ml.

TABLE V.—Effect of Ageing at 0° C. and Heating at 60° C. on the Precipitin Reaction of Uncompacted Sediment.

Treatment.	Dilution of antigen (1:1 = 1 mg./ml.).									
	1:1.	1:2.	1:4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	
Freshly prepared uncompacted sediment	+	+	+	+	+	+	+	+	+	+
Uncompacted sediment kept for 1 week at 0° C.	+	+	+	+	+	+	+	+	+	+
Uncompacted sediment kept for 3 weeks at 0° C.	+	+	+	+	+	+	+	+	+	+
Uncompacted sediment heated for 10 minutes at pH 5.9 and 60° C.	+	+	+	+	+	+	+	+	+	+



in their sedimentability (B and C) are also intermediate in their infectivity and serological behaviour. Similar fractions come from both types of extract, but in general those from the mincer extracts are rather more infective.

The properties of all fractions are altered by heating at 60° C. and pH 5.9, or by incubation with trypsin. All then show anisotropy of flow even at 0.5 g. per litre, precipitate rapidly with antiserum over a wide zone, give similar titres and typical flagellar type floccules. The most striking change in serological behaviour is given by fraction A, uncompacted at 40,000 r.p.m. Heating this causes the separation of a coagulum, which contains about 60 per cent of the total solid material. The remainder has the physical and serological properties of aggregated tobacco mosaic virus, but the production of these properties is not accompanied by any increase in infectivity. The difference in infectivities between the various fractions is too great to be accounted for simply by the presence of different quantities of material other than virus. Thus about 40 per cent of uncompacted sediment may be isolated as serologically active virus, yet its infectivity may be less than one-thousandth of that of pellets compacted at 24,000 r.p.m. To cause such a reduction in infectivity, the contaminating material would need to be a powerful inhibitor of infectivity, and of this there is no evidence. First, there is no increase in infectivity when these materials are removed and, secondly, the addition of uncompacted sediment to pellet gives no great reduction in infectivity.

After such aggregating treatments as heating or incubation with trypsin, the infectivity of all the fractions is reduced by about one-half when they are compared with the original preparations at dilutions of  $10^{-5}$  and  $10^{-6}$  g. per litre. This is presumably because aggregation leads to a smaller number of infective units, but the change in infectivity seems small compared with the striking changes in physical and serological properties.

The virus responsible for the slight infectivity of the uncompacted sediments produces symptoms in both tobacco and *N. glutinosa* identical with those produced by virus in the other fractions. Tobacco plants have been inoculated separately with fractions A and D, and there were no significant differences between the amounts of the four fractions that could later be separated from the infected plants.

#### *Chemical Constitution.*

The main pellets compacted at 24,000 r.p.m. (Fraction D, Table I) usually have analytical compositions falling within the range we have given for tobacco mosaic virus purified by precipitation methods (Bawden and Pirie, 1937*a*), but a few have contained 0.9 per cent. to 1.2 per cent of phosphorus and 5 per cent to 7 per cent of carbohydrate—about twice the normal values. Repeated ultracentrifugation has no effect on their constitution unless they have been allowed to age *in vitro* or they have been heated or incubated with trypsin. Then highly aggregated preparations are obtained with the normal content of phosphorus and carbohydrate. This phenomenon has been noticed only with preparations made from plants grown in the winter, but we have not studied a sufficiently extensive series to define the conditions that bring it about.

The analytical composition of the intermediate fractions, B and C (Table I), also generally falls within the usual range, but that of fraction A is more variable. The figures for all these uncompacted sediments lie in the ranges nitrogen 12 per cent to 15 per cent, phosphorus 0.1 per cent to 0.7 per cent, and carbohydrate 3 per cent to 10 per cent. Part of the material in this fraction is a component of the normal leaf, but when uninfected leaves comparable with infected ones are fractionated only about

one-fifth as much uncompact sediment is obtained. After incubation with trypsin, the amount of material that can be obtained from this fraction with the physical and serological characters of aggregated tobacco mosaic virus varies from 10 per cent to 40 per cent in different preparations. Thus the virus in this fraction is accompanied by, or combined with, much material which has chemical properties widely different from the virus itself, but there is little evidence on the nature of the material. A preliminary study has been made of the distribution of phosphorus in a series of fractions treated in various ways to differentiate between virus-phosphorus and phosphorus in other forms. This shows that after incubation with trypsin at pH 6.8 in glucosamine buffer, all the phosphorus except that associated with serologically active virus remains uncompact when ultracentrifuged. Glucosamine buffer is useful when phosphorus is to be determined, because its pK is 7.8 (Miles and Pirie, 1939), and it is sufficiently stable for use in these short periods of incubation.

### *Physical Properties.*

Aggregated tobacco mosaic virus precipitates optimally from dilute salt solutions at pH 3.4 and is soluble at pH values greater than 4, although in the absence of salts there is precipitation at pH 4.2 (Bawden and Pirie, 1937a). Centrifugally isolated virus behaves differently, and the different fractions differ from one another. Material that compacts at 24,000 r.p.m., fraction D, precipitates completely at pH 4.4, even in the presence of salts. The precipitates do not have the characteristic fibrous structure of aggregated virus, and they are difficult to see when separating from dilute solutions. They are highly hydrated, and even after centrifuging for 30 min. at 3000 r.p.m., the precipitate from 1 mg. of virus occupies 0.2 ml. As the pH is lowered, a denser and more easily visible precipitate separates, but it is only after standing for several hours that it develops the characteristic shimmer. Fractions such as B and C, which sediment slowly on ultracentrifugation and contain significant amounts of material that cannot be converted into aggregated virus, are precipitated at higher pH values. The precise value varies, but there is generally complete precipitation at pH 5. These fractions are not stable at this pH, and the changes that proceed are accompanied by a pH drift so that, in unbuffered solution, repeated addition of acid is needed to keep the pH at 5. After exposure to pH's between 4 and 5, and subsequent neutralization, part of a fraction that previously did not compact on ultracentrifugation at 24,000 r.p.m. will do so. It is obvious from the method of separation used that these fractions differ from one another in their sedimentation constants, and measurements on 11 fractions are given in the accompanying paper (Johnston and Ogston, 1945). They also differ in the readiness with which they can be orientated. Under our conditions of testing, the fractions A, B and C do not show anisotropy of flow even in 2 per cent solution; D shows it at this concentration, but on further dilution it disappears. The precise concentration at which anisotropy of flow is no longer readily elicited depends on a number of factors, among which are the age of the infected plants and the speed with which the fractionation, especially in its first stages, was carried through. The limit is usually between 2 and 5 g. per litre. The pellets show a similarly graded birefringence when examined in the ultracentrifuge tube without stirring: pellets like D resemble those from fully aggregated virus preparations, whereas those from B are brown and show little or no birefringence. The fact that fractions with different antecedents may have similar sedimentation rates, although their other properties are widely different, suggests that fractions differ from one another in more factors than particle size.

The process of pellet formation in the ultracentrifuge, even when not accompanied by any fractionation, leads to some aggregation even if the centrifuge is chilled and the pellet is dispersed in water immediately after centrifuging. There is still more aggregation if the dispersing is delayed for a few days.

#### *Treatments Causing Aggregation.*

All the centrifugally isolated fractions are unstable, and on ageing pass into forms that show anisotropy of flow more strongly and give precipitin reactions more characteristic of flagellar type antigens. The change occurs more rapidly in slightly acid than in alkaline conditions, and more rapidly at room temperature than at 0° C. The effects of ageing and heating on a sediment uncompacted at 40,000 r.p.m. (fraction A) are shown in Table V.

We have studied the aggregation of these fractions by exposing 0.2 per cent solutions to various conditions and observing the changes in precipitin behaviour and in appearance of anisotropy of flow. The two criteria are correlated, but in our conditions of testing cover different ranges of aggregation. Significant changes in precipitin behaviour can be detected before aggregation has proceeded far enough for the solutions to show anisotropy of flow, and by the time the solutions show significant anisotropy of flow they are fairly typical of flagellar type antigens. Further aggregation leads to little change in precipitin behaviour, but to considerable increases in anisotropy of flow. The first stages of aggregation are therefore followed serologically and the later ones by observations of anisotropy of flow. The changes are also associated with an increase in ease of sedimentation in the preparative ultracentrifuge, but measurements of the changes in sedimentation constant during aggregation have not been made.

Preliminary electron micrographs of our preparations, made by Dr. E. M. Crook and Dr. F. M. L. Sheffield, show that fractions such as A (Table I), which show no anisotropy of flow and behave like somatic type antigens, contain few rod-like particles, whereas rod-like particles predominate in fractions showing anisotropy of flow, and their average length increases as a result of treatments that increase anisotropy of flow and change the serological behaviour to the flagellar type.

Treatments leading to aggregation are conveniently studied with preparations showing no anisotropy of flow at 0.5 per cent and very little at 2 per cent (i.e. C or D in Table I). D already contains some aggregated virus, but it is most suitable for tests on aggregation as it is relatively stable. It appears to be free from those substances that cause aggregation, and spontaneous precipitates do not form in it nor do bacteria grow readily. Even so, only agents that cause aggregation in a few hours at 37° C. have been studied, for we have found no satisfactory antiseptic that does not itself cause aggregation, and products of bacterial growth are also powerful aggregating agents. Reproducible results are obtainable only with precisely controlled pH and salt content.

Aggregation is rapid in sap and is less in mincer extracts, which do not contain the juice that comes from frozen unminced leaves. The small amount of virus in this juice is highly aggregated. The reason for this is suggested by Table VI, which shows the effect of exposing the centrifugally fractionated virus to various dilutions of the supernatant prepared by centrifuging the juice from frozen unminced infected leaves at 40,000 r.p.m. to remove the virus. It will be seen that even when diluted tenfold this causes some aggregation. The juice has only about 1/20 of the protein content of the mincer extracts; it is essentially a leaf ultra-filtrate, and ultra-filtrates made by passing infective sap through a "cellophane" membrane had similar aggreg-

TABLE VI.—*Aggregating Effect of the Unsedimentable Part of the Fluid from Frozen but Unminced Leaves.*

Dilution of fluid.	Anisotropy of flow.	Time of first precipitation with antiserum.	Dilution of antigen at the optimum.
Undiluted	+++	2 minutes	1 : 1,000
1 : 1	++	2 "	1 : 1,000
1 : 3	+	4 "	1 : 4,000
1 : 10	—	8 "	1 : 8,000
Water	—	20 "	1 : 16,000

0.1 ml. lots of a 20 g./l. solution of virus fraction D were added to 0.9 ml. of fluid made up by mixing the requisite amount of juice prepared from frozen leaves as described in the text with 2 g./l. phosphate buffer at pH 5.8. This was also the pH of the juice. All contain 2 g. virus per litre and they were kept for 2½ hours at 37°. The symbols +++, ++, + correspond approximately to the anisotropy of flow shown by 1.0, 0.3 and 0.1 g./l. solutions of fully aggregated virus in m/20 pH 6 phosphate buffer.

gating effects. Fluids prepared in the same way from uninfected leaves behaved similarly.

At pH values below 5.5, anisotropy of flow increases rapidly even at room temperature, but on returning to pH 6.5–7 it is reduced or disappears, depending on whether exposure has been prolonged or short. As the pH is raised above 7, the amount of anisotropy of flow shown diminishes even with products already highly aggregated. This is reflected in the serological behaviour, for when precipitin tests are made at pH 8 precipitation is slower, and there is a greater zone of antigen excess than when tests are made below pH 7. Between pH 6 and pH 7 preparations are not apparently affected by exposure for a few hours to 37°. After partial aggregation by any of the agents that have been studied the properties shown depend on the thermal history of the product. Fully aggregated preparations show less anisotropy of flow at high temperatures than at low, but the change takes place within a few minutes, or even seconds, of the change in temperature (Bawden and Pirie, 1937a). By contrast, products partially aggregated by treatments at 37° C., which show moderate anisotropy of flow in 0.2 per cent solution when examined immediately, may lose this in a minute or two if cooled to 0° C. In part this is due to the smaller rates of shear attained in cold fluids, because of increased viscosity, but anisotropy of flow is not restored when the viscosity is lowered again by warming. On the contrary, anisotropy of flow which, though reduced, was still perceptible at 0° C. may disappear when the temperature is raised to 37° C. The rate at which it returns on incubation depends on the treatments used to cause the partial aggregation. The effects of cooling are also reflected in the precipitin behaviour. When tested immediately after incubation, partially aggregated preparations precipitate rapidly with antiserum over a wide range of antigen-antibody ratios, but if first cooled to 0° C. so that the anisotropy of flow has disappeared, they precipitate more slowly and give zones of antigen excess. In assessing the ability of different treatments to cause aggregation, therefore, tests were made on fluids immediately after they were removed from the incubator.

Aggregation, or partial aggregation, during incubation may be responsible for the production of double zones of precipitation which often occur when fractions such as A and B are tested serologically. Precipitation first appears with dilute antigen and is restricted to a narrow zone around the optimum, but after 2 or 3 hours in the water bath precipitation begins in the most concentrated antigen solutions, and these floccules are typical flagellar type. Sometimes the precipitates in these tubes disappear if the tubes are left at room temperature overnight, presumably because cooling has led to disaggregation, even though the virus is combined with antibody, and has

re-established the condition of antigen excess. When again heated to 50° C. these precipitates reappear, though not immediately. No such reversible precipitation has ever been noticed with fully aggregated virus or with the small precipitates formed by dilute solutions of unaggregated preparations.

Aggregation is affected by the salt concentration in two ways ; when sufficient is present it proceeds without the addition of any other material, and when insufficient is present the other agents investigated do not cause aggregation. This is illustrated, with phosphate and trypsin, in Table VII. In the absence of salts changes do go on,

TABLE VII.—*Effect of Phosphate on the Aggregation.*

Concentration of phosphate.	Concentration of trypsin.	Duration of exposure at 37°.	Anisotropy of flow.
10 g. per l.	0	1 hour	+++
5 g. per l.	0	1 "	++
2.6 g. per l.	0	3 hours	+
2.0 g. per l.	0.13 g. per l.	2 "	++
0.6 g. per l.	0.13 g. per l.	2 "	+
0.0 g. per l.	0.13 g. per l.	2 "	—

2 g./l. solutions of virus were incubated at pH 6.0 under the conditions stated. + + +, etc., have the same connotation as in Table VI.

for preparations that have been incubated with trypsin alone, and which have not therefore shown any increase in anisotropy of flow, will develop anisotropy of flow within a few minutes of the addition of sodium chloride or phosphate. A close comparison of the relative efficiencies of different ions has not been made, and most experiments have been made in the presence of M/40–M/60 sodium chloride, sodium phosphate, sodium acetate or glucosamine.

Various enzyme preparations cause aggregation at pH 6.7 in M/40 phosphate or glucosamine buffer. Trypsin and chymotrypsin, purified by the method of Northrop (1939), are the most active, but the soluble and indiffusible component of commercial "trypsin" (B.D.H.), takadiastase, ribonuclease (Kunitz, 1940) and the mixed snail stomach carbohydrases also work to an extent that is roughly proportional to their protease content. Commercial pepsin also is effective at pH 7.8 ; when tested at pH 2.5 there is much more rapid aggregation than at the same pH without pepsin, but this aggregation is apparently reversed by subsequent neutralization. The hypothesis that the aggregation under all these conditions is an effect of proteolysis seems reasonable, but we have no definite evidence for it.

A wide range of other substances will also cause aggregation at 37° C. in the presence of salts ; among these may be mentioned saturated solutions of amyl alcohol, benzene, chloroform, ether, octyl alcohol, thymol and toluene, and 0.3 per cent solutions of benzoate or salicylate and 1 per cent fluoride.

These treatments will convert part of the less rapidly sedimenting fractions, e.g. A or B in Table I, into rapidly sedimenting nucleoprotein precipitable by virus anti-serum, but the fractions have a variable content of material that cannot be so converted. This extraneous material contains both protein and carbohydrate, and part of it separates as a coagulum if the fraction is heated to 70° in M/20 pH 5.7 buffer, but this treatment will not fully aggregate a preparation with a low virus content. Even at this pH, heating seems to bring about stable combination between the virus and some of the contaminants ; at pH 7.0–7.5 there is pronounced combination and little or no aggregation. In a similar way, if the pH of the sap from infected leaves is adjusted

to 7.0–7.5 the amount of sedimentable material may be doubled by heating, and the pellets that separate when this heated fluid is ultracentrifuged are brown and only slightly birefringent. Presumably these phenomena are comparable with the combination that occurs when aggregated virus is heated with serum proteins (Bawden and Kleczkowski, 1941).

Full aggregation can be achieved by incubation with trypsin, but the greater the content of material other than serologically active virus the greater the amount of trypsin needed to bring about full aggregation. Fractions containing 30–40 per cent of extraneous material when incubated in 0.2 per cent solution with 0.1 per cent trypsin at pH 6.8 show the disaggregating effect of cooling most clearly, for anisotropy of flow will appear after several minutes' incubation, and rise to an apparent maximum in an hour or two. At this stage it will disappear completely on cooling to 0°, but if the incubation is continued for several hours the aggregation becomes less readily reversible, and anisotropy of flow no longer disappears on cooling.

#### *Tobacco Mosaic Virus as an O-type Antigen.*

The most obvious difference between the serological behaviour of anisotropic and isotropic preparations of tobacco mosaic virus is that shown when the two are tested over a range of dilutions against dilute antiserum. Preparations showing anisotropy of flow precipitate quickly over a wide range of dilutions and give a high precipitin titre, whereas preparations not showing anisotropy of flow precipitate only after prolonged incubation and then only over a narrow zone, giving a smaller titre and large regions of antigen excess in which no precipitation occurs. If the concentration of antiserum is increased the speed of precipitation is increased, the optimum moves to a greater antigen concentration and precipitation occurs over a wider range. The floccules produced in these conditions are dense and granular, resembling those formed by spherical antigens more than the fluffy, open floccules given by anisotropic preparations of tobacco mosaic virus.

The serological reactions of somatic and flagellar type antigens differ in other ways than in the forms of precipitate, especially in the ability of their antisera to cause precipitation after various treatments. For example, antisera to somatic antigens lose their ability to cause precipitation more easily than antisera to flagellar antigens on ageing or heating, and formolized antisera to somatic antigens do not cause precipitation, whereas formolized antisera to flagellar antigens do. From these facts it was widely believed that the two types of antigen give rise to antibodies with different stabilities. Kleczkowski's (1941*a* and *b*) work with heated antisera disproved this, and showed that heating antisera produces antibody-albumin complexes which still combine with their antigens, but whereas flagellar antigens precipitate after combination with such complexes, somatic antigens remain in solution. Experiments with tobacco mosaic virus preparations which do and do not show anisotropy of flow confirm the view that precipitation by heated or formolized sera depends on the antigen and not on the antibodies, for the former behave in every way like somatic antigens and the latter like flagellar antigens; in other words, treated antisera which will not precipitate tobacco mosaic virus in its shorter forms, precipitate it when aggregated.

After heating for 10 minutes at 80° C. at a dilution of 1 : 10 in saline, antiserum to tobacco mosaic virus failed to precipitate either uncompact sediment or a freshly prepared mill extract, although it still precipitated these strongly after they had been aggregated and rendered anisotropic by heating at pH 5.5 or incubation with trypsin. Similarly, antiserum which had been stored at 1° C. for 10 years still precipitated aggregated virus strongly, giving a precipitin titre of over 1 : 1000, but it would only

precipitate mill extract when used at 1 : 50. With partially aggregated preparations of the virus precipitation is reduced by such treatments, but not prevented completely. This is shown in Table VIII, where the results of formolizing tobacco mosaic virus antiserum are given. Antiserum at 1 : 4 in M/20 pH 8 phosphate buffer was treated with 0.2 per cent formaldehyde for three hours at 18° C., and then diluted 1 : 10 for comparison with untreated antiserum for its ability to precipitate these preparations of tobacco mosaic virus. Formolization had little or no effect on the ability of the antiserum to precipitate a fully aggregated preparation, reduced the precipitation of the partially aggregated preparation (possibly because only the aggregated fraction was precipitated) and failed to precipitate the unaggregated preparation.

Antigens, like antibodies, can form complex aggregates with serum albumin while undergoing the preliminary stages of heat denaturation. Complexes of somatic antigens, such as serum proteins and tomato bushy stunt virus, still combine with their antibodies but are not precipitated by them, whereas complexes with aggregated tobacco mosaic virus still precipitate normally (Bawden and Kleczkowski, 1941). Preparations of tobacco mosaic virus showing no anisotropy of flow, either mill extracts or uncompact sediments, behave like somatic antigens. It has already been shown that these change into anisotropic preparations if they are heated to 60–70° C. below pH 6 in the presence of salts. If heated at pH 7, however, there is no change in their optical properties and no coagulum separates. This heating narrows still further the zone of precipitation with dilute antiserum, and if the ratio of other proteins to virus is high, the heated solutions may even fail to precipitate with concentrated virus antiserum. Heating in the presence of salt to 80° C. at pH 7 with added albumin leads to complete loss of precipitability with virus antiserum.

The precipitin test, by determining either precipitin end-points or optimal precipitation points, has been used successfully in earlier work for quantitative measurements of tobacco mosaic virus (Beale, 1934 ; Bawden, 1943). This work was done with virus from infective sap, and which was already aggregated. From the results in this paper it is clear that the precipitin test cannot give even an approximation to the virus content of different virus preparations if their degree of aggregation differs greatly. A given weight of virus is rendered insoluble by combination with much less antibody if it is aggregated than if unaggregated, and a visible precipitate is given by much less aggregated virus. This is clearly shown by comparing the precipitin behaviour with constant antiserum of either mill extracts or uncompact sediments before and after aggregation by heating or trypsin ; there is a factor of over sixty in the concentration of antiserum with which the solutions containing equal weights of virus precipitate optimally and a factor of about eight in the precipitation end-points. There is competition between aggregated and unaggregated virus for the available antibody, and in mixtures of the two the presence of unaggregated virus inhibits the normal precipitation of the aggregated virus. This is shown in Table IX, which gives the results of precipitin tests with mixtures containing various amounts of aggregated and unaggregated virus. From this it is clear that the apparent uniform behaviour of a virus preparation in precipitin tests, with a single optimum, is no evidence that the preparation is homogeneous.

#### DISCUSSION.

The conflicting results that have been published for the size and shape of tobacco mosaic virus (Pirie, 1945) were most simply explained by assuming that the virus could occur in a range of different sizes. The data presented in this paper confirm this, but show that the properties of different preparations can vary much more than was previously suspected, and they suggest that all previous work has probably been

TABLE VIII.—Ability of Formolized Antiserum to Precipitate Tobacco Mosaic Virus in Different States of Aggregation.

Antigen.	Time.	Control serum 1 : 50.								Formolized serum 1 : 50.									
		Antigen dilution.								Antigen dilution.									
		1 : 1.	1 : 2.	1 : 4.	1 : 8.	1 : 16.	1 : 32.	1 : 1.	1 : 2.	1 : 4.	1 : 8.	1 : 16.	1 : 32.	1 : 1.	1 : 2.	1 : 4.	1 : 8.	1 : 16.	1 : 32.
Fully aggregated (trypsin treated)	2 min.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	10 "	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Partially aggregated	10 "	-	+	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
(pellet at 24,000 r.p.m.)	20 "	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fraction D	90 "	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Uncompacted sediment	20 "	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
at 40,000 r.p.m.	45 "	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(Fraction A)	90 "	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Antigen dilution 1 : 1 = 0.5 mg./ml.

TABLE IX.—Precipitin Behaviour of Mixtures Containing Different Proportions of Aggregated and Unaggregated Tobacco Mosaic Virus.

Mill extract.	Antigen.	Heated mill extract.	Time of first precipitation.	Antigen dilution.									
				1 : 2.	1 : 4.	1 : 8.	1 : 16.	1 : 32.	1 : 64.	1 : 128.			
1		0	120 minutes	-	-	-	-	-	-	-	-	-	-
0.95		0.05	60 "	-	-	-	-	+	+	+	+	+	+
			120 "	-	-	-	-	+	+	+	+	+	+
0.85		0.15	30 "	-	-	-	+	+	+	+	+	+	+
			60 "	-	-	+	+	+	+	+	+	+	+
			120 "	-	+	+	+	+	+	+	+	+	+
0.5		0.5	3 "	-	+	+	+	+	+	+	+	+	+
			30 "	+	+	+	+	+	+	+	+	+	+
			120 "	+	+	+	+	+	+	+	+	+	+
0		1	3 "	+	+	+	+	+	+	+	+	+	+
			30 "	+	+	+	+	+	+	+	+	+	+

Antiserum used at 1 : 400. The mill extract was heated undiluted for 10 minutes at 60° C. at pH 5.5.



done with virus far removed from the state in which it is produced in the infected plant. The virus seems to have few or no fixed properties, for the properties of any given product depend on its method of preparation. By different methods of purification, preparations can be made with comparable infectivities but widely different physical and serological properties; similarly, preparations can be made with widely different infectivities but similar physical and serological properties. Many of these differences in properties can be simply accounted for on the basis of a linear aggregation of a basic particle to give rod- or thread-like particles of various lengths. This would explain the change from somatic to flagellar type of precipitin behaviour and the development of anisotropy of flow; there is ample evidence that linear aggregation does occur, but there is little evidence about the precise nature of any primary particle. We have suggested before (Bawden and Pirie, 1937*a*) that this may be small and not greatly elongated. On the evidence that has accumulated since we still incline to this view, though there are undoubtedly difficulties in it. Foremost among these is the slight infectivity of the least aggregated virus fractions. So little is known about changes which destroy infectivity without altering physical and serological properties, however, that it is impossible to assess the importance of this fact.

In this work we have laid most emphasis on conditions that lead to aggregation, and from the evidence adduced it is clear that in leaf extracts aggregation is the change most likely to occur. We have shown, however, that cooling to 0° C. leads to disaggregation in contaminated, partially aggregated preparations. The slowly sedimenting material might therefore be an artifact, produced during the freezing of the leaves, for there is a greater ratio of aggregated to disaggregated material in sap from minced unfrozen leaves than in mincer extracts from the same leaves. This probably comes about because of aggregation proceeding in the sap rather than because of disaggregation on freezing, for the ratio is also great when the sap from frozen leaves is fractionated without the preliminary removal of the diffusible leaf components. Furthermore, the ratios given by mill extracts from well washed fibre are the same whether the fibre has been frozen or not. This is not conclusive, however, for there is some disaggregation when aggregated virus is mixed with fibre from healthy leaves and passed four times through the triple roller mill. This treatment, although much more severe than the single passage used in making mill extracts, has never produced material with the properties of fractions A and B; it produces some reduction in anisotropy of flow, and changes the serological behaviour slightly towards that characteristic of disaggregated preparations. We cannot exclude the possibility that the material with small particles and slight infectivity is produced from larger and more infective particles by mincing, milling or freezing, but on balance the evidence suggests that they exist in the leaf as such and are not artifacts.

The average length of the particles in a virus preparation is controlled by the past history and present environment of the preparation; it is a compromise between the forces leading to an end-to-end adhesion and the processes, such as thermal agitation, that tend to break this adhesion. Nothing is known of the forces holding the particles together in the rods. Aggregation in the more slowly sedimenting fractions is associated with the coagulation or digestion of a large amount of material other than virus nucleoprotein. In fractions such as A, which give sedimentation constants of from 20–28 S, virus nucleoprotein amounts to only 10–40 per cent of the fraction; the size of this material cannot therefore be determined, for if it were originally polydisperse, it might not show as an entity in the sedimentation diagram. On the other hand, fractions such as B contain 70–90 per cent of material that can be converted into aggregated virus, and this material, from the results given in the accompanying paper (Johnston and Ogston, 1945), must have a sedimentation constant in the range

41 to 122 S. In some leaf extracts the greater part of the material capable of reacting with virus antiserum is present in these forms. When the more rapidly sedimenting fractions such as D are incubated with trypsin, they aggregate further. We have no evidence that these changes are also associated with the removal of extraneous material from the virus nucleoprotein, but this is possible, as our tests have not been sufficiently refined to detect the loss of less than 5 per cent of the original preparations.

The various phenomena are most simply explained by postulating that the primary virus particles are unable to exist free in solution. At its poles there are probably groups which tend to combine with other materials, so that in the plant the virus may occur in forms chemically more complex than nucleoprotein. In the absence of other suitable material it seems that the particles combine with one another to form the characteristic rods, whose average length depends on the extent to which linear aggregation is impeded by extraneous material covering the polar parts of the primary particles. Some of these states appear to be more probable than others, for the ultracentrifugal data suggest that a large proportion of the virus present in some preparations has the same sedimentation constant.

This picture of extraneous material interfering with a tendency towards aggregation affords a ready explanation of the at first sight surprising fact that treatment with a proteolytic enzyme leads to the aggregation of a preparation that is predominantly protein. If these extraneous materials are destroyed, for example, by incubation with trypsin or heating, the aggregation is extensive and stable. On the other hand, if they are merely separated from the nucleoprotein, but not destroyed, they compete for combination with the active groups on the virus. Thus the reversal of incomplete aggregation by cooling could come about because of changed conditions leading to a change in the proportion of virus nucleoprotein that is combined with itself rather than with other material. This picture is, of course, largely hypothetical, but it is now well established that tobacco mosaic virus can combine with a wide range of different materials. In this paper evidence has been given that much of the virus in the leaf is associated with other leaf components, and complexes of the virus with clupein, nicotine, arginine, ribonuclease and proteolytic enzymes have been described (Bawden and Pirie, 1937*a*, 1940 ; Loring, 1942 ; Kleczkowski, 1944).

Infective virus particles presumably have a lower limit of size, but this has not been established. Of our fractionated preparations, those containing the smallest particles are weight for weight much less infective than those with larger particles. It has already been shown that this is unlikely to be accounted for by other materials present in the preparations, and it is clear that either most of the particles with the physical and serological characters of the virus are non-infective, or all the particles in these preparations have a lower capacity for causing infection. Three possible explanations can be offered for this lower infectivity : the material may be virus that has become inactivated without losing serological activity, for this is a common phenomenon *in vitro* (Bawden and Pirie, 1937*a*) ; it may be incompletely formed virus, that is it may have developed to the stage at which it has approximately the structure of a virus particle, but lacks some essential feature ; or it may be in some way malformed virus. If either of the first two explanations were correct it might be expected that the ratio between this material and the more infective fractions would vary with the length of time the plants had been infected ; if it is inactivated virus it might be expected to increase with age, if developing virus to decrease with age. We have found no such consistent variation although we have studied leaves infected for periods varying from 10 days to 10 weeks ; up to four weeks or so the total virus increases, but the ratio of the different fractions, and their behaviour, remains much the same. We have made no extensive series of comparisons of the infectivity of

similar fractions made from plants infected for different times, but those we have made have shown no striking differences. Spencer (1942) found that the duration of infection and the nutrition of the host plant affected the infectivity of centrifugally prepared virus, preparations from old lesions and from plants receiving nitrogen being from two to five times as infective as preparations from young lesions or nitrogen-deficient plants. These differences are small compared with those between our extreme fractions, and could probably be explained on the basis of his preparations containing different amounts of materials other than nucleoprotein and being in different states of aggregation. The facts that his least infective products were always those from plants containing least virus and were those giving two sedimentation constants support this interpretation.

It is not easy to decide the extent to which the preparations of virus made hitherto contain the various fractions that we have separated, but clearly they must have been heterogeneous and have contained much aggregated material. Little of the uncompact sediment (fraction A, Table I) would occur as such, for some of this would have aggregated while the remainder would have been discarded when the ultracentrifuge supernatant was poured off. This will certainly have happened to the intermediate fractions B and C in our earlier experiments on the centrifugation of sap (Bawden and Pirie, 1937*a*), for the maximum centrifugal field was only 16,000 g., and this was attained in a rotor so much heated by air friction that convection would seriously have interfered with sedimentation. With the more intense sedimentation used in some other work, e.g. 3 hours at 60,000 g. (Ross and Stanley, 1938), 1½ hours at 50,000 g. (Loring, Lauffer and Stanley, 1938), or 1½ hours at 60,000 g. (Stanley, 1938), much of this material could have been compacted and so carried into the final preparation. In more recent preparations (Lauffer, 1944) the ultracentrifugation has been somewhat less intense. The subsequent inhomogeneity would not be expected to show in ultracentrifugal analysis, for this is not well adapted for the recognition of contaminants unless each sub-component is homogeneous and constitutes at least 5 per cent of the material present, and each of our separated fractions contains material giving different sedimentation constants. However, it is likely that the exposure to conditions in sap would have led to much aggregation before sedimentation, so that the pellet might contain few unaggregated particles though the elongated ones would be variable in their infectivity. This is even more probable with preparations made by precipitation with acid and salt, for these conditions favour aggregation; though it is likely to be still incomplete unless the sap has been heated or the preparation has been incubated with trypsin. Indeed such preparations may be the only ones that are chemically homogeneous and contain nothing but virus nucleoprotein, and aggregation may be an unavoidable sequel to purification. No detailed study of the contaminants present in virus preparations has yet been made, but it is perhaps significant that samples tested for normal leaf proteins (Chester, 1936; Beale and Lojkin, 1944) have been found to contain them unless they have been incubated with trypsin (Bawden and Pirie, 1937*b*).

#### SUMMARY.

It is shown that tobacco mosaic virus aggregates when exposed to constituents of sap and to many other agents. A method is described for extracting virus from infected leaves which greatly minimizes aggregation. The virus in such extracts is inhomogeneous, and can be separated by differential ultracentrifuging into fractions with widely different properties. The most slowly sedimenting fractions contain much material other than virus nucleoprotein; the virus in them shows no anisotropy of flow, has serological behaviour resembling that of somatic antigens and only small

infectivity. The most rapidly sedimenting fractions contain little except virus nucleoprotein, show anisotropy of flow and have serological behaviour characteristic of flagellar type antigens.

All the fractions are unstable and readily pass into forms that sediment rapidly, show intense anisotropy of flow and have a serological behaviour characteristic of flagellar antigens. In most fractions this change is accompanied by the destruction of material other than virus nucleoprotein. It is suggested that the primary virus particle is small and not greatly elongated, and that it occurs in the plant combined with extraneous materials, the removal of which sets free groups capable of combining with one another. Reasons for the variation in infectivity of different fractions are discussed, but no definite conclusions reached.

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