BAWDEN, F. C. & PIRIE, N. W. (1956). *J. gen. Microbiol.* **14**, 460–477

Observations on the Anomalous Proteins Occurring in Extracts from Plants Infected with Strains of Tobacco Mosaic Virus

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

Rothamsted Experimental Station, Harpenden, Hertfordshire

SUMMARY: When extracts from plants infected with various strains of tobacco mosaic virus were ultracentrifuged, the non-infective supernatant fluids still contained 0·5–5 % of the protein serologically related to the viruses. The small, mostly spherical, particles aggregated to form short rods as the antigen was progressively purified by precipitation with acid or salts. It formed long rods when heated in pH 5·5 buffer or when incubated with trypsin. As the particles increased in length, their serological behaviour in precipitation tests changed from 'somatic' to 'flagellar' type.

Purified preparations of the unsedimented antigen from plants infected with either of two virus strains contained 0·1–0·2 % phosphorus, seemingly in the form of a ribose nucleic acid. No evidence was obtained that the preparations were mixtures containing some particles with the 0·5 % phosphorus characteristic of infective virus and some particles of protein free from nucleic acid.

One virus strain produced a higher ratio than the others of unsedimented to sedimented antigen. The amount of unsedimented antigen was correlated with the total content of anomalous protein when the protein was increasing rapidly, but later it fluctuated unpredictably. No conditions were found that consistently favoured its accumulation, but when plants systemically infected with the type strain were kept at 36°, the total amount of antigen decreased, while the amount unsedimented sometimes increased.

The proportion of the total antigen now obtained as poorly infective nucleoprotein is much less than 10 years ago, when a third of it sedimented in the ultracentrifuge but failed to compact into a pellet. Now the uncompacted sediment, with all the host plants and virus strains used, contains only a trivial part of the total antigen.

The virus released into sap when leaves are minced is, weight for weight, more infective than the virus that remains in the leaf residues until it is released by fine grinding.

The early work on the anomalous protein contained in virus-infected plants was done primarily to identify material that could be related to the viruses themselves, and its main aim was to make preparations with the maximum infectivity and the minimum of different components. Little attention was given to non-infective material, and the emphasis laid on the homogeneity of purified preparations served only to strengthen the general assumption that viruses multiply by the replication of an infecting particle to give a result that is uniform except for occasional variants regarded as equivalent to mutations. When we (Bawden & Pirie, 1945*a*, *b*) found that plants infected with the Rothamsted tobacco necrosis virus or with tobacco mosaic virus (TMV) contained more than one type of anomalous protein, we questioned the validity of this assumption, and have since argued (Bawden & Pirie, 1950*a*, *b*, 1952, 1953) that infection is more usefully regarded as a change in the protein metabolism

of the host cells that leads to a variety of related though not identical particles. This idea at first received little support but is now becoming more widely accepted, and other workers (Takahashi & Ishii, 1952, 1953; Commoner, Newmark & Rodenberg, 1952; Jeener & Lemoine, 1953) have found that plants infected with TMV contain more than one type of specific particle. These different particles have different sizes and so can be separated by differential centrifugation.

The small TMV particles we (Bawden & Pirie, 1945*b*) found were antigenically similar to the larger ones, but had little infectivity and precipitated with antiserum only over a narrow range of antigen/antibody ratios. Various treatments aggregated them linearly; their morphology, physico-chemical properties and serological behaviour then resembled those of the largest particles present in infective extracts, but aggregation did not enhance their infectivity.

The small particles described by other workers have many properties in common with those we separated, but they differ in one major character. Whereas our particles contained similar amounts of phosphorus to those in infective virus preparations and seemed to be nucleoprotein, all the other workers describe their particles as containing no nucleic acid. This difference could be explained by the fact that, whereas we studied material that sedimented but did not compact into a pellet when sap was ultracentrifuged, they have studied material that does not sediment.

The report of specific proteins free from nucleic acid brought infections with TMV into line with those with turnip yellow mosaic virus, which also lead to a mixture of proteins only some of which contain nucleic acid (Markham & Smith, 1949). The attention now being given to non-infective particles that are protein only is in its turn tending to oversimplify the problems concerned in infection, for the assumption is now being increasingly made that infectivity depends solely on the presence of nucleic acid. Certainly all the evidence points to its presence being essential, but specific nucleoproteins that are not infective also occur, not only in plants infected with TMV, but also in those infected with the Rothamsted tobacco necrosis virus (Bawden & Pirie, 1945*a*, 1950*a*).

The significance and origin of these various specific but not infective proteins is obscure. Equally they could be stages in the synthesis of virus particles, break-down products of virus particles, or concomitant products of protein synthesis that were never destined to become infective. While studying extracts from plants infected with the tobacco necrosis virus, we (Bawden & Pirie, 1950*a*) observed that purified preparations made from sap allowed to age at room temperature, or that were treated in some other ways, were more infective than preparations made rapidly from fresh sap kept cold. We interpreted this as evidence that the virus was gaining infectivity *in vitro*; we now know that this interpretation was wrong, for we have found that pellets sedimented from fresh sap contain systems that inactivate this virus (Pirie, 1952, 1953). These systems are unstable and soon destroyed in fresh sap, but they sediment with the virus and remain active when ultracentrifuge pellets from fresh sap are suspended in water.

With these effects in mind, we examined again sap from plants infected with TMV, to see whether components of leaf sap were acting *in vitro* to affect the infectivity of this virus and the amount of non-infective protein serologically related to it. We found no effects similar to those observed with the tobacco necrosis virus, and the infectivity of extracts containing TMV varied no more with the various treatments than could be accounted for by changes in the extent to which the particles became aggregated. In the course of these experiments we found we could no longer duplicate the results we got 10 years ago. Slowly sedimenting protein then accounted for a third of the total anomalous protein extracted from systemically infected leaves. Now it accounts for only about 1 % of it. Not only can we not repeat our own early results, but we also fail to repeat the results of other workers who describe their antigens as free from phosphorus. We have never isolated a protein that is serologically related to TMV and that is free from phosphorus.

For these reasons, we have again investigated the effects on the proportion and constitution of the small particles, of varying the duration of infection, the strain of virus used as inoculum, the age and species of the host plant, and the conditions under which the infected plants were kept. These experiments have not explained why we now get so much less slowly sedimenting material than we did 10 years ago, and although they have provided abundant evidence that its quantity can vary considerably, they have failed to provide adequate explanations for the variations.

METHODS

Viruses and host plants

Unless otherwise stated, the plants infected were of the Judy Pride variety of White Burley tobacco (*Nicotiana tabacum*), a variety that resists black root rot. We also used White Burley in our earlier work, but a variety susceptible to black root rot. We have been unable to check whether this change explains some of the differences, because we can no longer get seed of the older variety. We have also used recently another variety of White Burley, two varieties of Turkish tobacco, and tomato (*Lycopersicon esculentum*), but with all these the results resembled those with Judy Pride.

Most of our work has been with two strains of TMV, which we shall call the type strain and the Datura strain. Neither is a single strain of the virus, for if single local lesions in *Nicotiana glutinosa*, or small pieces of tobacco leaf which show different types of lesions, are used as inocula they produce a range of symptoms in tobacco plants. When continued as bulk cultures both give reasonably constant symptoms in tobacco, both deforming the leaves and producing mottles of various shades of green, with occasional yellow or necrotic spots. The type strain is derived from that used in 1945 and has been maintained continuously in glasshouse plants since then. It still causes the same gross symptom-picture and, although it may have altered during the years, we have no evidence that the unwitting selection of a new variant is responsible for the current small yields of non-infective material. We have on occasion

used as inoculum purified preparations of this strain made at various intervals since 1943, but the plants infected with these behaved like those infected at the same time from the stock-culture plants maintained in the glasshouse. The Datura strain is closely related to the type strain serologically and resembles it in most of its chemical and physical properties. It differs by giving smaller lesions in *N. glutinosa* and by giving a systemic mosaic disease in *Datura stramonium*, a plant that is killed by the type strain. Other strains we have used are those called U1 and U2 by Ginoza, Atkinson & Wildman (1954), and tomato aucuba mosaic virus. U1 is closely related serologically to the type strain and behaves like it in all the properties we have studied. U2 has only few antigens in common with the type strain and gives only local lesions in Judy Pride tobacco; it is the only strain of TMV we have encountered that infects tobacco but does not infect Kondine Red tomato plants; we have studied systemic infections in Turkish tobacco. Ginoza *et al.* (1954) reported various other differences between U2 and U1, of which perhaps the most relevant to our work is that U1 combines readily with some coloured components of tobacco sap, whereas U2 does not. Tomato aucuba mosaic virus also usually gives only necrotic local lesions in tobacco, and this we have studied in systemically infected tomato plants, which show a bright yellow mottle.

Extracts of plants systemically infected with all these strains contained slowly sedimenting components that were, weight for weight, much less infective than those that sedimented readily, but none contained them in the quantity that was usual in our earlier work. The amount of unsedimented antigen varied when different virus strains were compared on the same batch of plants at the same time, but it generally paralleled the amount of sedimented antigen, so that the ratio between the two was approximately constant. Only the Datura strain gave a consistently higher ratio than the type strain; although the total anomalous protein in extracts of plants infected with it was usually only half that with the type strain, two to four times as much antigen usually remained in the supernatant fluid after ultracentrifuging.

With influenza virus multiplying in the chick embryo, the ratio of non-infective (often called 'incomplete') to infective particles depends on the inoculum and is increased by increasing the ratio of the two in inocula (von Magnus, 1953). With this in mind, we have used inocula of the type strain made from slowly sedimenting particles, but the resulting infections did not differ from those caused by highly infective inocula, and extracts from the infected plants contained no greater proportion of slowly sedimenting components.

Plants were grown in compost and received supplements of inorganic nitrogen and phosphorus as needed. The total yields of anomalous protein from systemically infected plants were increased when the supplement produced a response in plant growth, but nutrition did not consistently affect the ratio of components that sediment differently.

The mean temperature in the glasshouses was about 22°. This was higher than in our earlier work, when the houses were heated only at night because fuel was scarce, but the results of experiments in which plants were kept at

different temperatures do not suggest that a difference of a few degrees in mean temperature would alone account for the change in ratio of slowly to rapidly sedimenting components. The plants were kept under natural light, but blinds were drawn according to usual glasshouse practice so that they were rarely exposed for long to full sunlight. When experiments required a constant temperature, the plants were kept in glass-walled thermostatically controlled chambers.

For routine work, plants with three or four well-developed leaves were inoculated on two lower leaves and systemically infected leaves were harvested from 2 to 6 weeks later, when they were showing pronounced symptoms. When extracts from inoculated leaves were studied, the leaves were rubbed uniformly over their whole upper surfaces with inoculum and then rinsed thoroughly with water.

Infectivity tests were made by the local-lesion method, with *Nicotiana glutinosa* as a test plant: preparations were usually tested at two dilutions, at least six half-leaves were inoculated with each dilution, and the different inocula were distributed over the test plants so that each occurred equally often on left- and right-hand half-leaves and at each leaf position.

Serological tests were made by adding 1 ml. of homologous antiserum at a constant dilution to each of a series of tubes containing 1 ml. antigen solution at various dilutions. The tubes were placed in a water-bath at 50°, with the fluid columns half immersed to ensure continuous mixing by convection. The highest dilution of antigen at which a precipitate separated after incubating for 3 hr. was taken as the precipitating end-point. When the end-point was to be used as a measure of the relative antigen content of different preparations, the preparations were all extensively aggregated before the titrations were made. The effects of aggregation on serological behaviour are described later.

Preparation of leaf extracts

To decrease the amount of fibre and avoid diluting the extracts with fluid of a relatively low virus content, the mid-ribs were cut away from the leaf blades when the infected leaves were picked. To get a uniform starting material in experiments made to compare different methods of making extracts, the leaf blades were heaped together, and the heap was cut into strips $\frac{1}{2}$ in. wide; aliquots were taken for the different treatments after the strips had been thoroughly mixed by shaking in a large vessel.

The leaf blades were usually minced in a domestic meat mincer, the sap expressed and the residue minced and pressed again. The sap was pressed out by hand through finely woven cloth into a cylinder cooled by ice. Sometimes the leaves were ground with a pestle in a mortar, or by dispersing them in a suitable fluid in a high speed mixer (MSE Top-drive macerator), or by forcing them through a slot about 0·001 in. wide (Pirie, 1956*a*). After grinding in the meat mincer, the residues were in a convenient state for further subdivision by the macerator or by forcing through the slot.

In attempts to prevent changes in the state of virus particles during extrac-

tion from leaf cells, we added a range of substances, such as neutralized ascorbic acid, formaldehyde, sodium azide, magnesium hydroxide, and maleate or citrate buffers in amounts enough to raise the pH value from the usual 5·5–6·1 to 6·3–6·6. None of these additions consistently affected either the ratio of sedimented to unsedimented antigen or the infectivity of the virus after isolation.

In 1945 we found that the aggregation of small particles in sap could be partly avoided by freezing the intact leaves and then, after thawing, washing away the diffusible components before grinding the leaves. We have repeated this treatment and also treated the intact leaves in other ways, such as exposure to chloroform vapour at 0° or 18°, and infiltration *in vacuo* with water, ascorbic acid or buffer solutions. None of these treatments consistently affected the distribution of antigen when the extracts were ultracentrifuged, and, when freezing the leaves had any effect, in striking contrast to our experience in 1945, it decreased the amount of unsedimented antigen.

As a routine treatment, the fresh extracts were clarified by centrifuging for 16 min. at 8000*g* and 0° and the supernatant fluids were then immediately ultracentrifuged. Experiments in which the time between making the extracts and ultracentrifuging were varied, showed that long delays invariably decreased the amount of unsedimentable antigen, whereas short delays gave variable results. Thus, when immediate ultracentrifugation produced a supernatant fluid with a precipitation end-point of 1/64, the end-point was usually 1/32 when centrifugation was delayed by a day spent at 0° and only 1/8 with a delay of several days. The precipitation end-point diminished sooner at 18° or 30°, but exposures of a few hours to these temperatures, whether or not air was excluded, did not decrease the amount of unsedimented antigen. On the contrary, in several experiments more antigen was found in the supernatant fluids when extracts were kept for 3–5 hr. at 0° before ultracentrifuging than when they were centrifuged fresh. This phenomenon is elusive, and the increase may be apparent rather than real; it may reflect the failure of our routine serological procedure, which involves aggregating the antigen by heating, to detect all the antigen in some supernatant fluids from fresh extracts, rather than an increase in the antigen in the supernatant fluid of the stored sample.

Ultracentrifugation of extracts

Our standard centrifugation was: 30 min. acceleration to reach 40,000 r.p.m. (80,000*g*), 30 min. at this speed, deceleration for 30 min. down to about 25,000 r.p.m. and then more rapid deceleration to stop in 15–20 min. The rotor was cooled to 0° before a run and was usually 4° or less after it. Centrifugation at lower speeds or for a shorter time left considerable amounts of infective material in the upper 6 ml. of the 7·7 ml. contained in the tubes.

The tubes are inclined at 10° to the axis of our centrifuge (Masket, 1941), so that sedimentation is across rather than down the tubes. Consequently, in one sense, a particle is sedimented when it has moved about 1 cm., but its final position depends on the readiness with which it slides down the wall of the

tube. Provided the initial fluid is optically clear and contains nothing that sediments readily and sticks to the walls, we have no reason to think that TMV ever fails to slide down completely.

The pear-shaped pellet of compacted antigen is overlain by an uncompacted layer that is obviously denser and darker than the bulk of the supernatant fluid. In our previous work this layer contained much of the antigen, but now its antigen content does not differ greatly from that of the upper supernatant fluid. Nevertheless, we have kept the uncompacted layer separate from the pellet and the rest of the fluid. Immediately the ultracentrifuging was ended, the top 6 ml. of fluid was siphoned off carefully, and the remaining fluid poured into a different container. The pellet was allowed to drain for a few minutes, the open end of the tube rinsed with water, and the pellet suspended in water. Further subdivision of the supernatant fluid seemed unnecessary, for, as would be expected from the lateral movement of particles during ultracentrifuging, we have never found any differences between the top 3 ml. and the next 3 ml. when these portions were siphoned off separately.

RESULTS

The measurement of antigen content by precipitation tests

Commoner & Rodenberg (1955) commented on the fact, for which they said they could offer no explanation, that preparations of the fraction they called B 8 yielded smaller precipitates with its homologous antiserum than infective preparations of TMV yielded with this serum. The explanation probably lies in the different average particle size of the antigens in the two types of preparation, for although they described their B 8 as a polymerized protein, their method of aggregation (exposure to pH 5) would probably still leave many particles smaller than those in normal preparations of TMV purified by the usual method. Similarly, the slight differences they described between the behaviour of infective preparations could be explained by the fact that the preparations were in different states of aggregation at the time of testing. We found (Bawden & Pirie, 1945*b*) that precipitation of this antigen by antibodies depended on the size and shape of the particles.

The TMV antigen preparation ranges from approximately spherical particles about 15 mμ. in diameter to rods more than 1 μ. long and, with preparations at extremes of this range, precipitation with one antiserum will give the contrasting features usually considered typical of 'somatic' and 'flagellar' antigens. Preparations containing only small particles give dense white floccules that separate slowly and with dilute antiserum separate only over a small range of antigen/antibody ratios around a sharply defined optimum (Table 1). Preparations containing long rods give fluffy open precipitates that separate quickly over a wide range of antigen/antibody ratios, and there is no sharp optimum unless antiserum is used very dilute. Both kinds of preparation precipitate more rapidly below than above pH 7 (Table 1).

When antigen preparations contain mixtures of particles of different sizes, or of those intermediate between the extremes, the precipitation behaviour is also

intermediate. Most of the particles in ultracentrifuge pellets that are resuspended in water at pH 6–7 have lengths distributed around 300 mμ. and behave in this intermediate way. In our conditions of testing, precipitation is usually apparent within 5 min., but in these conditions, with antiserum diluted less than 1/100, the optimum for first precipitation is poorly defined.

Table 1. *The effect of antiserum concentration and* pH *values on the precipitation of unaggregated and aggregated antigen by antibodies*

Antiserum dilution	pH value	Time (min.)	Dilution of unaggregated antigen 1/2	1/4	1/8	1/16	1/32	1/64	
1/25	6	5	+	−	−	−	−	−	
		15	++++	+++	++	+	−	−	
		45	++++	+++	+++	++	++	+	
1/100	6	15	−	−	+	−	−	−	
		45	++	+++	+++	+++	++	+	
1/400	6	45	−	−	−	−	+	−	
			Dilution of aggregated antigen 1/2	1/4	1/8	1/16	1/32	1/64	1/128
1/400	5	5	++++	+++	++	+	−	−	−
		15	++++	+++	+++	++	++	+	−
		45	++++	+++	+++	+++	+++	++	+
1/400	6	5	+++	++	+	−	−	−	−
		15	++++	+++	++	+	−	−	−
		45	++++	++++	++++	+++	++	+	+
1/400	7	15	+	+	−	−	−	−	−
		45	+++	+++	+++	++	+	−	−

+ signs indicate the amount of precipitate. The precipitates of the unaggregated antigen were white and opaque, and those of the aggregated were grey and translucent. The time is the interval between putting the antigen-antibody mixtures in the water-bath and taking the reading.

Preparations of TMV antigen that differ greatly in their average particle size behave so differently in precipitation tests that, from a knowledge only of this behaviour, it would be reasonable to assume that they also differ widely in their content of specific antigens. This assumption is made unlikely by the fact that a preparation, which is precipitating as a typical 'somatic' antigen, will behave in a typical 'flagellar' manner with the same antiserum after the particles have been aggregated. However, as aggregation might alter the antigenicity by obscuring, freeing or destroying some antigens, we have injected rabbits separately with preparations of small particles that did not sediment at 40,000 r.p.m. and with preparations made by aggregating infective preparations. No qualitative differences were found between antisera prepared against the two types of antigen and each antiserum could be fully adsorbed by the heterologous antigen.

There is, then, nothing to suggest that the small particles differ in their antigenicity from large ones, or that increasing the length of the particles alters their antigen content. The differences in precipitation behaviour seem attributable

solely to the physical state of the antigen, which will obviously influence the measurements customarily used in serological assays. We have stressed (Bawden & Pirie, 1946) that measurements either of optimal precipitation point or of precipitation end-point can be used to assess the antigen content of TMV preparations only when these preparations contain particles in the same state of aggregation. The qualitative behaviour during precipitation tests gives some information about the state of aggregation, but the only way in which reasonable uniformity can be assured is to aggregate the preparations as fully as possible before the test is made. This, too, has the advantage that precipitation will occur more rapidly and the tests can safely be done with much more dilute antiserum.

Methods of causing aggregation

There are various ways in which preparations can be aggregated; the most reliable is to incubate them with trypsin. When many samples are to be tested, however, this is laborious, because of the need to adjust the pH value, for whereas aggregation by trypsin requires a pH value above 7, the precipitation test is best done about pH 6. With most kinds of preparation, heating at 60° and pH 5·5 is as useful as is incubation with trypsin. When using heat to aggregate the small particles in the supernatant fluids from ultracentrifuged saps, the conditions need controlling carefully, because prolonging the time at 60°, or increasing the temperature, can lead to the loss of much antigen. Our standard procedure has been to heat for 3 min. at 60° in 0·1 M-phosphate buffer (pH 5·5).

This heating aggregates the antigen adequately in unfractionated sap, in preparations made by resuspending the ultracentrifuge pellets, and in the supernatant fluids from the sap of green leaves; the titres from such preparations after heating are the same as those obtained when the preparations are incubated with trypsin. With other kinds of preparation, however, the standard heating can cause loss. Samples of supernatant fluid from ultracentrifuged sap of yellow leaves when heated fresh often give lower titres than do samples incubated with trypsin; their titres increase to those of trypsin-incubated samples when the fluid is stored for a day or more before being heated. A similar phenomenon occurs more often with the antigen present in the 1·5 ml. of fluid that overlies the pellets, and in this fraction it may happen with sap from green or yellow leaves. Some samples that gave precipitation end-points of 1/64 after incubation with trypsin, or when stored for a day before being heated, failed to precipitate with antiserum when heated fresh, but the difference is more commonly a factor of four between the samples treated differently.

We have not studied this phenomenon in detail, because, once recognized, the possible sources of error it introduces are easily avoided. All the fluids in which it occurs contain much material that coagulates at 60° and partly precipitates on ageing. This precipitation is particularly noticeable in the 1·5 ml. of fluid that overlies the pellet. We assume that the heat coagulum carries with it more antigen than does the precipitate that forms slowly in the cold, but we

have been unable to recover the antigen by incubating the heat-denatured coagulum with trypsin and there are other possible explanations. The antigen may be more than usually sensitive to heat in these fluids and be destroyed by 3 min. at 60°; or it may combine with some material in the fresh preparations to form a complex not precipitable by the antiserum, for there are other examples of 'somatic-type' antigens which form non-precipitating complexes when heated in the presence of much other protein (Bawden & Kleczkowski, 1941; Kleczkowski, 1945).

The infectivity of preparations

As a routine all the fractions separated from ultracentrifuged extracts were assayed both for their relative infectivities and their antigen contents. In addition to the regular comparisons between the sedimented pellets and the unsedimented antigen, many comparisons were also made between the pellets sedimented from different lots of sap or from leaf extracts made in different ways. Pellets from all lots of sap were highly infective, producing lesions at 100 times the dilution needed to give a visible precipitate with antiserum. By contrast, the unsedimented antigen was always poorly infective. Some supernatant fluids with precipitation end-points of 1/64 produced no lesions, and those that produced a few lesions per leaf always gave a ratio of antigen content/infectivity that was 50 or more times greater than with the sedimented antigen. Infectivity in the supernatant fluids can be avoided provided great care is taken to ensure that the cones which shut the tops of the ultracentrifuge tubes do not trap any sap, but some contamination from this source is usual when making bulk preparations. Consequently, when the antigen is concentrated by methods described below, the preparations usually contain some infective particles that should have sedimented when the sap was ultracentrifuged.

Infectivity per unit weight of the sedimented virus varied with different batches of leaf sap, but rarely by as much as a factor of two, differences too small to attempt to correlate with other variables such as duration of infection, age of leaf or cultural treatments. Purified preparations of the Datura strain were always, weight for weight, less infective than those of the type strain when tested on the same *Nicotiana glutinosa* plants.

Another consistent difference found with both strains of virus, and confirming earlier results with the type strain (Bawden & Pirie, 1945*b*, 1946), is that virus released into the sap when leaves are minced is more infective than the virus that remains in the leaf fibre until released by fine grinding. More of the total virus contained in leaves is released into the sap made by mincing leaves now than 10 years ago, but when tested at 1 mg./l. the virus sedimented from the sap has again produced three to four times as many lesions as the virus sedimented from extracts of the finely ground residual fibre. The same difference is found whether the fibre is ground with a pestle and mortar, a high-speed macerator, or by pressing hydraulically through a fine slot. As such different mechanisms produce the same result and the infectivity of purified virus is not affected by adding it to fibre from uninfected plants and

subjecting the mixture to the various treatments, it seems unlikely that infectivity is decreased by changes occurring during the grinding. This possibility cannot be excluded, but it seems more probable that the virus in different sites in the leaves differs in infectivity.

Variations in the yield of unsedimented antigen

Some samples of ultracentrifuged sap have given supernatant fluids with precipitation end-points of 1/256, after aggregating the antigen. These have been commoner with sap from plants infected with the Datura strain than with the type strain, but even with the Datura strain precipitation end-points of 1/64 and 1/32 are more usual. The end-points are not always correlated with the total antigen in the initial sap, for although high end-points are rare unless the sap also contains much sedimentable antigen, some samples of sap with high antigen contents have yielded supernatant fluids with little unsedimented antigen. Most often the unsedimented antigen has been less than 1 % of the total antigen, though with the Datura strain it has sometimes been as much as 5%. Differences in the amount of unsedimented antigen can only be measured in the supernatant fluids. Many of the variations may be caused by antigen under some circumstances appearing in the supernatant fluid and in others being sedimented, but the amount sedimented is so large that this possibility cannot be tested experimentally, because differences too small to measure in the sedimented antigen would account for the whole of the unsedimented antigen.

The yields of unsedimented antigen from successive batches of leaves intended to be similar have varied considerably. We think it unlikely that differences in centrifugation account for these variations, because extending the period of centrifuging much beyond that needed to sediment all the infective particles, or recentrifuging a supernatant fluid, does not decrease the amount of antigen that remains unsedimented. It is more likely that some uncontrolled variation in the condition of the plants affects the state of the antigen or the composition of the extract.

We have studied several variables that affect the total amount of antigen in leaf extracts without being able to correlate the amount unsedimented with any one, except with duration of infection in newly infected leaves. As found by Commoner & Rodenberg (1955), infective virus and sedimentable antigen occur in inoculated leaves before any unsedimented antigen is detectable. This does not necessarily mean that the virus is produced first; if sedimentable and unsedimented antigen were being produced simultaneously in the ratio in which they occur later, the unsedimented antigen would not be detected by our methods until the precipitation end-point of sap exceeds 1/100. Clearly the unsedimented antigen is not formed in bulk as a precursor for the large particles; if it is in any way a precursor, as suggested by van Rysselberge & Jeener (1955), it soon changes to larger particles when the virus content of infected leaves is increasing rapidly. As the total virus content of infected leaves increases, so also for a time does the content of unsedimented antigen,

but then it is apt to fluctuate in successive batches from the same plants. When we have studied plants systemically infected for different periods, however, we have usually obtained more from those infected for 3 weeks than from those infected for 6 weeks.

Table 2 shows the results of one experiment comparing the antigen content of systemically infected leaves occupying different positions on the main stem and picked 3 weeks after the plants were inoculated. Consistently, sap from

Table 2. *The content of sedimented and unsedimented antigen in systemically infected leaves of different ages*

Leaves were picked 24 days after plants were inoculated with the type strain of TMV, the sap extracted and ultracentrifuged. The supernatant fluids were titrated against TMV antiserum and their antigen content is given as the reciprocal of the maximum dilution at which precipitation occurred. The sedimented virus was purified and weighed.

Type of leaf	Unsedimented antigen (relative contents)	Sedimented antigen (g./l. sap)
Youngest, < 5 cm. broad	64	3·6
Intermediate, 5–8 cm. broad	64	2·2
Intermediate, > 8 cm. broad, green	8	1·9
Oldest, > 8 cm. broad, yellow	4	1·2

the oldest leaves contained least total antigen, but the diminution in the amount of unsedimented antigen from young to old leaves shown in this experiment was not reproduced in other experiments. The reporting of results as antigen/ml. sap can be misleading, for those in Table 2 may suggest that young leaves contained more virus than did old ones. This is not so, the oldest leaves weighed five to ten times more than the youngest and also gave more sap for equal fresh weights, so that their total antigen content was much greater.

The fluctuations in content of unsedimented antigen suggest that this antigen is unstable and that conditions in the host cells before the leaves are picked may affect its amount. Exposure to temperatures around 36° can free infected plants from some viruses, and Kassanis (1954) showed that leaves systemically infected with TMV contain less virus when plants are kept at 36° than when kept at 20°. As our results seemed to vary more in the summer, when the temperature sometimes reached 30° or more in the glasshouse, than in the winter, we have kept systemically infected plants at different temperatures before they were harvested. Plants kept at 36° for a week or more before the leaves were picked have always given less sedimentable antigen than those kept at mean temperatures of 24° or lower, but they have sometimes given more unsedimented antigen. To quote one experiment in which plants already systemically infected with the type strain were kept for 10 days in three different conditions, the yield of sedimented antigen was 4·1 g./l. sap from plants at 17°, 3·8 at 24° and 1·7 at 36°, whereas the relative yields of unsedimented antigen (expressed as reciprocals of their precipitation end-

points) were 32, 64 and 128. In other experiments, however, though the amount of sedimented antigen decreased, the amount unsedimented was not increased by high temperatures.

Purification of the unsedimented antigen

The starting material for purifying the antigen was, perforce, the supernatant fluids from leaf extracts centrifuged at 40,000 r.p.m., and producing the antigen in the amounts needed for critical examination entailed re-loading the centrifuge many times. To avoid this tedious ultracentrifuging we tried to concentrate the unsedimented antigen by such treatments as pressing water from unminced leaves that had been frozen and thawed, or concentrating the total antigen in extracts by partial freezing, by dialysis under pressure, or by precipitating it with acid or salt and resuspending in a smaller volume. None of these treatments was successful, and when such preparations were ultracentrifuged, although their supernatant fluids usually contained some unsedimented antigen, this was never more concentrated than in the original extract. These treatments presumably either aggregate the small particles so that they sediment in the ultracentrifuge or attach them to some leaf or sap component.

After centrifuging at 40,000 r.p.m., the upper 6–6·5 ml. of fluid in each tube were collected and kept at 0°. By the time three or four loads had been centrifuged, the first fluids contained some precipitate, but all were mixed, adjusted to pH 4·6 and centrifuged at 8000 r.p.m. immediately. On adjusting the fluid to pH 3·3 a shimmer developed but when centrifuged at this stage the precipitate packed badly; after standing at 0° for a few hours the fluid was stirred briefly and left undisturbed overnight for the precipitate to settle. Most of the fluid was poured off. The white precipitate contained 80–90 % of the original antigen and was collected by centrifuging at 8000 r.p.m. The remainder of the antigen was mainly in the first precipitate that separated at pH 4·6, though some was in the fluid poured off at pH 3·3. The antigen in the precipitate can be obtained by incubation with trypsin at pH 7, separating the material that still precipitates at pH 4·6, and then precipitating at pH 3·3. The antigen in the fluid poured off at pH 3·3 can be precipitated by adding $(NH_4)_2SO_4$ at the rate of 400 g./l.

The main precipitate at pH 3·3 was suspended in a volume of water equal to 1/100 the original supernatant fluid, neutralized and clarified by low-speed centrifugation. The precipitation at pH 3·3 was repeated to remove contaminants carried over mechanically at the first precipitation. The antigen solution was then ultracentrifuged; although none of the antigen originally sedimented at 40,000 r.p.m., some then sedimented, for the small particles progressively aggregated during precipitation with acid. When ultracentrifuged between pH 6·5 and 7·5, about 10 % remained in the upper 6 ml. of supernatant fluid, and the bulk was equally divided between a compacted pellet and an uncompacted sediment overlying it. As observed by Commoner & Yamada (1955), the antigen sediments more readily from acid solutions, and at pH 5·5 almost all of it compacts into the pellet. The addition of salts, e.g. 0·1 M-ammonium

acetate, also makes sedimentation from neutral solutions almost complete. Antigen that has compacted in these conditions is not permanently altered and when again ultracentrifuged from water at pH 6·5–7·5, part again remains uncompacted.

Any infective particles that may have been present as contaminants in the original supernatant fluid are concentrated into the pellet, leaving an almost non-infective, uncompacted layer. Typically, a solution of the uncompacted layer containing 50 mg. antigen/l. may give an average of fewer than 1 lesion/half-leaf, when the pellet at the same concentration gives 10 or more lesions and the virus that sedimented from sap 20 or more lesions at only 1 mg./l. For most purposes this fractionation is unnecessary, because except for slight differences in serological behaviour, which suggest that the pellet contains particles of a slightly larger average size, we have found no other differences between the compacted and the uncompacted sediment. However, all results obtained with unfractionated preparations were confirmed on preparations that were either uninfective from the beginning or were made so by fractional centrifugation.

We have used this method with equal success to make preparations of the small antigens contained in extracts from plants infected with either the type strain or the Datura strain of TMV. There is no call to distinguish between the two, for although each contains some specific antigenic groups, their general physical and chemical properties are closely similar. We have made no preparations of the small antigens from the other strains.

The purified preparations of small antigens precipitate more rapidly with TMV antisera than do the small particles in the supernatant fluids from ultracentrifuged samples of fresh sap, but less rapidly than normal virus preparations. Few or no rods are seen when the supernatant fluids are examined in the electron microscope, whereas purified preparations contain few spheres and most of the material occurs as rods approximately 15 mμ. wide and of various lengths between 30 and 200 mμ. The distribution of particle lengths was determined in some preparations; the mean was about 75 mμ. and the most common length 50 mμ. When such preparations were heated with pH 5·5 phosphate buffer, or were incubated with trypsin, the particles aggregated linearly and their physical and serological behaviour was much altered. Solutions containing 1 g./l. then showed anisotropy of flow strongly and electron microscopy showed many long particles, with an average length of more than 300 mμ. Morphologically, and serologically, the preparation was now indistinguishable from one of aggregated infective virus. It precipitated rapidly with virus antiserum, precipitated over a wide range of antigen-antibody ratios and gave a precipitation end-point of about 3 mg./l., compared with 12 mg./l. given by the same preparation before aggregation. Like infective TMV, the non-infective antigen resisted hydrolysis by commercial preparations of proteolytic enzymes and by air-borne bacterial contaminants.

Preparations of infective virus contain 0·5 % phosphorus. Our preparations of the non-infective antigen contained between 0·1 and 0·2 % P, and we gave considerable attention to finding how this is combined because other workers

(Takahashi & Ishii, 1952; Commoner *et al.* 1952; Jeener & Lemoine, 1953) were unanimous that their unsedimented antigen was not a nucleoprotein and contained no phosphorus. Repeated precipitation at pH 3·3 or from neutral solutions with ammonium sulphate, or sedimentation in the ultracentrifuge, did not alter the P content of preparations, and when sedimented in conditions which gave a compacted pellet and an uncompacted sediment, the P content of the two fractions did not differ by an amount exceeding the experimental error of determinations on small amounts of material containing so little P (Holden & Pirie, 1955*b*). Preparations were incubated with commercial trypsin, or with pancreatic or leaf ribonucleases in the presence of citrate, and when the aggregated antigen was recovered by ultracentrifugation its P content was unchanged. These treatments did not separate the phosphorus in a form unprecipitable by trichloroacetic acid; they destroyed the normal leaf nucleoprotein that often contaminates preparations of TMV (Pirie, 1956*b*), and so it is unlikely that the phosphorus is present as this type of contaminant.

The phosphorus appears to be present as nucleic acid because it is split off by heating at 100°, or by extracting at 20° with N-$HClO_4$, in the same manner as nucleic acid is split from TMV (Pirie, 1956*b*). These extracts have a characteristic UV absorption maximum at 260 mμ. The absorption maximum of the antigen itself is at 275 mμ., which is not unexpected considering the preponderating part that absorption at 280 mμ., the usual maximum for proteins, will play in a protein with such a small content of nucleic acid. These observations exclude the possibility that the phosphorus is present as a contamination by phosphate, metaphosphate or most of the phosphoric esters. As they do not exclude deoxyribonucleic acid, which is present in some TMV preparations (Hoff-Jørgensen, 1952; Holden & Pirie, 1955*a*), $HClO_4$- extracts of the antigen were neutralized with KOH, filtered, evaporated to dryness, and tested for deoxyribonucleic acid by the Dische (1955) method. Using conditions under which 20μg. deoxyribonucleic acid can be measured satisfactorily, none was found in samples with a P content and UV absorption corresponding to 300μg. nucleic acid. In comparisons between this type of extract and extracts with the same P content made from normal TMV, the same colours were given by the Bial reaction for pentoses (Dische, 1955). We have had too little material to be able to isolate nucleic acid and recognize its characteristic physical properties, but the P in extracts made by boiling the antigen was largely precipitated by acid and lost this property after incubation with leaf ribonuclease.

All this evidence suggests that the phosphorus in these antigen preparations is present as ribonucleic acid and that the link between this and protein is similar to the one in TMV, for each is stable in a range of conditions that disrupt many other nucleoproteins. An obvious interpretation for a content of 0·2 % P is that our preparations of unsedimented antigen were mixtures, consisting of material that is two-fifths nucleoprotein with the usual content of 0·5 % P and three-fifths of protein free from nucleic acid; but we have been unable to get any evidence to support this. Not only have we failed to frac-

tionate the preparations by many different methods of precipitation, but when examined electrophoretically they gave a single peak, whereas mixtures of the antigen and infective preparations of TMV gave two peaks.

DISCUSSION

The main points that call for discussion are the differences between our present and past results and between our results and those of other workers. There is no certain explanation, but it seems likely that the range of anomalous proteins in plants infected with strains of TMV can be greater than previously recognized and that the extent to which the different types accumulate differs greatly in different conditions. The main component is always a nucleoprotein with large, readily sedimentable particles and, except for some differences in particle length, this seems reasonably uniform. However, there are many methods (Bawden, 1950) whereby virus preparations can be rendered non-infective without any obvious change in their physico-chemical properties or serological behaviour, and preparations partly inactivated by such treatments have not yet been separated into infective and non-infective portions. Until some method of fractionating such a mixture is developed, the possibility cannot be excluded that ordinary preparations of TMV are mixtures of infective and non-infective particles. Indeed, the fact that preparations made at different times by similar methods differ in their infectivity per unit weight, although they resemble each other in other properties, suggests that the preparations contain different proportions of infective and non-infective particles. In our earlier work we demonstrated that much of the nucleoprotein had little infectivity. This protein did not compact into a pellet when sap was ultracentrifuged and so could be separated from the more-infective protein that did. There has been little of this uncompacted sediment in extracts we have centrifuged recently, but this does not necessarily mean that there is also only little of the non-infective nucleoprotein. It may occur in the same amounts as previously, but in a different condition so that it now compacts and the previous method of fractionation is no longer effective. The change may be in the composition of our plants or extracts, which previously allowed this material to accumulate as small particles but now does not.

Although we can not define conditions that encourage the smallest particles to accumulate, our results show that their amount varies considerably with the virus strain used, the duration of infection and with the conditions under which the plants are grown. Our results, too, agree with those of other workers in that this unsedimented protein differs chemically from the sedimentable protein, even though we find some phosphorus and they found none. Different conditions of the host plant or different virus strains may also account for this difference, because specific products of virus multiplication may accumulate preferentially in different conditions.

None of the results of detailed examination of the anomalous proteins present in extracts of plants infected with TMV fits with the idea of virus multiplication leading to a uniform, stable end-product. They are more easily

interpreted by regarding infection as a change in the protein metabolism of the host, with the conditions in the cells determining the equilibrium between the various products of metabolism. Harrison (1956) found that the Rothamsted tobacco necrosis virus can multiply at 30°, and that at this temperature it is also inactivated in the leaves. The results obtained when plants systemically infected with TMV were kept at 36° suggest that this virus, too, is inactivated at high temperatures, and that the concentration at any given time probably represents an equilibrium between synthesis and degradation. In this respect, at least, viruses probably resemble the normal leaf proteins, which also vary with different conditions of growth. In the course of synthesizing and degrading virus particles, the infected cell produces proteins of a variety of types; one type becomes detectable only when it differs in some way that allows it to be separated from the bulk product and when it is stable enough to accumulate. A change in conditions will change the relative activity of different enzyme systems in the cells, and the types of protein that are stable in one set of conditions will not necessarily be the same as those stable in another.

We thank Dr K. V. Shooter for making electrophoretic measurements on our preparations and Mr H. L. Nixon for taking electron micrographs; also Dr S. P. Capoor and S. Wildman for supplying us with strains of TMV.

REFERENCES

BAWDEN, F. C. (1950). *Plant Viruses and Virus Diseases*, 3rd ed. Waltham, U.S.A.: Chronica Botanica.

BAWDEN, F. C. & KLECZKOWSKI, A. (1941). Some properties of complexes formed when antigens are heated in the presence of serologically unspecific proteins. *Brit. J. exp. Path.* **22**, 208.

BAWDEN, F. C. & PIRIE, N. W. (1945*a*). Further studies on the purification and properties of a virus causing tobacco necrosis. *Brit. J. exp. Path.* **26**, 277.

BAWDEN, F. C. & PIRIE, N. W. (1945*b*). The separation and properties of tobacco mosaic virus in different states of aggregation. *Brit. J. exp. Path.* **26**, 294.

BAWDEN, F. C. & PIRIE, N. W. (1946). The virus content of plants suffering from tobacco mosaic. *Brit. J. exp. Path.* **27**, 81.

BAWDEN, F. C. & PIRIE, N. W. (1950*a*). Some factors affecting the activation of virus preparations made from tobacco leaves infected with a tobacco necrosis virus. *J. gen. Microbiol.* **4**, 464.

BAWDEN, F. C. & PIRIE, N. W. (1950*b*). The varieties of macromolecules in extracts from virus-infected plants. In *Viruses* 1950, p. 35, ed. by M. Delbrück, Calif. Inst. Technology.

BAWDEN, F. C. & PIRIE, N. W. (1952). Physiology of plant virus diseases. *Ann. Rev. Plant Physiol.* **3**, 171.

BAWDEN, F. C. & PIRIE, N. W. (1953). Virus multiplication considered as a form of protein synthesis. In *The Nature of Virus Multiplication*, Symp. Soc. gen. Microbiol. **2**, 21.

COMMONER, B., NEWMARK, P. & RODENBERG, S. D. (1952). An electrophoretic analysis of tobacco mosaic virus biosynthesis. *Arch. Biochem. Biophys.* **37**, 15.

COMMONER, B. & RODENBERG, S. D. (1955). Relationships between tobacco mosaic virus and the non-virus proteins. *J. gen. Physiol.* **38**, 475.

COMMONER, B. & YAMADA, M. (1955). The non-virus proteins associated with tobacco mosaic virus. *J. gen. Physiol.* **38**, 459.

DISCHE, Z. (1955). Color reactions of nucleic acid components. In *The Nucleic Acids*, **1**, 285, ed. by E. Chargaff and J. N. Davidson. New York: Academic Press Inc.

GINOZA, W., ATKINSON, D. E. & WILDMAN, S. G. (1954). A differential ability of strains of tobacco mosaic virus to bind host-cell nucleoprotein. *Science*, **119**, 269.

HARRISON, B. D. (1956). Studies on the effect of temperature on virus multiplication in inoculated leaves. *Ann. appl. Biol.* **44**,

HOFF-JØRGENSEN, E. (1952). A microbiological assay of deoxyribonucleosides and deoxyribonucleic acid. *Biochem. J.* **50**, 400.

HOLDEN, M. & PIRIE, N. W. (1955*a*). The preparation of ribonucleic acid from yeast, tobacco leaves and tobacco mosaic virus. *Biochem. J.* **60**, 46.

HOLDEN, M. & PIRIE, N. W. (1955*b*). A comparison of leaf and pancreatic ribonuclease. *Biochem. J.* **60**, 53.

JEENER, R. & LEMOINE, P. (1953). Occurrence in plants infected with tobacco mosaic virus of a crystallisable antigen devoid of ribonucleic acid. *Nature, Lond.*, **171**, 935.

KASSANIS, B. (1954). Heat-therapy of virus-infected plants. *Ann. appl. Biol.* **41**, 470.

KLECZKOWSKI, A. (1945). Conversion of non-precipitating and inhibiting protein complexes into forms again precipitable by the antisera to the original proteins. *Brit. J. exp. Path.* **26**, 33.

MAGNUS, P. VON (1953). 'Incomplete' forms of influenza virus. In *The Dynamics of Virus and Rickettsial Infections*, p. 36. New York: The Blakiston Co. Inc.

MARKHAM, R. & SMITH, K. M. (1949). Studies on the virus of turnip yellow mosaic. *Parasitology*, **39**, 330.

MASKET, A. V. (1941). A quantity type rotor for the ultracentrifuge. *Rev. sci. Instrum.* **12**, 277.

PIRIE, N. W. (1952). The infectivity of tobacco necrosis virus. *Rothamst. Exp. Sta. Ann. Rep.* p. 77.

PIRIE, N. W. (1953). Some host components that affect viruses during isolation. In Symposium 'Interaction of Viruses and Cells', VI. *Congr. int. Microbiol.* p. 11.

PIRIE, N. W. (1956*a*). A disintegrator for use with small quantities of fresh leaves. *J. agric. engng Res.* **1**, 81.

PIRIE, N. W. (1956*b*). Some components of tobacco mosaic virus preparations made in different ways. *Biochem. J.* (in the Press).

RYSSELBERGE, C. VAN & JEENER, R. (1955). The role of the soluble antigens in the multiplication of the tobacco mosaic virus. *Biochim. Biophys. Acta.* **17**, 158.

TAKAHASHI, W. N. & ISHII, M. (1952). An abnormal protein associated with tobacco mosaic virus infection. *Nature, Lond.*, **169**, 419.

TAKAHASHI, W. N. & ISHII, M. (1953). A macromolecular protein associated with tobacco mosaic virus infection: its isolation and properties. *Amer. J. Bot.* **40**, 85.

(*Received* 18 *October* 1955)