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Some Components of Tobacco Mosaic Virus Preparations made in Different Ways

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(*Received* 7 *October* 1955)

Tobacco mosaic virus (TMV) is being studied in many different laboratories for many different reasons, and with results that are not always identical. Some of the diversity is probably caused by the existence of many virus strains, but much of it comes from differences in the conditions under which the host plants are grown and in the technique used for isolating the virus. Bawden & Pirie (1945) demonstrated that several different types of fraction could be made from infected leaves, each containing material related serologically to TMV but differing in infectivity, physical properties and chemical composition.

The different fractions in extracts of the infected leaf differ in their rate of sedimentation in the ultracentrifuge and in the readiness with which they form a coherent pellet in the tube. If therefore the leaf extract is ultracentrifuged for the minimum time needed to sediment most of the virus, and if as much as possible of the supernatant fluid is poured off, the preparation will contain only part of the total material related to TMV, but that part will be relatively uniform because it will contain only one of the fractions described by Bawden & Pirie. The preparations of TMV used in most laboratories are of this type, but even with them there are some inconsistencies, and changes go on during storage.

The object of this paper is to describe some of these changes and to suggest techniques for testing TMV preparations to determine the extent to which they have occurred. These changes had gone nearly to completion in preparations made by the original brutal methods (Bawden & Pirie, 1937*a*, 1943); the gentler ultracentrifugal methods that are now generally used leave components in the preparations that are not essential for infectivity. Significance can be attached to differences in the physical properties or analytical composition of virus strains only if it is certain that all are in the same state. It is clearly convenient that this state should be the simplest with all dispensable material removed, even though there is reason to think (cf. Pirie, 1949, 1953) that complexes between the simplest form of TMV and other materials are not artifacts but exist in the infected plant.

EXPERIMENTAL

Materials and methods

All the preparations of TMV used in these experiments were the pellets made when obtaining ultracentrifuge supernatant fluid for an examination of the properties of the very slowly sedimenting forms of TMV. The technique of growing and harvesting the plants is described in another paper (Bawden & Pirie, 1956). Three virus strains—type strain, *Datura* and U2—were used; their relationships to one another are discussed in that paper. The technique of mincing and ultracentrifuging was that used for making normal leaf nucleoprotein (NP) (Pirie, 1950). Unless otherwise stated, the preparations were sedimented for 30 min. at 65 000–80 000 *g*.

The serological activities and infectivities of these preparations were measured (Bawden & Pirie, 1956) and no preparation used here was less than half as active as the best preparations. Tests were also made on material that had been exposed to the various forms of incubation. These showed that there had been no significant fall in infectivity, and the serological activities, when measured after the aggregation procedure described by Bawden & Pirie (1956), were also unaltered.

The techniques of precipitating nucleic acid with uranyl nitrate and trichloroacetic acid (TCA) and of determining total P were those described by Holden & Pirie (1955*c*).

The measurements of ultraviolet absorption were made at about 20° in 1 cm. quartz cells on a spectrophotometer (Unicam Instrument Co.).

Yeast nucleic acid was used as substrate in the determinations of nuclease activity, and was made by the method of Holden & Pirie (1955*b*).

An air-driven ultracentrifuge of the type described by Masket (1941) was used; in this the tubes are inclined at only 10° to the axis. Sedimentation is therefore effectively complete when the material has moved about 1 cm. across the tube rather than the much greater distance down it. The rotor was cooled to 0° immediately before each run, and was still at less than 5° at the end of it.

RESULTS

Determination of the nucleic acid content

General aspects of nucleoprotein fission. If preliminary treatments, followed by adequate tests, show that a TMV preparation is substantially free from phospholipid, normal leaf nucleoprotein, deoxyribonucleic acid, etc., clearly its P content can be used as a measure of the amount of ribonucleic acid present. In practice the absence of presumed contaminants cannot be demonstrated satisfactorily, and it is necessary to separate the nucleic acid or its breakdown products and to attempt their identification. This is one facet of the general problem of determining the nucleic acid content of tissues, but with TMV special factors arise. Nucleic acid is more easily separated from TMV than from many other ribonucleoproteins (Pirie, 1954; Holden & Pirie, 1955*b*). This makes it possible to differentiate between ribonucleic acids occurring in the same preparation but linked in different ways. It also makes it easier to separate the nucleic acid in a macromolecular form, and when in this form it can be further characterized by following the effect of enzyme preparations on it. This possibility is lost if the nucleic acid has been extensively degraded by separation from the protein with alkali, TCA or $HClO_4$, as in the methods of Schmidt & Thannhauser (1945), Schneider (1945) and Ogur & Rosen (1950). These methods are obviously convenient in use and are well adapted to routine estimation, but the advantages are gained at the expense of some loss in discrimination.

Some possible methods for splitting TMV have already been discussed (Pirie, 1954). Holden & Pirie (1955*b*) found that, after fission by boiling, only 2–3 % of the total P remained associated with the main protein coagulum. Not all the virus protein is precipitated with the coagulum, and treatments with other deproteinizing agents, e.g. 'colloidal iron' (Bawden & Pirie, 1937*a*) or chloroform and pentanol (Holden & Pirie, 1955*b*), are needed to remove it completely. This non-uniform behaviour of the protein suggests that even the most highly purified preparations of TMV may contain more than one type of protein, and this should be borne in mind in investigations of the structure or amino acid composition of TMV. It is also of interest in conjunction with the suggestion made by Elson & Chargaff (1955) that an intact ribonucleic acid chain is normally associated with a polypeptide chain, and that the double system is the fundamental structural particle. The amount of protein that remains soluble after heating TMV under suitable conditions is 5–8 % of the total, so that protein and nucleic acid are present in the solution in approximately equal amounts, which is in accord with Elson & Chargaff's suggestion.

Nucleic acids have their maximum absorption at about 260 mμ., whereas most proteins have it at 280 mμ. The ratio of density at 260 mμ. to density at 280 mμ. can therefore be used as a measure of the amount of protein present. Thus for purified nucleic acid the ratio is nearly 2, whereas it is 1·65–1·8 on these heated fluids. This is also the range found with extracts made by heating with $HClO_4$, so that, although there is some evidence of contamination, it is not serious and is no worse after heating than after $HClO_4$ extraction.

Fission by heating

The conditions of heating are not critical. The solution must not be acid, or nucleic acid is lost on the protein, and some salt must be present, or the protein will not coagulate (Bawden & Pirie, 1937*a*). A convenient mixture contains 2·5 g. of virus/l., 0·005 M citrate buffer (pH 6) and 0·025 M-NaCl. This is heated in a boiling-water bath for 3 min., cooled and centrifuged, and the precipitate is stirred up with half the volume of 0·025 M-NaCl and centrifuged again. The fluids are mixed for P determination and measurement of ultraviolet absorption; total P is determined on the washed precipitate. It is necessary to work with about 10 mg. of TMV, i.e. 4 ml. of solution, if the figure for the P content of the precipitate is to have any significance. If significance is being attached only to the density at 260 mμ. and to the P content of the fluid, one-fifth or one-eighth of this weight of virus is sufficient. Preparations of the different strains of TMV that have been used behave differently on coagulation. Thus the *Datura* strain has been found to flocculate more quickly than the type strain, and strain U 2 coagulates more slowly and remains gelatinous and bulky. It cannot therefore be washed satisfactorily, so that it carries down more P than the proteins from the other strains.

In spite of these differences in the texture of the denatured protein, nucleic acid appears to separate similarly from all the strains examined, provided that they have received the same type of pretreatment. But the nucleic acid does not separate completely when preparations contain significant amounts of the labile P that is discussed below.

Incubation with citrate removes this labile P, and after this treatment the coagulum is free from P. If, as is likely, the labile P comes from NP associated with the TMV, this behaviour is to be expected because, even under the best conditions that could be found (Holden & Pirie, 1955*b*), protein and nucleic acid did not separate completely when NP was boiled. These phenomena are probably responsible for much, if not all, of the effect described by Cooper (1953) and Cooper & Loring (1954). They found that only two-thirds of the nucleic acid separated from the protein when their preparation of TMV was denatured by heat in the fresh state, but that separation was more complete when the preparation had aged for a few weeks. In the absence of any other evidence it seems probable that the fresh preparations contained a considerable amount of normal leaf nucleoprotein, and that this did not release its nucleic acid on denaturation by heat. On ageing, however, the normal leaf nucleic acid would have been destroyed by the ribonuclease that accompanies it (Pirie, 1950), and so would no longer be present in the protein coagulum.

Fission by perchloric acid

Extraction with $HClO_4$ (Ogur & Rosen, 1950) is now becoming a standard method for separating the nucleic acids from tissues, and it is generally accepted that ribonucleic acid can be extracted in 24 hr. at 4° with N-$HClO_4$, whereas the deoxyribonucleic acid separates from the protein only after heating for 20 min. in 0·5N-$HClO_4$ at 70°. With TMV the cold treatment releases only two-thirds of the nucleic acid, but it is all released on exposure to N acid at 20°. An experiment illustrating the release on heating and with $HClO_4$ is set out in Table 1. This shows clearly the complete or nearly complete separation of P from the protein except when the fresh preparation is heated. It also shows that the heated fluids are as suitable for measurement of ultraviolet absorption as the $HClO_4$ extracts, and that the amount of absorption is about the same under the two conditions. The density at 400 mμ. is included as an indication of the clarity of the extracts.

Material that absorbs at 260 mμ. and is not extracted by cold $HClO_4$ but is by hot, is commonly considered to be deoxyribonucleic acid (DNA). By this criterion that part of the nucleic acid which is not extracted from TMV in 24 hr. at 4° could be looked on as DNA, and the presence of small amounts has already been demonstrated in some preparations of TMV by more trustworthy methods (Hoff-Jørgensen, 1952; Holden & Pirie, 1955*b*). No DNA is, however, detectable by the Dische (1955) procedure in TMV preparations that have been incubated with citrate, under the conditions described below, and then recovered by ultracentrifuging. Though it is a common component of TMV preparations, DNA is not therefore essential for virus activity.

Recognition of extraneous ribonucleic acid

Young infected leaves of tobacco and other plants contain NP, and it can be isolated by methods similar to those generally used in the preparation of viruses by ultracentrifuging (Pirie, 1950). This will contaminate virus preparations, but the extent of contamination is diminished by many of the pretreatments given to leaves and leaf extracts because NP is less stable than the plant viruses generally investigated. Freezing, heating to 60°, addition of ethanol or phosphate, and precipitation by $(NH_4)_2SO_4$ or acid all modify it so much that it no longer resembles TMV, and all these treatments are used in different methods of preparing TMV.

When young infected tobacco leaves are the source of TMV, and when gentle isolation procedures are used so as to get evidence about the original state of the virus in the leaf, the amount of material with properties similar to NP in a pre-

Table 1. *Separation of nucleic acid from the protein of two types of TMV preparation*

Two 10 mg. lots of freshly prepared Rothamsted culture of TMV and two 10 mg. lots of the same preparation after it had been incubated with citrate and isolated again by ultracentrifuging were used. One of each pair was heated to 100° and the other extracted at 20° with $HClO_4$ as described in the text. Phosphorus determinations were made on the whole protein precipitate and on fractions of the supernatant fluids. Ultraviolet-light-absorption measurements were made in a 1 cm. cell on another fraction of each fluid diluted to be the equivalent of 18 ml. for the whole 10 mg. of virus. The control solutions were the appropriate citrate–NaCl mixture or $HClO_4$.

Type of preparation and method of extraction	Phosphorus (μg.) from 10 mg. in the		$E_{1\,cm.}$ of the fluid diluted to 0·555 g. of initial TMV/l.		
	Protein ppt.	Fluid	260 mμ.	280 mμ.	400 mμ.
Freshly prepared and then heated	5·2	56	1·40	0·77	0·04
Same lot extracted with $HClO_4$	0	61	1·46	0·94	0·015
Incubated with citrate, ultracentrifuged, and then heated	1	54	1·10	0·61	0·04
Same lot extracted with $HClO_4$	0	55	1·14	0·72	0·01

paration becomes large. The results given in the preceding section suggest that this is also true of preparations made in other Laboratories. So far there is no method for distinguishing between NP that is merely mixed with TMV and similar material combined with TMV. That question is discussed elsewhere (Pirie, 1949, 1953, 1956*a*); here discussion is about the determination of one form of P, which will be called labile P, in a TMV preparation and with the factors that affect the quantity of it.

When preparations that have been made by two cycles of differential ultracentrifuging by the method already outlined are ultracentrifuged again, at a concentration of about 7 g./l., from neutral water, the supernatant fluid may contain only 1 mg. of P/l. This is less than one-fortieth of the P sedimented, and on the repeated ultracentrifuging there is little further removal of unsedimentable P. The pellet can be resuspended easily in water or dilute buffer solution, and nothing can be sedimented from it in 10 min. at 10 000 *g*.

Preparations like this are comparable to those commonly called TMV. They are stable if protected from bacterial contamination and contain 0·5–0·6 % P, all of which is precipitated by 50 g. of TCA/l. Every preparation made in this way has contained material with many properties of NP. Outstanding among these is the ability to autolyse if incubated for a few hours, particularly with citrate ions, at 37° and in the presence of an antiseptic such as chloroform. When NP prepared from young uninfected leaves is incubated thus, most of the protein coagulates and the nucleic acid is degraded so far as to be no longer precipitated by TCA (Pirie, 1950). Citrate is more convenient for use here than phosphate, which Bawden & Pirie (1945) had used to bring about a similar action and also to aggregate TMV preparations, because the action can then be followed by total P determination. When the proportion of NP in a TMV preparation is small there is no visible coagulation of protein. The mechanism of this effect of citrate is not known, but TMV preparations made by ultracentrifuging contain 0·3–0·5 % of Ca, and Ca inhibits the action of leaf ribonuclease on NP (Pirie, unpublished work). It is therefore probable that citrate acts by sequestering the Ca.

For routine determination of the labile P in a virus preparation, an amount of the solution containing 3 mg. of dry matter is made up to 1 ml. in 0·02 M citrate buffer (pH 6·0) and incubated for 16–24 hr. at 37° in the presence of a drop of $CHCl_3$. It is cooled and 1 ml. of TCA (100 g./l.) is added; after standing for 5–10 min. on ice the precipitate is centrifuged down, the fluid poured off and total P determined on both fluid and precipitate. A few representative figures are collected in Table 2. Five

Table 2. *Amount of labile phosphorus in different types of TMV preparation*

Methods used for making the leaf extracts and separating the virus from them are described in the text.

Type and duration of infection	Breadth of leaf and type of extract	Method of preparation	Percentage of total P in the preparation	Percentage of total P that is labile
Rothamsted strain, 20. ix. 53 to 14. x. 53	<8 cm. frozen leaves minced	Ultracentrifuged twice	0·52	17
	Unfrozen leaves minced	Ultracentrifuged twice	0·80	41
	Fibre residue from last milled	Ultracentrifuged twice	0·74	29
Strain U2, 7. i. 55 to 14. ii. 55	<8 cm. fresh leaves minced	Ultracentrifuged twice	0·71	47
	Fresh leaves pressed through slot	Ultracentrifuged twice	0·50	22
Rothamsted strain, 7. iii. 55 to 31. iii. 55	<5 cm. minced	Ultracentrifuged twice	0·9	55
	>5 cm. still green, minced	Ultracentrifuged twice	0·81	45
	>5 cm. lower yellow, minced	Ultracentrifuged twice	0·71	38
	<5 cm. minced	Sap left 24 hr. at 16° before ultracentrifuging	0·56	37
Rothamsted strain, 31. iii. 55 to 2. v. 55	<8 cm. minced	Ultracentrifuged twice immediately	0·49	20
	<8 cm. minced	Ultracentrifuged after $(NH_4)_2SO_4$ precipitation	0·51	2
Datura strain, 30. vi. 55 to 15. viii. 55	8 cm. minced	Ultracentrifuged twice immediately	0·51	24
	8 cm. minced	Sap left 48 hr. at 21° before ultracentrifuging	0·47	13

different batches of plants were used, and each was subdivided into 2, 3 or 4 for comparison of different types of treatment. Several points stand out clearly: The three virus strains are essentially similar. There is material resembling NP in TMV preparations from yellow leaves, although very little NP can be made from uninfected yellow leaves (Pirie, 1950). Labile P can amount to half the total, but the analyses in Table 2, and all the others that have been made, show that this happens only when the total P content of the preparation is anomalously high. But one-quarter of the P in a preparation with the accepted P content (0·50–0·56 %) can be labile. The proportion of labile P in a preparation is diminished by most variations in the preparative technique. Thus freezing the leaves before mincing, freezing the sap, allowing the sap to age at room temperature before ultracentrifuging, and a single precipitation with $(NH_4)_2SO_4$ all remove labile P, and all are already known to destroy NP.

The effect of varying the technique used in grinding up the leaf is also of interest. Leaf blades are generally passed through a domestic meat mincer. An alternative in small-scale work is to force the leaf through a slot 5–25 μ. wide between a steel cylinder and plug. This requires a pressure of 1–2 tons/sq.cm. (Pirie, 1956*b*). Preparations can be made from the residues after mincing and pressing out the sap by grinding in a roller mill (Bawden & Pirie, 1944), or in a high-speed macerator, or by pressing through the slot. These variations diminish the amount of labile P, and preparations made from the fibre residues generally have only 10 % of their P in this form. TMV made from fibre residues is consistently less infective than that made from sap (Bawden & Pirie, 1956), but diminished infectivity is not an attribute of preparations that for other reasons contain little labile P. Infectivity is not, for example, significantly affected by incubation with citrate.

There is no reason to think that these methods of making TMV preparations with diminished NP content are all effective for the same reason, but there is good reason to think that an enzyme is acting during the incubation with citrate. Labile P is split off at about the same rate throughout the pH range 5·5–7·5 with citrate concentrations in the range 0·1–0·003 M. But it is not split off on simple contact with citrate, nor on prolonged exposure at 0°, nor on incubation in the presence of formaldehyde (5 g./l.). This much can be shown when solubility in TCA (50 g./l.) is used as a criterion of labile P. Exposure to citrate at 0° does, however, cause changes that can be recognized by ultracentrifuging. Thus a third ultracentrifuging from 0·02 M citrate increases the P content of the supernatant fluid three- to ten-fold, compared to the value in water, with a corresponding diminution in the labile P content of the pellet. But the P in the supernatant fluid is still precipitable by TCA if the temperature during the ultracentrifuging is kept low. From this precipitate ribonucleic acid is easily prepared by the method already described for the preparation from NP (Pirie, 1950). This is the main reason for looking on labile P as ribonucleic acid, but there is no evidence that all the labile P is in this form. The facts are, however, compatible with the idea that ribonuclease in the preparations is the effective agent in these changes, and in a later section it is shown to be present in the preparations.

These results agree in many respects with those of Siegel & Wildman (1954) and Ginoza, Atkinson & Wildman (1954). They were particularly interested in the association of coloured material with TMV preparations, in the dissociation of the complex when it is exposed to phosphate and citrate, and in differences in the abilities of different virus strains to associate with the colour, but they also observed the presence of some labile P. The amount of labile P was not stated, but it was presumably small because they were working with virus preparations made from leaves that had been through a colloid mill, and the extracts had been frozen.

Presence of extraneous protein

Preparations of TMV made by salt precipitation and ultracentrifuging still carry enough normal leaf protein to elicit an anaphylactic response in guinea pigs sensitized to normal protein. This ability can be removed by incubating heated preparations with trypsin (Bawden & Pirie, 1937*b*). The amount of normal protein in these preparations is probably small. If the labile P in TMV preparations is contributed by material like NP the amount of normal protein present will be much larger because NP consists mainly of protein. A few comparisons of the effect of incubating TMV with citrate and with citrate in the presence of trypsin have therefore been made.

The supernatant fluid from preparations that have been ultracentrifuged after incubation with citrate contains only 2·6–3·2 atoms of N for each atom of P. The reason for this discrepancy from what would be expected if the action were a simple and complete breakdown of nucleic acid has not been determined, but when trypsin (Armour 'crystalline') is also present during the incubation the amount of N in the supernatant is doubled. There is no reason to doubt that, during the incubation without trypsin, breakdown of nucleic acid is predominant and the protein of the NP accompanies the TMV into the pellet, but that it is split

during trypsin incubation. The results were similar when TCA precipitation was substituted for ultracentrifuging.

These TMV preparations appear to contain enough ribonuclease to bring about complete fission of the NP when they are incubated with citrate, for no more P becomes unsedimentable when they are incubated with trypsin or with the other commercial enzyme mixtures that have been tried. Preparations made from sap aged at room temperature or by precipitation with $(NH_4)_2SO_4$ contain only 2% or less of their N in a form that does not sediment after incubation with trypsin.

Precipitation of TMV by ammonium acetate

The number of salts suitable for precipitating most proteins from aqueous solution is limited, and $(NH_4)_2SO_4$ is used more commonly than any other. It is also generally used with TMV, but there is no reason for the restriction because TMV is precipitated reversibly by a wide range of substances. Best (1940) found that the precipitate separating from ammonium acetate solution under critical conditions, like the precipitates brought out by nicotine and arginine (Bawden & Pirie, 1940), dispersed completely when the solution was shaken, and re-formed again slowly. Because the ammonium ion is particularly effective at displacing other cations from colloidal systems, and because ammonium acetate evaporates easily from a preparation *in vacuo*, the conditions for precipitation by ammonium acetate have been examined more carefully than those for the other salts, and differences between virus strains and between virus preparations in different states have been found.

With a solution of a fresh preparation of the Rothamsted type strain of TMV isolated by ultracentrifuging only and containing 5 g. of TMV/l. in 0·01 M sodium citrate (pH 6) a shimmer is immediately apparent when enough ammonium acetate solution has been added to make the solution 1·3 M. At 1·6 M concentration the shimmer slowly increases to an opacity which disappears if the solution is shaken briskly. It reappears if the solution is left quite still for a few minutes, but appears more quickly if the solution is gently agitated. Even after many hours, precipitation is incomplete at 1·6 M. A 2 M concentration gives complete precipitation, but the intermediate stages cannot then be completely dispersed by shaking.

The phenomena are similar, but more sharply demonstrable, with part of the same preparation of TMV after incubation at 37° for 16 hr. in 0·01 M citrate (pH 6) in the presence of $CHCl_3$, or with a preparation isolated from the infected sap by precipitation with $(NH_4)_2SO_4$ and at pH 3·3 (Bawden & Pirie, 1943). The shimmer is then apparent with 0·8 M ammonium acetate and precipitation is, after a time, apparently complete with M ammonium acetate. More prolonged incubation, or incubation with the mixture of enzymes in commercial trypsin, does not lead to a virus preparation that begins to be precipitated at a lower concentration than 0·8 M, but the precipitates formed are perceptibly more compact and less easily dispersed.

Under the same conditions the *Datura* strain of TMV is less easy to precipitate. Even at 2·5 M ammonium acetate the precipitation of unincubated preparations is incomplete, and with incubated preparations 1·3 M concentration is needed for precipitation. These precipitates also disperse on shaking, and the re-formation is hastened by gentle shaking.

Other salts, including $(NH_4)_2SO_4$, illustrate the same phenomena, but less dramatically. Conditions have not been found in which a properly flocculated precipitate can be dispersed, but if a partially precipitated mixture is shaken vigorously and then quickly centrifuged for 5 min. at 600 *g* the initially clear supernatant will again be partially precipitated on standing. The fact that the amount of precipitate in a system, or even the very existence of two phases in it, is so greatly affected by small rates of shear goes far to explain the difficulty often experienced in attempts to centrifuge a precipitate that appears to be settling well under gravity (cf. Pirie, 1955). The phenomenon may also be in part responsible for the scatter of the results of Loring (1940) for the 'solubility' of TMV in $(NH_4)_2SO_4$ solutions of varying concentration.

This type of phenomenon is, however, mainly of interest because it offers another possibility for following the progressive changes that go on in virus preparations during storage. In this respect changes in precipitability by certain salts under critical conditions are comparable with the change from 'flagellar' to 'somatic' type of precipitation with antiserum (Bawden & Pirie, 1945). The properties of these materials are of interest in all the forms in which they can exist, but it is the properties exhibited before *in vitro* changes have taken place that are most relevant to the biological properties of viruses.

Ribonuclease activity

The changes that take place in TMV preparations when incubated in citrate suggest that leaf ribonuclease (LRNase) is present, and a series of measurements of the LRNase activity of different types of preparation has been made. The activities are always rather low and the material that is being tested as an enzyme contains nucleic acid, part of which is undergoing fission. This introduces some difficulty in interpreting the results, but in most experiments the amount of labile P added along

with the material being tested can be kept below 6% of the P present in the yeast nucleic acid substrate. This is, for example, so in all but one of the tests set out in Tables 3 and 4.

At first the conditions defined by Holden & Pirie (1955*a*) for the assay of LRNase were used, but to get greater sensitivity the concentration of substrate yeast nucleic acid was later lowered from 400 mg. to 50 mg. of P/l. and the duration of incubation was increased to 24 hr. The experiments quoted were carried out under these conditions. Unstoppered tubes or tubes with glass or polythene stoppers were used because rubber stoppers are one cause of erratic results. Holden & Pirie found that zinc was an exceptionally powerful inhibitor of LRNase, and the zinc oxide used as a filler in many types of rubber probably dissolves slowly.

Differences between preparations of TMV. It is clear from Table 3 that the LRNase activity of a TMV preparation diminishes as the sap ages, and is greatly diminished by precipitation with $(NH_4)_2SO_4$. Preparations made from the fibre after pressing out the leaf sap have also a low activity. The presence of LRNase in a preparation therefore parallels the presence in it of the labile P discussed above, and this increases the probability that the labile P comes from NP associated with the TMV, for NP carries LRNase with it (Pirie, 1950). The precision of the enzyme assays is too small to allow a strict correlation with labile P content, but more than 100 preparations have been assayed for LRNase and labile P without a gross departure from parallelism being found. The effect of citrate incubation on the enzyme activity is therefore of

Table 3. *Comparison of the LRNase activities of preparations of the Rothamsted strain of TMV made in different ways*

Methods used for making the virus preparations and for determining labile P are given in the text. For the LRNase assay the indicated amount of material to be tested was mixed in 2 ml. final volume of 0·01 M citrate (pH 6) containing yeast nucleic acid (50 mg. of P/l.) and a little thymol. Samples (0·5 ml.) were withdrawn at the start and after 5 and 24 hr. incubation at 37°, and pptd. with the uranyl nitrate and TCA (UrTCA) reagent (Holden & Pirie, 1955*c*). Phosphorus was determined on the supernatant. To get strictly comparable figures it is necessary to subtract half of the figure in the labile P column from the 24 hr. figure because that amount of P/ml. is not contributed by the substrate YNA.

Source of preparation	Amount added (mg.)	Amount of P in prep. (μg.)	Amount of labile P in 2 ml. (μg.)	P soluble in UrTCA, in mg./l. after 0 hr.	5 hr.	24 hr.
Fresh sap	0·7	5	1·5	1	13	46
One-day-old sap	0·7	4	1·0	1	10	34
Extract from finely ground washed fibre	0·7	4	0·8	1	5	13
	2·3	13	2·6	1	7	31
Ammonium sulphate ppt. from sap	3	14	0	1	2	11

Table 4. *A comparison of the LRNase content of supernatants and pellets after ultracentrifuging TMV treated in various ways*

Four 64 mg. lots of the Rothamsted strain of TMV purified by two cycles of ultracentrifuging were recentrifuged after the treatments specified. The volume of fluid in each tube was 7·7 ml.; after the run 5 ml. was pipetted off, and the remainder of the fluid was poured off. The drained pellets were suspended and samples analysed for dry matter, total P, labile P and RNase activity. The figure put down for 'labile P' on the four supernatant fluids is the amount of P not precipitable by uranyl nitrate and TCA (UrTCA) after incubation but, unlike labile P proper, much of it was already unprecipitable as a result of the earlier incubation. To get comparable figures, half of this value should, however, be subtracted from the mg. of P/l. value at 24 hr. LRNase assay as in Table 3.

Treatment before the third ultracentrifuging	Quantity of supernatant (s) or pellet (p) tested	Weight of P in the material tested: Total (μg.)	Labile (μg.)	P in mg./l. soluble in UrTCA after different times of incubation: 0 hr.	5 hr.	24 hr.
In water at 0°	0·5 ml. (s)	1·5	(1·5)	1	11	44
	1·7 mg. (p)	13	5	1	16	51
In water for 16 hr. at 37°	0·3 ml. (s)	15	(15)	8	18	33
	1·7 mg. (p)	9	3	1	5·6	28
In 3 ml. of 0·02 M citrate (pH 6·0), for 16 hr. at 37°, then diluted to 7·7 ml.	0·1 ml. (s)	6	(6)	4	10	28
	1·7 mg. (p)	9	0	1	2	7
	9·0 mg. (p)	47	1	2	6	37
Citrate added at 0° immediately before ultracentrifuging	0·1 ml. (s)	1·5	(1·5)	3	10	46
	1·7 mg. (p)	12	4	1	5·2	22

interest, and an experiment is set out in Table 4. It shows that TMV sedimented from citrate has a diminished LRNase activity compared to TMV sedimented from water, and that a period of incubation causes still greater diminution. There is also a diminution after incubation in water but it is not so great. Table 4 also shows that much of the enzyme activity that disappears from the TMV pellet as a result of these treatments is present in the supernatant fluid. A balance sheet cannot be drawn up because LRNase shows an enhanced activity in the presence of TMV; this is discussed below. A comparison between tests 1 and 4 makes it clear that contact with citrate at 0°, which has already been shown to make much of the labile P unsedimentable, is sufficient to increase the enzyme activity of the supernatant and diminish that of the pellet. Test 3 (Table 4) shows that the activities of both fractions are diminished by incubation, and this is confirmed in separate tests with TMV incubated for various periods. The instability of LRNase on incubation at 37° has already been commented on (Holden & Pirie, 1955*c*). Experiments of this type cannot be interpreted in detail because of the conflicting effects of enzyme destruction and activation; but, during many repetitions with different incubation procedures, conditions have not been found in which the loss of enzyme activity by the pellet has not been accompanied by loss of labile P and increased ease of precipitation by ammonium acetate.

When TMV preparations, made by ultracentrifuging only, are subjected to further cycles of ultracentrifuging there is always a layer of sedimented but uncompacted material lying over the pellet in the centrifuge tube. This material has a higher content of labile P and LRNase than the pellet but, although sedimenting more slowly than the TMV pellet, it contains material related to TMV and it is generally only slightly less infective and serologically active than the pellet underlying it (Bawden & Pirie, 1956). The amount of this material is greatly diminished by any of the treatments—heating, exposure to dilute phosphate or citrate, precipitation by acid or $(NH_4)_2SO_4$ etc.—that aggregate TMV (Bawden & Pirie, 1945). By ultracentrifuging at only 30 000 *g* the proportion of the material remaining uncompacted is increased and the LRNase content of the pellet correspondingly diminished. Although the uncompacted material contains TMV, its physical and enzymic properties resemble closely those of a fraction that can be made by similar methods from NP preparations from uninfected plants. The attempt to systematize the effects of varying both the sequence of sedimentations and the ionic environment will be dealt with in a later paper on NP.

Effects of TMV on LRNase activity

So far it has not proved possible to make an infective TMV preparation showing no LRNase activity. Activities even lower than that shown by preparation 3 in Table 4 can be made by $(NH_4)_2SO_4$ precipitation and repeating the ultracentrifuging, this time from 0·05 M ammonium acetate containing 2 g. of yeast nucleic acid/l. Centrifuging in this way from yeast nucleic acid is also effective in removing LRNase from NP (Pirie, unpublished observations). LRNase is very easy to detect in the presence of TMV, for the virus has an activating or protective effect. Thus in tests similar to those in Table 4, 5 mg. of a TMV preparation that had been sedimented from YNA made 2 mg. of P/l. soluble in 5 hr. and 5 mg. in 24 hr., whereas with 0·03 unit (Holden & Pirie, 1955*a*) of pea-seedling LRNase the figures were 3 and 7 mg. of P/l. at these times. With TMV and pea LRNase together, however, they were 29 and 38 mg. of P/l. again at the same times. It is unlikely that this effect is caused by the TMV protecting the enzyme from destruction because it is also shown in brief experiments and in experiments at 25°. It is more probable that it is an example of the promotion of enzyme–substrate complex formation by a third substance, and is therefore similar to the activation of cellulase by inert protein discussed by Whitaker (1952).

TMV that has been precipitated with TCA gives another example of what is probably a similar activation. Equal volumes of the solutions, 10 g. of TMV/l. and 100 g. of TCA/l., are mixed and left for 5–15 min. at room temperature. The precipitate is centrifuged down hard, washed once with water, suspended, and neutralized with dilute KOH; nearly all the P of the original TMV is still present. If a sample of the neutral suspension is immediately reprecipitated with TCA the P is reprecipitated, but if the suspension is incubated the P becomes unprecipitable to an extent depending on the components of the incubation mixture. A few results are set out in Table 5, and from this it is clear that, as in the action of LRNase on other substrates, the presence of ions is needed for the action, and citrate ions are among the more effective. It is also clear that the action is inhibited by formaldehyde and goes faster in the presence of very small amounts of LRNase. After a second precipitation with TCA there is no action.

There is therefore no reason to look on this action as anything but an unusually sensitive test for LRNase; it is probably not akin to the nucleic acid fission that takes place in TMV treated with 0·033 N-NaOH (Grégoire, 1950). The sensitivity may be the result of intimate association of enzyme and substrate in the coagulum, but it is at least partly the result of the use of TCA as precipitant.

Table 5. *Effect of incubating TMV that has been precipitated with TCA*

0·1 ml. lots of a suspension of TMV (20 g./l., pH 6) that had been precipitated with TCA were diluted to 1 ml. in the manner stated, and incubated with a drop of $CHCl_3$ for the time stated. TCA solution (1 ml. of 100 g. of TCA/l.) was added and P determined on both fluid and precipitate.

Suspending fluid	Temp.	Percentage of P soluble in TCA (50 g./l.) after incubation for 5 hr.	24 hr.	48 hr.
Water	35°	0	30	45
0·005 M citrate (pH 6)	35°	25	45	—
0·02 M citrate (pH 6)	35°	35	70	90
0·02 M citrate (pH 6)	0°	0	0	10
0·02 M citrate (pH 6) +0·01 unit of LRNase	35°	80	90	—
0·02 M citrate (pH 6) +5 g. of formaldehyde/l.	35°	0	5	10
0·02 M ammonium acetate	35°	20	50	—
0·1 M ammonium acetate	35°	30	60	—
0·1 M-NaCl	35°	15	45	—
0·003 M ethylenediaminetetraacetate	35°	20	45	—

TCA precipitates nucleic acid incompletely from dilute solution (Holden & Pirie, 1955*c*), but in these experiments, presumably because the nucleic acid is being carried down along with the TMV protein, TCA is satisfactory. When it is replaced by the uranyl nitrate and TCA reagent there is more complete precipitation of nucleic acid fission products; hydrolysis is then shown only after more prolonged incubation and with some preparations of TMV it remains incomplete. This suggests that, as in other actions where many links of the same type are being hydrolysed enzymically, the first stages of the action are disproportionately easy to detect. Preparations of TMV that have been purified so far that the hydrolysis of yeast nucleic acid by them is only just perceptible do not have their enzymic activity against yeast nucleic acid enhanced by precipitation by TCA. There is, therefore, no reason to think that nuclease is being produced here from an inactive precursor.

DISCUSSION

From these observations it is clear that preparations of tobacco mosaic virus made by quick and gentle methods contain material that is not present in preparations that have been made by methods that are rougher or more dilatory. There is no conclusive evidence as yet that these other materials are combined with the virus and not simply mixed with it, and it is probable that it would be wisest to regard many of the preparations described here as contaminated with adventitious components of the leaf not necessarily associated with the virus. This is a question that cannot be settled by the technique used here, and the only relevant evidence is that preparations made from leaves so old that little or no nucleoprotein would have been isolated had they been uninfected, contain a significant amount of material with an instability and enzyme activity similar to that of normal leaf nucleoprotein (NP). The fact that tobacco mosaic virus made from leaves that have been frozen or very finely ground in a roller mill or by pressing through a narrow slot contains less labile phosphorus does not give positive support to either point of view. Freezing destroys much of the NP, but fine grinding does not. The interpretation of this particular piece of evidence is complicated by the fact (Bawden & Pirie, 1956) that tobacco mosaic virus made by fine grinding is invariably less infective than that made normally.

Whether or not it is adventitious, material similar to NP is present in tobacco mosaic virus preparations made by the method used here, and it is probably present in preparations made by the methods used in many other Laboratories. Its recognition is therefore a matter of importance, and a few tests may be proposed that should be applied to every tobacco mosaic virus preparation destined for a study of amino acid, purine, or pyrimidine composition or for the measurement of any physical properties. After incubation with citrate and chloroform the amount of phosphorus and nitrogen appearing in forms not precipitable by trichloroacetic acid should be negligible, otherwise it is reasonable to conclude that material like NP is present. On incubation with trypsin no nitrogen should become soluble in trichloroacetic acid except the 0·5% that would come from threonine split off if enzymes able to catalyse the action studied by Harris & Knight (1955) are present.

When freed from these materials, preparations of tobacco mosaic virus appear to contain only the cations needed to maintain neutrality, and protein and nucleic acid bonded in such a manner that they resist attack by those nucleases and proteases that have been tested. This is the limit beyond which fractionation has not been carried without loss of

infectivity. To avoid ambiguity, preparations in this state need a designation, and this should distinguish them from those in general use without raising the issue of whether the removal of all the other materials from the preparation can properly be called a purification (cf. Pirie, 1949, 1953). The term TMV (*L*) is suitable because of the limiting character of this type of preparation.

Experience with the strains of tobacco mosaic virus studied here suggests that by the time fractionation has been carried so far as to have removed these extraneous forms of phosphorus and nitrogen, the virus will no longer be in its original state. There is an increase in the readiness with which tobacco mosaic virus shows anisotropy of flow; this has been commented on repeatedly, but elaborate measurements would be needed to put it on an objective basis. Two other properties are easier to define. The behaviour on precipitation with ammonium acetate is described here, and it is probable that any tobacco mosaic virus preparation that does not become precipitable at a lower concentration after incubation with citrate and chloroform, or trypsin, has already been modified during the process of purification. The criterion for native tobacco mosaic virus has to be put in this slightly roundabout way because the different strains of virus precipitate with different concentrations of ammonium acetate; it is the change in precipitability that is significant. Parallel with this change is the change in the concentration at which a neutral and relatively salt-free solution of tobacco mosaic virus deposits a liquid–crystalline layer. It is unusual to find a fresh preparation, made by ultracentrifuging only, that will deposit this layer even when as concentrated as 60 g./l., but as *in vitro* changes proceed, liquid–crystallization becomes easier. Preparations that have been ultracentrifuged after being incubated with citrate generally form the layer at 20–25 g./l., and when they have matured further they form it at even lower concentrations. The limit found with the preparations studied here was 6·8 g./l.

It is tempting to try to simplify the picture and interpret all these phenomena in terms of two variables: the extent to which tobacco mosaic virus is contaminated in a purely mechanical way by extraneous substances, and the extent to which a hypothetical fundamental unit, perhaps 300 mμ. long, becomes broken or aggregated linearly. These attempts may yet succeed and it may be a coincidence that the treatments which remove NP from a preparation are those that promote aggregation. But it is also possible that the removal of material like NP from the ends of the tobacco mosaic virus rods is an essential preliminary to aggregation.

SUMMARY

1. Part of the phosphorus in many tobacco mosaic virus preparations becomes soluble in trichloroacetic acid when the preparations are incubated. There is evidence that this is caused by the fission of normal leaf nucleoprotein by ribonuclease.

2. Some steps in the purification remove the normal nucleoprotein but other properties of the virus, notably its precipitability by ammonium acetate, are altered at the same time.

3. Plant ribonuclease is more difficult to remove and it is detectable in all preparations. The smallest amount is found in those that have been ultracentrifuged from salt solutions in the presence of nucleic acid. Tobacco mosaic virus that has been denatured by trichloroacetic acid is a particularly sensitive system for detecting this residual nuclease.

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