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The Isolation from Normal Tobacco Leaves of Nucleoprotein with some Similarity to Plant Viruses

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Thirty years ago various lines of evidence defined the limits of size within which the viruses fell, and it became obvious that viruses were larger than other soluble materials that had by that time been studied biochemically. It was generally assumed that they were largely protein, and it was therefore probable that they would be dense enough to be separable by centrifugation; this was achieved with tobacco mosaic virus by Bechold & Schlesinger (1933). Infective material has been sedimented from extracts of diverse origin in many different laboratories, and the products so obtained are generally spoken of as if they consist largely or even exclusively of virus. This usage depends on the underlying assumption that particles with physical properties similar to those of viruses are rare in uninfected tissues. Claude (1940) showed that the assumption was unfounded and,

using the same techniques on normal tissue that were being used to make preparations of Rous sarcoma virus from tumour tissue, he prepared virus-like material from a range of tissues. His investigations laid the foundations of our knowledge of macromolecular tissue components. The need for adequate controls is so well recognized in biochemistry that it seems odd at first sight that preparations from uninfected tissues should not have been made earlier and more extensively. Much of our knowledge of viruses has, however, come from work that was not primarily concerned with their biochemistry, and it is often by no means easy to see which normal tissue would be most suitable for use as a control, and in what nutritional state it should be. Whatever the reason, no work on plant material has so far been published that is at all comparable in

thoroughness to Claude's work on animal tissues. Detailed knowledge of plant viruses is limited to the small fraction of their number robust enough to withstand the ordinary procedures of fractionation (cf. Pirie, 1946). The leaves on which viruses are generally cultivated do not, however, contain significant amounts of extractable macromolecular protein with this stability. The normal macromolecules only obtrude themselves when methods adapted to the unstable plant viruses are being used. Information about them is not only of intrinsic interest, but it is an essential preliminary to any work on the unstable majority of plant viruses.

In this paper attention will be confined to material that could be carried through into a virus preparation made by the ultracentrifugation of extracts of tobacco leaves. The main component is a nucleoprotein. There is every reason to think that it is inhomogeneous and it may be derived from different parts of the leaf; it will however, for convenience, be treated as if it were a single substance, and will be referred to as NP.

METHOD OF PREPARATION

The midribs are cut out from tobacco leaves immediately after picking, and the laminae are passed through a domestic meat mincer and squeezed on madapolam into a cylinder cooled with ice. The residue is reminced and squeezed again. Starch grains and the coarser cell fragments are removed by centrifuging for 10 min. at about 8000 rev./min. (6000 g) in a chilled angle centrifuge and the slightly green and opalescent fluid is ultracentrifuged in a chilled head of the type described by Masket (1941). 30 min. at 40,000 rev./min. (80,000 g) is sufficient. Sedimentation aggregates most of the chromoprotein in leaf sap so that a pale-yellow opalescent extract results when the ultracentrifuge pellets are stirred up with water at pH 6, using one-tenth of the original volume of sap, and centrifuged for a few minutes at 8000 rev./min. The green residue is re-extracted with water. Each of these extractions should be allowed to proceed for about 1 hr. before the residue is centrifuged off; the mixture should be kept cold at all times. The combined extracts are ultracentrifuged again and the pellet is extracted with water, this time the volume of water used should be one-fiftieth or one-hundredth of the volume of sap. The product is a colourless or pale-green opalescent solution. The chromoprotein can be removed completely by centrifuging for 30 min. at 8000 rev./min., or by ultracentrifuging again. Some NP is lost along with the chromoprotein by either procedure because it also undergoes aggregation when sedimented. Twice centrifuged material was used in the experiments to be described and it was always used within a few days of preparation. Most experiments have also been repeated on material sedimented three or more times so as partially to exclude effects due to traces of sap carried mechanically through two sedimentations.

Tobacco (*Nicotiana tabacum*) plants grown in a heated glasshouse have been used as the source of NP in all the experiments quoted here, but preparations with similar properties have also been made from tomato (*Lycopersicon esculentum*), French bean (*Phaseolus vulgaris*) and white

dead nettle (*Lamium album*). The extracts from these other species are more difficult to handle and the yields of NP have been no better than from tobacco.

Yield. As will be obvious from the precautions used during the preparation, leaf sap is an environment in which NP is unstable. This is not altogether surprising for sap is not a physiological fluid, but is a mixture that is made during the process of mincing, and it is at that time that NP is first exposed to it. If sap is allowed to stand at room temperature or even at 3° for a few hours the yield falls, and if NP that has been isolated by the method described is added to leaf sap the amount recoverable falls if ultracentrifugation is delayed. This fall is especially marked if sap from leaves more than 10 cm. long is used, and after 24 hr. at 3° little or nothing can be separated by the method described here. Sap from leaves less than 8 cm. long gives a good yield even after several days at 3°. It is well known that the dry matter, and especially the Ca content of tobacco-leaf sap increases with the age of the leaf. This may well account for the loss, for, as will be shown, Ca ions precipitate the protein. Delay in the preparation or inadequate cooling will therefore cause loss. The yield is greatly diminished if the sap is frozen.

No study has been made of the effect of cultural conditions on the yield of protein, but there is a systematic fall with increasing size of leaf. This is illustrated in Table 1 (a) where the

Table 1. *The effect of the size of the leaf on the nucleoprotein content of the sap*

	Length of leaf (cm.)	Average wet weight of leaf (g.)	Yield of NP (g./l.)
(a)	Under 16	1.2	1.1
	16-21	4.0	1.5
	22-26	5.5	1.2
	26-30	7.0	0.45
	Over 30	8.8	0.19
(b)	Under 24	2.4	2.7
	24-30	5.1	1.0
	30-35	7.4	0.8

yields from batches of leaves of different sizes, all taken from the same group of plants, are set out. These leaves were taken from plants about 2 months old and not yet sending up a flower shoot. Only the largest were perceptibly yellow; if yellowing or wilting goes further the yield is still smaller. Table 1 (b) sets out the results of a similar experiment with the lanceolate leaves of 3-4-month old plants already carrying flower buds. The high yield got from these lanceolate leaves suggested that the axillary leaves which develop on the stem after the first leaves have been harvested would be a good source of the protein, and several batches, with leaves up to 10 cm. long, have given yields of 1.5-2.0 g./l.

The instability of the protein in sap appears to be partly due to the low molecular weight components of the sap. Attempts have therefore been made to remove these by washing them away after destroying the osmotic control of the leaf by freezing or exposure to CHCl_3 or ether. These treatments, however, diminish the yield; freezing the intact leaf indeed causes a greater diminution than freezing the mince from a control sample of leaves. The yield is also diminished if the sap is dialysed before ultracentrifuging. It can, however, be increased by the addition of several agents, during or just after mincing, or even to the sap. No attempt has yet been made to systematize these effects, but with

mature leaves (15–30 cm. long) azide, thioglycollate and citrate increases the yield, whereas fluoride diminishes it. Thus the addition of 0.01 and 0.05 M-citrate and 0.01 and 0.05 M-azide, both at pH 5.8, to samples of the same sap immediately after expression, raised the yield of protein from 1.1 to 1.9, 3.5, 3.7 and 4.0 g./l., respectively. Yields are not often as large as this, but with large leaves, or with small leaves if the sap is allowed to lie for some hours before ultracentrifuging, the proportionate effect is of this order. This effect suggests that the lower yield of NP from large leaves is due to its greater instability in this sap, and to the greater firmness with which it adheres to the fibre rather than to an actually lower content in the leaf, but this has not yet been proved.

other materials, but measurements have been made of the distribution of P in the different fractions into which a leaf is divided, and the results of one such experiment are set out on Table 2. Three things are clear from this table; (1) most of the P, other than PO_4^{---} initially present in the sap, remains attached to the fibre after mincing and washing and more than half of this appears as PO_4^{---} on incubation; (2) 80% of the P that can be precipitated from the sap by TCA is sedimented on ultracentrifugation; (3) there is more P that can give rise to PO_4^{---} on incubation in the two types of sediment that separate at 8000 rev./min. than in the final preparation of NP. These facts suggest that, although most of the NP in the sap is being separated from it, only a small part of the total NP of the leaf may be being isolated. Mention has already

Table 2. *Partition of phosphorus from 100 g. of leaf*

(Leaves 12–15 cm. long, without midribs, were minced and pressed as described in the text, and the fibre residue washed three times with water. The washed fibre, the washings, the undiluted centrifuged sap and the sediment got by centrifuging at 8000 rev./min. were analysed for P separately. Samples from the fibre and 8000 rev./min. sediment were incubated at 38° for 48 hr. with two lots of water in the presence of CHCl_3 ; PO_4^{---} was determined on the fluid.

P precipitable by TCA was determined on the sap and on the supernatant fluid from an ultracentrifugation at 40,000 rev./min. and PO_4^{---} was determined on the filtrate from the TCA precipitate. The 40,000 rev./min. sediment was worked up in the manner described in the text, and P was determined on the purified NP fraction and the 8000 rev./min. sediments separated during the preparation. The PO_4^{---} produced by the latter on incubation at 38° in the presence of CHCl_3 was also determined.)

	Quantity of each fraction	Amount of P in the whole of the fraction (mg.)	Partition of P in the fraction	
				(mg.)
Washed fibre	5.5 g. dry matter	15	P appearing as PO_4^{---} on incubation	9.9
Pooled washings of fibre	310 ml.	4.3		
Sap centrifuged at 8000 rev./min.	78 ml.	13.6	Inorganic P	6.8
			P precipitable by 4% TCA	4.1
			P precipitable by 4% TCA from ultracentrifuge supernatant	0.78
			P in the final NP preparation	1.7
			P in the sediments at 8000 rev./min. separating during the preparation	1.6
			P in this sediment appearing as PO_4^{---} on incubation	1.3
8000 rev./min. sediment from sap	0.96 g. dry matter	2.1	P appearing as PO_4^{---} on incubation	0.95

Changes of pH in the mince in the range 5.0–7.5 do not significantly affect the recovery of phosphorus in the preparation, but from sap that has been made slightly alkaline there is a larger yield of sedimentable material, with a reduced phosphorus content.

Distribution of NP in the leaf. The technique of mincing used is so crude that it is to be expected that much of this protein will remain in the leaf fibre after pressing out the sap, some may precipitate along with the chloroplasts and starch during the initial centrifugation, some may escape sedimentation on the ultracentrifuge, and some may fail to resuspend after ultracentrifugation. At present isolation is the only certain method of recognizing NP, but P estimations can set limits to the amount that may be present. Two properties, which will be described more fully in later sections of this paper, enable the limits to be defined more closely; P in the form of NP is precipitated by trichloroacetic acid (TCA), and it is liberated as PO_4^{---} when NP is incubated at 37° at neutrality. It is obvious that NP shares the properties with

been made of the increased yield caused by the addition of azide and some other agents during the preparation; it is probable that these agents act not only by protecting NP from destruction, but also by mobilizing part of this bound NP. Unsuccessful attempts have been made to increase the yield by other means. If the grinding is made more thorough, e.g. by use of a roller mill (Bawden & Pirie, 1944), more P is extracted, and this sediments on ultracentrifugation. But fine grinding also increases greatly the amount of chromoprotein in the extract, and satisfactory separation of the two has not been achieved. Little P that sediments on the ultracentrifuge is extracted from the fibre residue by water, and only a little more is extracted by NaCl. Extracts made with 2–10 g. NaCl/l. contain significant amounts of protein sedimentable in the ultracentrifuge, but this contains only 0.1–0.5% P. Further work on the nature of the P associated with the fibre and the more readily sedimented fractions of the leaf is in progress, and will be reported later.

Properties

Composition. The analytical composition of the protein depends on the number of times it has been ultracentrifuged, for, as will be described later, it dissociates to some extent and the fractions differ in composition. When made by the standard method it contains 13–15% N, 2.0–3.0% P and 10–16% carbohydrate, estimated by the colour developed on

Behaviour on repeated centrifugation. Even the freshly prepared protein is not homogeneous; a fraction with diminished P content can be sedimented from it slowly on an angle centrifuge at 8000 rev./min. An experiment is set out in Table 4 which shows that this sedimentation takes place more readily in acid solution.

The fraction of a preparation that sediments in this way at any pH is substantially independent of

Table 3. *The protection of nucleoprotein by azide from the deleterious effects of ageing in sap*

(Half of a uniform batch of leaves was minced and pressed as described in the text, half the sap was ultracentrifuged immediately, and half after 5 hr. at 25°. The other half of the leaves were treated in the same way except for the addition of NaN_3 during the mincing. The yield and composition of the products from these four types of sap are set out.)

		Yield (g./l.)	P content (%)	Carbohydrate content (%)
Minced normally:	Centrifuged immediately	1.03	2.1	13
	Centrifuged after 5 hr. at 25°	0.24	1.7	12
Minced with 1 mg. NaN_3 /g. of leaf:	Centrifuged immediately	1.4	2.0	12.3
	Centrifuged after 5 hr. at 25°	1.3	2.0	12.0

heating with orcinol in H_2SO_4 , and expressed as glucose (Pirie, 1936). From the rate of colour development with orcin and from the positive Bial test this appears to be pentose. No deoxypentose has been detected by the methods of Stumpf (1948) or Cohen (1944). Preparations made from sap that has been dialysed or allowed to age, or from large leaves, contain less P and carbohydrate. Agents, such as azide which protect the protein from destruction on ageing in sap, also protect it, to some extent, from loss of phosphorus. This is illustrated in Table 3. If a solution that has been centrifuged for 30 min. at 8000 rev./min. is run into 10 vol. of an ethanol-ether mixture containing 0.1N-HCl, less than 5% is soluble and only part of this is afterwards soluble in CHCl_3 . This lipid contains only traces of P and carbohydrate. The pellet at 8000 rev./min. may, on the other hand, contain as much as 0.7% of phospholipid P. 5–8% of residue is left after combustion; the metal in this residue is predominantly Ca even after the preparation has been ultracentrifuged from 0.05M-KCl or NaCl. No other component of NP has, as yet, been recognized.

Table 4. *Sedimentation of nucleoprotein at different pH's*

(A 9 g./l. solution was divided into four 3 ml. lots, and these, after adjustment of the pH, were centrifuged simultaneously for 30 min. at 8000 rev./min. The fluids were poured off and the sediment was dried, weighed and analysed.)

pH	Percentage in sediment	P content of sediment (%)
5	90	1.7
6	30	1.3
6.9	23	1.05
8.1	19	0.93

the size of the leaf, or the age of the sap from which it was prepared. Material with a slightly enhanced P content can be ultracentrifuged from the supernatant fluid at 40,000 rev./min. in the usual way. This pellet is, however, still inhomogeneous, and can be separated as before by centrifuging again at 8000 rev./min. On each ultracentrifugation some material remains unsedimented, and some does not resuspend when the sediment is stirred up with water. Less of the protein becomes insoluble if it is sedimented for 30 min. at 26,000 rev./min. (35,000 g), and this lower speed is adequate for complete sedimentation when the suspending fluid is water, although not with the original sap. The amount

Table 5. *The solubility of ultracentrifuge pellets separated from salt solutions of different strengths*

(Four solutions containing 1.5 g./l. NP and different concentrations of NaCl were ultracentrifuged for 30 min. at 36,000 rev./min. The pellets were suspended in water and centrifuged at 8000 rev./min. for 10 min. The insoluble material was dried, weighed and analysed.)

Suspending fluid	Percentage of material not resuspending after sedimentation	Phosphorus content of this insoluble material (%)
Water	35	1.5
0.5 g./l. NaCl	12	1.15
2.0 g./l. NaCl	17	0.9
8.0 g./l. NaCl	59	0.77

becoming insoluble is also diminished if the protein is sedimented from a dilute salt solution instead of water. This is illustrated in Table 5. It is only after being twice sedimented from leaf sap that the protein is freed sufficiently from other material to make this

effect noticeable. There is, therefore, no advantage in suspending the pellets, from the first run on sap, in salt solutions rather than in water. On each ultracentrifugation part of the P of a preparation remains in the supernatant along with a smaller proportion of other material than in the original preparation. Material containing 4–5 % of P can be made in this way. Old preparations and preparations that have been centrifuged at pH 7–8.5 give more of this unsedimentable material. Its formation on incubation will be described later. Clearly centrifugation brings about partial dissociation into aggregated material with a low, and unsedimented material with a high P content. By further centrifuging there therefore appears to be no possibility of making a homogeneous preparation.

Two preparations were examined electrophoretically. In 0.02M-phosphate at pH 6.5 each contained two components moving at 15 and 11×10^{-5} cm.² sec.⁻¹ V.⁻¹, respectively; the faster accounts for about 70 % of the refraction increment. Both components contain P, the faster rather more than the slower, but facilities have not been suitable for the separation of a sufficient amount of protein for characterization of the two components. The electron micrograph shows particles with a greater tendency to adhere than the spherical plant viruses have. Here also the inhomogeneity shows clearly and, although preparations consist mainly of spheres 20–30 m μ . in diameter, there is much amorphous material. Drying this protein in bulk leads to complete loss of solubility even when the drying is carried out at low temperature. There is no means of telling whether the amorphous material is derived by the breakdown of some of the spheres or whether it is present in the undried preparations. Evidence has already been given that NP becomes more readily sedimentable at pH 5, in more acid solution it flocculates. Precipitation is complete in the pH range 4.3–3.6, but in more acid solution there is partial resolution. After acid precipitation, even at 0°, it does not dissolve again completely at neutrality. With (NH₄)₂SO₄ there is nearly complete precipitation at one-third of saturation and it is complete at half saturation, but these precipitates also do not redissolve completely in water. The amount of protein that remains insoluble can be diminished by working rapidly, but even if the cycle of operations is completed in 15 min., and at a low temperature the final product is so modified that it precipitates again from solution in a few days. Ca or Ba ions precipitate the protein from neutral solution; to get complete precipitation of a 5–1 g./l. solution of NP the concentration of divalent ion must be 0.01M. This is more than ten times the amount already present in the soluble NP preparation. The precipitate redissolves when the excess divalent ion is removed by washing with water or, better, with 0.002M-NaCl or NH₄Cl. After

this treatment it is as stable as before, and there is no evidence that the precipitation has brought about any fractionation. In the presence of Na salts greater concentrations of Ca are needed.

Attempts have been made to use these precipitants to concentrate NP from the initial leaf sap, but they have not proved satisfactory. Each causes serious losses, and they precipitate so many other sap constituents that it is not practicable to reduce in this way the volume being centrifuged.

Antigenicity. The protein has a low apparent antigenicity as would be expected from the success that has attended the use of unfractionated sap from plants, with various virus infections, in making specific antiviral antisera. Intravenous injection of over 100 mg. of protein, in 10–20 mg. lots, into a rabbit produced a serum with a dilution end point of 1:80. With smaller amounts of antigen the sera were even more feeble. The end point for precipitation of the antigen by serum under optimal conditions was 50 mg./l. Clearly the antigenicity, whether measured by the ability to produce antibody or by precipitation end point is feeble if compared with that of the plant virus preparations or the bacterial antigens. There are many possible causes for this feebleness. NP may be intrinsically a poor antigen, it may contain many different antigenic groups each of which is acting separately, its instability may lead to its destruction in the rabbit before antibody formation can take place, or to destruction during the serological test before union with antibody can take place.

Stability. Solutions of NP become more opalescent during a few weeks in the ice box and then flocculate. This process is accelerated by some antiseptics, e.g. CHCl₃, toluene, and octyl alcohol. Precipitation is not significantly affected by thymol. When flocculation occurs most of the P has been split off from the protein, but this is only the culmination of a continuous process. If samples are ultracentrifuged at intervals after preparation, the unsedimentable P increases steadily until, with flocculation, none of the P that is in solution can be sedimented on the ultracentrifuge. Ions, especially citrate and PO₄, facilitate flocculation. No fluid has been found in which NP can be frozen without becoming largely insoluble on thawing. There is at the moment, therefore, no method of preservation, and all observations that are reported here were made on preparations within a few days of harvesting the leaves.

Changes analogous to those that proceed during storage in the ice chest can be studied conveniently at 38°. At this temperature flocculation takes 6–10 hr. in the absence of salts and by the time it has taken place the separation of the P-containing moiety is substantially complete. In Table 6 an experiment is set out showing how both flocculation and fission take place more quickly in the presence of NaCl. In this experiment samples of the fluid were

withdrawn after the interval stated and precipitated with TCA so as to get a compact protein precipitate on centrifuging; total P was estimated on both fluid and precipitate. Similar results are given by experiments in which the coagulum is centrifuged off without adding TCA, but there is then more P-containing fluid carried mechanically in the precipitate. The agreement, which appears in experiments like those in Table 6, between fission and flocculation is, however, largely fortuitous; the two processes are independent. Thus CHCl_3 promotes flocculation without having a significant effect on fission, and citrate promotes fission but not flocculation. This is illus-

Table 6. *Percentage of phosphorus separated from protein after different times of incubation in different environments*

(The four tubes contained 1 g./l. NP and NaCl at the concentration stated. At intervals 0.5 ml. was withdrawn. After the stated periods of incubation at 38°, 0.5 ml. samples were withdrawn from each and precipitated with 1.5 ml. of 5.5% TCA. P was determined on the fluid and on the precipitate, and the figure set down is the percentage of the P that was in the fluid.)

Time (min.)	Concentration of NaCl			
	0	0.01 M	0.02 M	0.05 M
0	0	0	0	0
50	16	38	49	90
100	23	58	90	91
200	58	90	100	100
400	91	100	100	100
Time at which flocculation was apparently complete (min.)	400	120	90	45

trated in Table 7, which also shows that the rate of citrate-activated fission is not greatly affected by pH in the normal working range. In the presence of CHCl_3 , therefore, a P-containing coagulum separates and in the presence of citrate the protein can remain soluble for a short time although P-free. Other salts, e.g. NaF, NaN_3 , KI, increase the rate of action to approximately the same extent as NaCl. These effects, at first sight, conflict with the already

mentioned stabilizing action of citrate and azide on NP in sap, but it is probable that different mechanisms are involved. NP deteriorates more in 1 hr. in sap, than in 1 day in water; it is protected from this sap-catalysed deterioration by azide and citrate, and these agents can increase the yield because this effect is much greater than their stimulation of the intrinsic instability of NP.

This fission is most easily interpreted as an enzyme action in which both the enzyme and its substrate are present together in the NP particle. If a strong, e.g. 10 g./l., solution of NP is run into boiling water and kept boiling both during the addition and for a minute afterwards, there is no coagulation, and on ultracentrifugation in the usual way 70–80% of the P is found in the pellet. Only about half the pellet will resuspend in water so completely that it does not again sediment in 10 min. at 8000 rev./min. Both parts contain 2.0–2.5% P and on incubation for a few hours in the presence of salts there is no flocculation and no P is liberated in a form that is soluble in TCA. This boiled material does, however, flocculate, with separation of the P, if it is incubated with as little as one-fifth of its weight of unboiled NP. Attempts have been made to separate the enzyme and substrate and so get a stable preparation of nucleoprotein without boiling, but when a series of preparations has been compared under the same conditions, the differences in their rates of fission have been small. There are often large differences in the rates of flocculation, but the conditions affecting the rate of flocculation have not yet been systematized. In these comparisons, preparations that have been ultracentrifuged four and five times from water or from 0.1 M-NaCl, preparations that have been precipitated fractionally with Ca, and preparations that have been recovered by ultracentrifugation of NP that has already undergone partial fission at 3° during 1–2 weeks, have been used.

By the time all the P-containing moiety has separated from the protein some of it has undergone further action with the liberation of PO_4^{---} . This action goes to completion slowly, but after 48 hr. at pH 6 in 0.02 M-citrate at 38°, 80% of the P is present

Table 7. *Separation of phosphorus from protein after different times of incubation in different environments*

(Seven tubes, each containing 1 g./l. NP, and the NaCl specified at 0.005 M. Two contained CHCl_3 . After the stated periods of incubation at 38°, 0.5 ml. samples were withdrawn from each, and precipitated with 1.5 ml. of 5.5% TCA. P was determined in the fluid and in the precipitate, and the figure given is the percentage of the P that was in the fluid.)

Time (min.)	NaCl	NaCl + CHCl_3	Citrate				
			pH 5.5	pH 6.0	pH 6.0 + CHCl_3	pH 6.6	pH 7.1
0	0	0	0	0	0	0	0
10	0	0	18	25	25	27	27
20	5	5	42	43	42	47	46
40	25	20	73	82	80	82	87
90	50	45	76	83	82	86	90
Time at which flocculation was apparently complete (min.):							
	200	70	40	100	60	150	200

in the form of PO_4^{---} . The formation of PO_4^{---} takes place more slowly in supernatant fluid from which the protein precipitate has been removed by centrifugation after incubation for 100 min. It appears, therefore, that, although the phosphatase action is mainly a property of the precipitate, some soluble phosphatase dissociates from it during the first stages of incubation. If no salt is present during the incubation, the precipitation of protein is so much delayed that it is not possible to get a sharp separation between the two actions; by the time the coagulum is free from P about one-third of the P is already in form of PO_4^{---} .

In this section the fission has been described in terms of P; substantially the same conclusions are come to if carbohydrate is estimated. By the time the protein coagulum is free from P its carbohydrate content has been reduced to less than 2%, measured by the orcin method, and carbohydrate appears in the fluid. Even after prolonged incubation, however, in the presence of an antiseptic, no reducing sugar is formed.

NP is an association between various constituents of the leaf. Changes in the technique of preparation are likely, therefore, to affect its properties; this has been observed and the results will be described in a later paper. The technique of preparation that has been used in work described here is extremely simple and is designed to remove NP as quickly as possible from the presence of substances that are known to affect it. No attempt has been made to separate the component enzymes from the association because an understanding of the changes that are going on during the 'purification' of a plant virus is the primary object of the investigation; it may be that information is also being gained about the behaviour of the material *in vivo*.

Properties of the fragments of NP

Separation of nucleic acid and its breakdown products. Nucleic acid can be prepared from NP by several methods, but even with fresh preparations the maximum yield has only accounted for 60% of the P in the NP taken. Older preparations give smaller yields probably because of changes of the type described in the preceding section.

Equal volumes of 10 g./l. NP and N-NaOH are mixed at 0°, and left for 30 min. at 0°; the mixture is taken to pH 7-8 by the addition of 5N-HCl with cooling. This treatment disrupts NP as it does other nucleoproteins and gives a coagulum of denatured protein, which contains a tenth or less of the P. The protein is centrifuged off and washed and the fluids taken to pH 4, when a further small amount of protein separates and is removed. This precipitate contains 1-2% of P. Nucleic acid separates as a characteristic resinous mass when enough HCl is added to bring the pH of the fluid to 1. It is washed once with water to remove NaCl, dissolved, and centrifuged clear at neutrality. About half the P initially present in the preparation appears in this fraction, and most

of the remainder can be precipitated from the acid mother liquor by the addition of one volume of ethanol.

This standard method of separating nucleic acid from nucleoprotein has the defect that an intensity of treatment with alkali sufficient to separate all the nucleic acid from the protein is also sufficient to degrade some of the nucleic acid. Schneider (1945) found that an acid-soluble derivative of nucleic acid was liberated when nucleoproteins are heated in 5% TCA for 15 min. at 90°. With NP a high temperature is not necessary. It is clear from the method used to follow the changes taking place during the incubation of NP, that TCA precipitates all the P in a fresh preparation. In the course of a few hours at 38°, or days at room temperature the P is released from the precipitate. By gentler treatment with TCA, nucleic acid can be separated from the protein in an acid-precipitable form.

Equal volumes of 100 g./l. TCA and 2-5 g./l. NP are mixed; if the precipitate is centrifuged off immediately, washed, and taken to pH 8 with NaOH almost all of it dissolves. The amount of protein that remains insoluble increases with the duration of exposure to TCA, and, after 15 hr. at room temperature, most of the protein has been so modified that it remains insoluble while only a small portion of the nucleic acid has been converted into the acid soluble form. The TCA precipitate is therefore centrifuged off after 15 hr. and washed twice by centrifuging after stirring up with one-fifth the original volume of fluid. It is now evenly suspended in water, taken to pH 8.5 by adding dilute NaOH with thorough stirring, left for an hour or more, and then centrifuged at 8000 rev./min. The gelatinous solid is resuspended in half the volume of fluid and centrifuged out again after soaking for 2-3 hr. These pH 8.5 extracts contain 60-70% of the P that was in the NP. More can be extracted by further washing at pH 8.5, or better, by resuspending the precipitate, reprecipitating it with TCA and repeating the extraction. On adjusting the pH to 4.5 a precipitate separates which carries out with it some P, this can also be partially recovered by repeating the TCA precipitation. Nucleic acid separates from the pH 4.5 fluid when it is acidified more strongly. Some P remains in the acid fluid and part of this can be precipitated by the addition of ethanol. The yield of nucleic acid, after washing and resolution at neutrality again, accounts for 60-70% of the P originally present in the NP used.

Mention has already been made of the stabilization of NP by boiling it to inactivate its enzymes; after this treatment most of the nucleic acid still sediments with the protein on ultracentrifugation. When boiled in the presence of salts the protein coagulates and most of the nucleic acid remains soluble. Thus 5 ml. of a 5 g./l. solution of NP was run into 2 ml. of a boiling solution that was 0.2M with respect both to NaCl and sodium citrate, at such a rate that boiling was maintained during the addition. The solution was centrifuged when cold and the fluid adjusted to pH 4.5 with acetic acid. It was again centrifuged and the fluid acidified with HCl to precipitate the nucleic acid. This technique of separation has proved less satisfactory than the other two. The separation of nucleic acid by addition of glacial acetic acid, which works well with tobacco mosaic virus (Bawden & Pirie, 1937) and some other nucleoproteins, fails completely with NP.

These three methods give nucleic acid preparations with similar properties. After being reprecipitated with acid and dissolved by the addition of KOH the analytical composition is P, 6.9–7.5%; N, 12–13% and carbohydrate, 30–35%, estimated by the orcin method using glucose as a standard, although the sugar has been identified as ribose by paper chromatography. The material has the physical texture of yeast nucleic acid and shows the same ultraviolet absorption maximum at 260 m μ . On acid hydrolysis this nucleic acid, like those from yeast and tobacco mosaic virus, releases free PO_4^{---} in two stages; half is set free in 30 min. at 100° in N-HCl , i.e. under the conditions that release PO_4^{---} from the 'high-energy phosphates', the other half is only released after many hours' hydrolysis.

Nucleic acid can be made in these ways only from fresh preparations of NP, and it is important that the technique of separation used should not encourage internal enzymic changes. With old preparations the results are variable and depend on the extent to which these changes have gone on. By the time a preparation is beginning to coagulate little or no nucleic acid can be made from it. After fission by any of the methods described and removal of the protein at pH 6 and 4.5, the fluid gives no precipitate with more acid, but nucleotides can be separated from it as Ba salts by precipitation with ethanol. After incubation either in water or in salt solution, nucleotides can be separated if the fluid is fractionated soon after the protein has coagulated, but, as the experiments already described on the liberation of PO_4^{---} would suggest, nucleosides are present if the incubation has been continued for 1–2 days.

The properties of the protein moiety of nucleoprotein. Incubation of NP in dilute salt solution at neutral pH leads to the separation of 55–60% of the preparation as a precipitate. Under conditions which favour precipitation, e.g. in the presence of CHCl_3 or at pH 5.5 or lower, the precipitate still contains P, but if incubation is continued after flocculation this is lost, and, after washing with water, the precipitate may contain less than 0.1% P and 1% carbohydrate. It contains 14.5–15.5% N, dissolves readily on incubation with trypsin (Armour Laboratories Ltd.) and gives the biuret, Millon and Sakaguchi reactions. It dissolves to give a viscous solution at pH's outside the range 3–9.

Attempted separation of nucleic acid from sap

The readiness with which nucleic acid can be prepared from NP after the denaturation of the protein by precipitation with TCA suggests that by the direct precipitation of fresh sap with TCA it might be possible to prepare the nucleic acid in bulk without the trouble of preliminary ultracentrifuging.

The 4% TCA precipitate, from sap that has been centrifuged at 8000 rev./min., is left for 24 hr. at room temper-

ature, centrifuged off at 3000 rev./min. washed three to four times by suspending in 2% TCA and centrifuging and finally washed with water. If it is then suspended in water at pH 8.5 and the extract adjusted to pH 4.5, as already described, much of the P in the extract precipitates along with the protein. It is better, therefore, to centrifuge after some hours' soaking at pH 8.5–9, adjust the extract to pH 6 and centrifuge off what protein precipitates at that pH before removing the last traces of protein at pH 4.5. The proportion of the initial P in the TCA precipitate that comes out on these three precipitates varies, but representative figures for the pH 9, 6 and 4.5 precipitates are 15, 8 and 10%, respectively. The supernatant fluid at pH 4.5 contains the remainder of the P in organic combination, but it gives no precipitate of nucleic acid when acidified strongly. This method, therefore, although satisfactory with purified NP, fails to separate nucleic acid from whole sap. It is probable that the large amounts of sap protein, other than NP, preferentially adsorb undegraded nucleic acid when they are denatured. The P compounds still present in the fluids after protein has been removed are, therefore, predominantly nucleic acid breakdown products partly derived by enzyme action in the sap or during the preparation and partly as a result of 24 hr. exposure of nucleic acid to 4% TCA. A reliable technique for separating the small amount of nucleic acid from these protein precipitates, and so from sap in bulk, has not yet been devised. The fractionation and characterization of these mixtures, and of the nucleotide mixture that is made from old preparations of NP has depended on the separation of any PO_4^{---} present with Ba at pH 9 followed by the ethanolic precipitation of nucleotides as Ba salts and, after removal of the ethanol, of nucleosides with HgSO_4 . A fuller account of this aspect of the problem will be published later.

The phosphatase and nuclease of sap and NP

Mention has already been made of the fact that the protein precipitate carries with it much of the phosphatase activity towards the nucleotide that separates from NP on incubation. It also acts on β -glycerophosphate, pyrophosphate, metaphosphate, adenosinetriphosphate, yeast nucleic acid and the nucleic acid from tobacco mosaic virus, giving PO_4^{---} as one product of the action in each case. Preparations of NP and the protein derived from it do not all have the same enzyme activity. This, taken in conjunction with the fact that these enzyme activities are also shown by tobacco leaf sap, suggests that the NP may be adsorbing enzymes from the sap. Once the protein has been isolated dissociation of enzymes from it does not take place readily. Thus enzyme activity is only slightly diminished in NP that has been precipitated by Ca or that has been ultracentrifuged several times from NaCl solutions of different concentrations within the stability range. Ultracentrifugation from strong salt solutions, e.g. 2M-NaCl, yields pellets with diminished activity, but, as has already been stated, these pellets will no longer disperse again in water. Dialysis greatly reduces the yield of NP, but, in agreement with this partial dissociation in strong salt, it enhances the phosphatase activity of the material that can be prepared.

A convenient and stable solution of leaf phosphatase and nuclease can be made by dialysing and centrifuging the supernatant fluid that is a by-product when sap is ultracentrifuged to prepare NP. The P content of this preparation is so low, about 0.1% of the dry matter, that no allowance need be made for it when enzyme actions are followed by P estimation. In experiments using NP as an enzyme, allowance is made for the rate, known from control experiments, at which NP itself gives rise to TCA-soluble P or to PO_4^{---} . When tested against the different substrates at a concentration giving 0.1 g. of P/l. in 0.02M-citrate pH 6 at 38° in the presence of thymol, the rates of initial phosphatase action on yeast nucleic acid, glycerophosphate, metaphosphate and adenosinetriphosphate were in the approximate ratios 1:3:5:10. There is no unequivocal difference in the relative rates of hydrolysis of these substrates, whether sap, NP, or the protein from NP is used as phosphatase. The experiments performed so far do not, therefore, show whether all these actions are due to the same enzyme.

Batches of sap differ in their activity, but an average preparation at 0.1 g./l. will bring about 30% hydrolysis of glycerophosphate in 1 hr. To achieve this rate of hydrolysis, NP must be used at 1–2 g./l., and the protein that flocculates from it on incubation at 2–3 g./l. These activity differences do not necessarily reflect differences in the amount of enzyme present, but may be due to the gross differences in dispersion. The enzyme in a sap preparation does not sediment on the ultracentrifuge, whereas the particles in the protein floccules are visible, and access of substrate to enzyme in the interior must be impeded.

The fact that NP contains nucleic acid and that this is all converted quickly on incubation into material soluble in TCA suggests, not only that NP carries nuclease, but that the nuclease resembles that of intestinal mucosa rather than of pancreas in that it carries the hydrolysis so far that all the nucleic acid becomes unprecipitable by TCA and no 'resistant core' is left. This is confirmed by experiments with commercial yeast nucleic acid. Using the same conditions of incubation as in the phosphatase experiments, but measuring the total P soluble in the uranyl and TCA reagent of MacFadyen (1934), the sap enzyme preparation hydrolyses 30% of the nucleic acid in 1 hr. at 0.1 g./l. and leaves only 10–20% of the nucleic acid precipitable after 7 hr. This rate of hydrolysis is also given by NP or the protein derived from it at 1–3 g./l. It is noteworthy that nucleic acid stabilizes NP so that the flocculation that usually accompanies incubation does not take place until after the nucleic acid has disappeared. Similarly, nucleic acid makes the protein particles derived from NP more easily dispersible in water, and pancreatic ribonuclease promotes the

flocculation of NP even if it has been stabilized by boiling. One of the factors involved in the maintenance of NP in its dispersed state is therefore the nucleic acid; this is a phenomenon that has been recognized with other proteins.

DISCUSSION

The main object of this work was the examination of the properties of the proteins in leaf extracts, that are sedimentable on the ultracentrifuge, in sufficient detail to enable them to be recognized in and removed from virus preparations. The results that have been described suggest that the presence of a phosphatase in a virus preparation is strong *prima facie* evidence for contamination. Any virus which is sufficiently stable to be recoverable from sap that has been allowed to age at room temperature for a day or which can be isolated by repeated precipitation with strong salt solutions or purified and stored at pH 4–4.5 is unlikely to be contaminated with intact nucleoprotein (NP). This probably explains the readiness with which tomato bushy stunt virus can be purified when all operations are carried out in the 4–4.5 pH range (Bawden & Pirie, 1943). Besides the interest that NP has for the virus worker, two points of general interest call for discussion. Is there any justification for looking on this complex of nucleoproteins and enzymes as a primary leaf component rather than an artifact? To what extent does the presence of such a component in the leaf account for some earlier observations on nucleic acids and their derivatives in leaves?

The search for enzyme activities in NP has so far been superficial and, to judge from the range of enzymes that has been found in microsomes and organelles in other tissues (cf. Claude, 1949; Bradford, 1950), many may be present. All those that have been recognized in NP are also present in the leaf sap from which NP has been separated. In this respect NP is peculiar, for most of the enzyme activity is associated with the fraction that is not sedimentable on the ultracentrifuge, whereas many of the enzymes in mammalian microsomes or mitochondria are present predominantly in the centrifuge pellet rather than the supernatant. Repeated centrifuging under different ionic conditions does not remove the enzyme activity from NP; the union between enzyme and nucleoprotein, if it takes place during the mincing and pressing of the leaf, is therefore relatively stable. Such stable, artificial, unions are already known, thus Hoagland, Ward, Smadel & Rivers (1942) found that vaccinia virus preparations would adsorb phosphatase, catalase and lipase, and retain them during subsequent stages of purification. On the other hand, those plant virus preparations that have had their enzyme activities investigated, although they also are made up predominantly of

pentose nucleoprotein, and associate themselves with various components of the sap (cf. Pirie, 1947), form loose complexes that dissociate readily on ultracentrifugation or precipitation. Artificial complexes, e.g. that between trypsin and tobacco mosaic virus (Kleczkowski, 1944), are also dissociable without destruction of the virus. It may well be that this difference between NP and plant viruses, other than potato virus X, is correlated with the fact that the latter are not attacked by those enzymes that have been recognized in sap, whereas the more stable union with the former is an enzyme:substrate complex that has been isolated before the action goes to completion. In making such a contrast the unrepresentative nature of the group of plant viruses that has been studied in detail must be emphasized. As has already been pointed out (Pirie, 1946), these viruses have been selected for study because of their stability both in the sap expressed from leaves and in more precisely defined fluids. Most of the known viruses have not been studied, and some of them may well resemble NP closely. Nevertheless, it is clear that if NP and the sap enzymes have access to one another in the intact cell, the NP particle will survive there for a relatively short time; whereas the viruses that have been studied retain their gross structural integrity in this environment for longer. There is as yet no hint of what qualities differentiate a virus from the proteins incapable of infecting a host, but among the desiderata, solubility and stability in the environment provided by the host cell are prominent. The argument has already been advanced (Pirie, 1946) that it is the first quality that distinguishes the viruses from chromosome-linked genes; it may be the second that distinguishes them from the microsomes.

Much work has been published on the nucleic acids and 'nucleoproteins' of uninfected plant buds and sprouts, but the leaf has received less attention. Bawden & Pirie (1937) prepared, by precipitation with ammonium sulphate, an unstable 'nucleoprotein' from tobacco sap that had been heated to 70°. The results varied from sample to sample, and it is probable that this was due to variation in the sizes of the leaves used, in the time for which the protein was exposed to expressed sap or ammonium sulphate, in the rate of heating and temperature at which fractionation was carried out. All these factors affect the yield and extent of destruction of NP, and the evidence that has accumulated since suggests that the protein was derived by the breakdown of NP. Bawden & Pirie (1938) made a series of protein fractions from unheated tobacco sap by precipitation with ammonium sulphate and found that part of each fraction was sedimented in 2 hr. at 12,000 g, but that the sediment contained less than 0.02 % P. In the work described in the present paper similar material was found in extracts made from the

fibre residue with salt solutions or at pH 8. It does not occur to any significant extent in fresh sap that has been kept cold. The unsedimentable proteins of sap aggregate when precipitated with ammonium sulphate so that after resolution they are sedimentable; they also undergo changes leading to precipitation when aged in sap. The fractions made in 1938 were probably derived from the native proteins by these changes.

Wildman, Campbell & Bonner (1949), and Wildman, Cheo & Bonner (1949) have studied the properties of tobacco-leaf extracts made by grinding the leaves in a cold colloid mill, filtering and freezing. That part of the protein, which remains in solution on thawing, was separated from leaf components of small molecular weight by evaporation and dialysis. In some experiments the extract was dried while frozen. A single electrophoretic component accounted for 75 % of the protein in the preparation and this, because of its purine, carbohydrate and phosphorus content was referred to as a nucleoprotein. NP is destroyed by several of these treatments and its fragments will appear partly in the fraction rendered insoluble by freezing and partly in the final preparation. It seems likely that the protein-linked phosphorus has its origin largely in the breakdown of NP. As has been emphasized elsewhere in this paper, there is no certainty that NP is not itself an artifact made during the course of the preparation, but the knowledge that has been gained of its properties has led to a method of preparation that avoids the more obviously damaging procedures. It is therefore more probable that the primary state of much of the nucleoprotein of the leaf, and most of the nucleoprotein of the leaf sap, is similar to NP than that it is similar to the products handled by Bawden & Pirie (1938) or by Wildman *et al.* (1949).

The methods that have been generally used for 'clarifying' the sap of virus-infected plants as a preliminary to the serological or chemical study of viruses include heating, precipitation with ethanol, ageing, freezing and exposure to phosphate. All these procedures are designed to remove chloroplasts and chloroplast fragments, but, to varying extents, they coagulate NP also. Preparations of those plant viruses stable enough to withstand these treatments should not, therefore, be greatly contaminated with NP and this is especially so with viruses that make up a considerable proportion of the total protein of the infected leaf. Furthermore, as has been pointed out, the amount of NP in leaf extracts diminishes as the leaves mature, either through age or virus infection.

The position is different with unstable viruses, with viruses that are present in only small amounts in the infected sap, and in work designed to shed light on the initial state of viruses in the leaf. Three examples may be given. From tobacco plants infected with tobacco ringspot virus Stanley (1939)

isolated 5–50 mg./kg. of a sedimentable nucleoprotein that carried infectivity. Its properties were in many ways similar to those of NP; thus it contained 3.2–4.1 % phosphorus in the form of pentose nucleic acid and preparations slowly precipitated on standing. This substance was made by differential ultracentrifugation of sap that was carefully kept cool and, although much of the NP would have been coagulated by the freezing and phosphate treatment to which the leaves were subjected, amounts of NP and its derivatives of the same order as the small yield of virus preparation would remain soluble and sedimentable. No evidence was given that the same preparative procedure, carried through on uninfected plants, yielded no product and, in the absence of this control, it is reasonable to assume that a considerable part of the final product was derived from NP. The methods used by Bawden & Pirie (1945) to separate fractions of tobacco mosaic virus with particles of different size and infectivity cannot be relied on to remove all the NP. These fractions were made from leaves that had been frozen while intact, and this would remove much of the NP; furthermore, the yields were sufficiently large (0.15–3.7 g./l. of sap) to make the contamination quantitatively less important than in the previous case. Some virus preparations did, however, contain twice the usual amount of phosphorus, and this excess phosphorus could be removed by treatments such as ageing and incubation with trypsin that destroy NP. Finally, Bawden & Pirie (1950) have used the sedimentable protein from uninfected tobacco plants to separate infective and uninfected components of preparations of a tobacco necrosis virus. The infective component is preferentially precipitated at pH 4 along with the normal protein, and this confuses the interpretation of results on different virus preparations when these are contaminated with normal protein to different extents. All infective preparations were to some extent contaminated.

The possibility that NP, or substances like it, are virus precursors has an immediate, but meretricious, appeal. Two facts could be used in favour of the idea; NP, like the plant virus preparations that have been intensively studied, contains pentose nucleic acid; during infection the NP content of the leaf diminishes as the virus content increases. The latter fact must be seen in the context of what little is known of the effect of the physiological state of the plant on the NP content of sap. As the leaf ages NP disappears from the sap, until by the time it is beginning to yellow none can be isolated. This happens without infection, but one of the effects of infection is to hasten these degenerative changes. In the absence of any other evidence, therefore, it would seem reasonable to regard the disappearance of NP as a consequence of the senescence of the leaf, and failure of the mechanisms concerned with NP production,

rather than as a consequence of the direct conversion of NP to virus. When NP disappears it is probable that much of it is converted into amino-acids and other metabolically available nitrogen compounds. Any protein synthesis which is going on at the same time will make use of this nitrogen and part of the nitrogen of the NP will therefore reappear as virus in an infected plant. But this does not make NP a virus precursor in any useful sense of the term. The term must be reserved for substances that are converted into virus by one or two simple changes and without radical reshuffling. Unequivocal evidence that this happens can probably only be got if the change can be brought about *in vitro*. If the term is not so reserved it must logically be extended still further so that our domestic plants and animals become human precursors, and the salmon's muscles become the precursors of its gonads. By overlooking the necessity for this distinction Wildman, Cheo & Bonner (1949) have argued that cytoplasmic protein, the phosphorus of which may well be derived by the breakdown of NP, is a precursor of tobacco mosaic virus. They recognize the necessity of supplementing the simple observation that as one material disappears another appears, by amino-acid analyses; but only very close similarities would be even relevant. Using the analogy of human feeding, the so-called 'first-class proteins', especially when mixed, agree closely in amino-acid composition with mixed proteins of tissues. The demonstration of long identical sequences of amino-acids would be necessary before the role of one protein as the immediate precursor of another could be considered seriously in the absence of *in vitro* evidence for the conversion.

Many tissues contain structures comparable to NP; these have been held to play a part in protein synthesis, and there is also histological evidence (cf. Newcomer, 1940) suggesting that mitochondria in plants have a synthetic role. Mitochondria are larger than the particles of NP in the state in which they appear in the final preparation, but one reasonable possibility is that NP has its origin in the break-up of these notoriously fragile particles. If, therefore, an attempt is being made to relate NP directly with virus multiplication it would seem more reasonable to study the possibility of it being part of the machinery making viruses and other proteins rather than the raw material from which virus is made.

This preliminary study of the unstable, submicroscopic, sedimentable nucleoprotein of the tobacco leaf attempts to co-ordinate observations made during work on plant viruses. Though it has had the immediate object of increasing our understanding of the changes that go on during the isolation of a virus several points of intrinsic interest have emerged. The association of enzymes with the nucleoprotein explains its instability in sap and

when isolated, and the concentration of this enzyme system, along with the virus during the first stages of a centrifugal virus isolation, may explain some of the changes taking place in virus preparations during isolation. This association does not yet shed any light on the *in vivo* activities of NP, but it is clear that there are structures in leaf extracts with many points of resemblance to the microsomes that can be separated by similar methods from animal tissues. Although they may well be fragments broken from a larger structure during the process of extraction, their existence should be borne in mind in assessing the cogency of any conclusions about the dynamics of the leaf based on the study of extracts. Few experiments have been made with plants other than tobacco and, for convenience, cultural conditions have been kept as uniform as possible. The results do not, therefore, necessarily apply to other species or conditions of growth, and it may well be that tobacco grown under the conditions used is far from being the best source of NP. The responsiveness of the chemical composition of the leaf to changes in cultural conditions is a continual source of trouble when a uniform starting material is required; this responsiveness does, however, give the leaf the biochemical advantage that systems can be studied under a much wider range of conditions than is possible with animal tissues, and this advantage has not yet been adequately exploited.

SUMMARY

1. A nucleoprotein has been prepared from the sap of young uninfected tobacco leaves by ultra-centrifugation. The yield varies from 3 g./l. with the sap of immature leaves to 0.1 g./l. or less with large mature leaves, or with sap that has been allowed to age before processing.

2. The nucleoprotein is unstable *in vitro* and autolyses in a few hours at 38° yielding denatured protein and either nucleotides or nucleosides depending on the duration of hydrolysis.

3. The denatured protein still retains some of the enzymes that occur in leaf sap, and different preparations have been compared using metaphosphate, glycerophosphate, adenosinetriphosphate and yeast nucleic acid as substrates.

4. Reasons are given for thinking that, although it bears some resemblance to them, the nucleoprotein cannot usefully be regarded as a precursor of the plant viruses.

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