The Fission of Tobacco Mosaic Virus and some other Nucleoproteins by Strontium Nitrate

By N. W. PIRIE
Rothamsted Experimental Station, Harpenden, Hertfordshire

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During the first few years of work on the chemical properties of preparations of tobacco mosaic virus, the view that activity was associated with a nucleoprotein was contested; this led to an examination of the conditions under which the nucleic acid and the protein in a virus preparation remained associated or were separated. As a rule the presence of a link was assessed by seeing whether the protein and nucleic acid sedimented together on ultracentrifugation and by the separation of a phosphorus-free coagulum of protein. The linkage was not apparently broken by high concentrations of the salts generally used in protein fractionation, e.g. sodium chloride and ammonium sulphate, nor by exposure for a few hours at room temperature between pH's 0 and 9. Separation was, however, easily effected by the traditional method of exposure to cold alkali, and also by boiling in dilute, neutral salt solutions, by running the nucleoprotein solution into several volumes of acetic acid or by treatment with pyridine (Bawden & Pirie, 1937). Sreenivasaya & Pirie (1938) separated the protein and nucleic acid by incubating virus preparations with 10 g./l. solutions of sodium dodecyl sulphate; this agent was used by Bawden & Pirie (1940*b*), who also got separation with concentrated phenol and various other substances, including urea (Bawden & Pirie, 1940*a*). These observations indicate that, very probably, the preparations studied are nucleoproteins rather than protein nucleates. That is to say, the linkage between the protein and nucleic acid is not a readily dissociable salt linkage, but only breaks when one or other partner is modified. The protein moiety, for example, is denatured when the virus is heated or dissociated by such agents as urea.

This conclusion gave an added interest to the observation that tobacco mosaic virus preparations dissociate when exposed for a few hours to molar solutions of strontium nitrate. No detailed study has been made because this is not the type of phenomenon with which we are primarily concerned; some rather casual observations on it may, however, be reported.

EXPERIMENTAL

Experiments with tobacco mosaic virus preparations. During a 10-year period, many different virus preparations have been used. Some were aggregated preparations made by precipitation with acid and $(NH_4)_2SO_4$ (Bawden & Pirie, 1943*a*) and some were fractions, with differing degrees of aggregation, made by differential ultracentrifugation (Bawden & Pirie, 1945). The two types of preparation behaved similarly with $Sr(NO_3)_2$. All preparations had lain for some months in a refrigerator and were ultracentrifuged again immediately before use. The preparations made by ultracentrifuging only are similar to those normally used by other workers on this virus, and although their properties differ widely from those of the bulk of the anomalous nucleoprotein in the infected leaf, they will for convenience be referred to as virus or *TMV*.

Solutions containing *TMV* and $Sr(NO_3)_2$ are initially clear and show the anisotropy of flow characteristic of *TMV* solutions. After an interval, which depends on the pH, the temperature, and the concentrations, the anisotropy of flow diminishes, an opalescence appears, and later a precipitate. The course of one such experiment is shown in Table 1. The separation of nucleic acid in this system, like the separation by boiling, is accompanied by a fall in pH; the borate buffer present is only sufficient to keep the fall from exceeding 1 unit. When the concentration of $Sr(NO_3)_2$ is smaller, or that of *TMV* greater, the precipitate that forms has a different texture from that separating under the conditions of Table 1. The latter is dense and centrifuges out easily, whereas the former compacts badly and, at least when newly formed, disperses completely when vigorously shaken, but re-forms on standing. This behaviour has been

Table 1. *Effect of* pH *on the fission of TMV by* $Sr(NO_3)_2$

(Solutions containing (final concentrations) 3·5 g./l. *TMV* and 1·6 M-Sr $(NO_3)_2$ adjusted to the pH stated and made 0·03 M with sodium borate buffer of the same pH. Kept at 21° and the appearance noted at intervals.)

	Time				
Initial pH	10 min.	30 min.	2 hr.	4 hr.	20 hr.
8·0	No change	No change	Opal.	Opal.	Ppt.
8·5	Faint opal.	Opal.	Ppt.	—	—
9·0	Opal.	Opal.	Ppt.	—	—
9·5	Ppt.	—	—	—	—

noticed in other systems containing *TMV* both *in vivo* and *in vitro* (cf. Bawden & Pirie, 1940*b*). In this system it is encountered with $Sr(NO_3)_2$ weaker than M and *TMV* stronger than 15 g./l.

The concentrations affect not only the physical character of the precipitate but also its composition; the dense type of precipitate is substantially free from nucleic acid, whereas that separating from concentrated *TMV* or dilute $Sr(NO_3)_2$ solutions carries much or all of the nucleic acid with it. Separation can be brought about by adding alkali, but not by a further few weeks of exposure to dilute $Sr(NO_3)_2$. A mixture that has precipitated without fission because of an inadequate concentration of $Sr(NO_3)_2$ does not undergo fission consistently if the concentration is increased later. Similarly, complete fission cannot be obtained under these conditions and with a final virus concentration greater than 15 g./l. by adding the virus gradually during several hours.

This system offers a method for preparing nucleic acid from *TMV* which seems to expose the nucleic acid to conditions less drastic than those generally used. In any study of the original state of the nucleic acid in *TMV* it may therefore prove useful.

Nucleic acid made in this way has the usual resinous texture when precipitated with acid. It is readily attacked by both pancreatic and tobacco-leaf ribonuclease, and, like preparations made in other ways from *TMV*, it is relatively ineffective as an inhibitor of streptococcal deoxyribonuclease (Bernheimer, 1953). The absorption spectrum could not be distinguished from that of nucleic acid made from *TMV* by other methods.

In choosing optimum conditions for the fission, several considerations have to be borne in mind. $Sr(NO_3)_2$ solutions strong enough to dissociate *TMV* are good solvents for nucleic acid even when acid. The nucleic acid may be partly precipitated by the addition of ethanol, but the concentration of ethanol needed makes some of the $Sr(NO_3)_2$ crystallize out, so that a mixture of nucleic acid with the salt results. This difficulty can be avoided to a large extent if the action is not allowed to go so far as to precipitate all the protein, for then the residual soluble protein precipitates from the strong $Sr(NO_3)_2$ solution on addition of acid, and brings with it nearly all the nucleic acid that had been separated. But under these conditions the separation from the original protein coagulum is incomplete. It is necessary to choose, therefore, between incomplete fission with nearly complete recovery of the nucleic acid, and complete fission with difficult, and generally incomplete, recovery. The course of a representative experiment giving good recovery of nucleic acid is set out.

20 ml. of a 20 g./l. solution of *TMV* (2·1 mg. P) were added to 30 ml. of 2 M-$Sr(NO_3)_2$, each previously adjusted to pH 8·5. The pH fell immediately and was restored to 8·5 by periodical addition of 0·1 M-KOH; altogether, this required 1·0 ml. In 10 min. at 20° there was a heavy opalescence and no anisotropy of flow nor shimmer was detectable; in 20 min. a precipitate settled. After 70 min. its texture did not appear to change. The mixture was evacuated briefly so as to remove the air entangled in the coagulum; this air would have prevented clean separation on the centrifuge. The coagulum was centrifuged off and washed with 20 ml. of water. Acetic acid was added to the combined supernatants and the precipitate separating at pH 4 was centrifuged off and washed with 3 ml. of water. 2 ml. of conc. HNO_3 were added to the chilled fluids and the gummy precipitate of nucleic acid and a little protein was allowed to settle at 0° for a few hours; it was then centrifuged off and washed with 2 ml. of water. Most of the protein was in the first coagulum, and this contained 160 μg. P, the smaller precipitate of protein, separating at pH 4, contained 470 μg., the nucleic acid precipitate 1·3 mg., and the final fluid about 100 μg. P (this last estimation is uncertain because of the large amounts of Sr present). Nucleic acid was separated from the small protein precipitate thrown out at pH 4 by suspending it in 5 ml. of water at pH 8·5 and adding 0·5 ml. of saturated $(NH_4)_2SO_4$ solution also at pH 8·5. The protein precipitates along with very little of the nucleic acid; this was then precipitated by adding a few drops of conc. HCl and the precipitate was washed on the centrifuge.

The combined nucleic acid precipitates were carefully dissolved by adding KOH solution to pH 6 and then acetic acid to pH 4. After lying for some days at 0°, a small amount of protein was removed by centrifuging at 8000 rev./min. and the nucleic acid was again precipitated from the clear fluid at pH 1. This precipitate was washed, dissolved by adding KOH to pH 5, centrifuged clear if need be, and dried while frozen. The yield was 19 mg. of potassium nucleate, containing 8·5% P, i.e. 1·6 mg. P or 76% of that present in the original *TMV*. Some more nucleic acid can be separated from the protein precipitates by dissolving at pH 8·5 and throwing out most of the protein with $(NH_4)_2SO_4$ as already described; it can also be separated from the acid $Sr(NO_3)_2$ solution by the addition of ethanol.

The P content of precipitates separating from acid solution was determined by a method based on that of Kuttner & Lichtenstein (1932). Other precipitates and fluids contain Sr and these were evaporated to small volume in an oven at 100° with an excess of H_2SO_4. They were then taken up in water, centrifuged after standing for an hour, and the acid solution poured off from the $SrSO_4$ for analysis. Control experiments with known amounts of nucleic acid mixed with Sr salts showed that the recovery of P by this method was satisfactory.

Experiments with other nucleoproteins. Under the conditions used here, the other available nucleoproteins have not undergone fission in the same way as *TMV*. Thus, tomato bushy stunt virus gradually goes opalescent in $Sr(NO_3)_2$ solutions which are more concentrated than 1·2 M and there is some precipitation, but there is little separation of nucleic acid from the protein. There is more separation from preparations of the Rothamsted strain of tobacco necrosis virus, but conditions have not been found that lead to such sharp separation as from *TMV*. With these two virus preparations it is necessary to dilute the reaction mixture to about 0·5 M-$Sr(NO_3)_2$ before centrifuging, for stronger solutions keep some of the denatured protein dissolved. The normal nucleoprotein from young tobacco leaves (Pirie, 1950) is easily separated into protein and nucleic acid by treatment with alkali, and with trichloroacetic acid, under conditions that do not cause fission of *TMV* and the other viruses, but it is relatively resistant to $Sr(NO_3)_2$. The nucleoprotein is precipitated by low concentrations, but if a solution is added to 4 vol. of 2·4 M-$Sr(NO_3)_2$, there is no precipitation immediately unless the mixture is diluted to about 0·5 M. After a few minutes an opalescence develops, followed by flocculation of most of the protein. Even after 5 hr. at pH 8, however, the precipitate still contains one-third of the nucleic acid and much of the remainder pre-

cipitates along with the small amount of protein that precipitates with acid. The nucleic acid can be separated from this if the protein is dissolved in dilute alkali and then precipitated by the addition of 0·1 vol. of satd. $(NH_4)_2SO_4$ sol.; from the supernatant fluid the nucleic acid can be precipitated by adding acid. This method of preparation may have advantages in some circumstances, but it does not have the simplicity of the preparation from *TMV*.

Experiments with nucleic acid. In an attempt to bring about fission of the more resistant nucleoproteins, some tests were run at 37° and in them a protein coagulum free from nucleic acid resulted, but no nucleic acid could be isolated from the fluid. Commercial yeast nucleic acid and *TMV* nucleic acid made by the method already described were therefore incubated with 1·6 M-$Sr(NO_3)_2$. At intervals, samples were withdrawn and diluted with 2 vol. of water, and 10 N-HCl was added to a final concentration 0·1 N. The dilution is necessary because nucleic acid is soluble in acid $Sr(NO_3)_2$ solutions which are more concentrated than 0·7 M. After a few hours' incubation, precipitation with acid was impaired and there was no precipitation after 24 hr. at 37° and pH 8·5. There is, however, precipitation in these fluids—to an extent depending on the duration of incubation—on the addition of ethanol and almost all the P is precipitated by the addition of uranyl nitrate. It is clear, therefore, that this type of fission resembles fission with alkali in that the conditions have to be controlled or the nucleic acid will be destroyed as well as being liberated.

Experiments with other salts. This fission by $Sr(NO_3)_2$ was noticed during work on the inactivation of tomato bushy stunt virus by freezing in different environments (Bawden & Pirie, 1943*b*), and on the crystallization of the virus from various salt solutions (cf. Pirie, 1945). A thorough search for other salts with the same property has not been made, but a few related ones have been tested.

$SrCl_2$ is more soluble than the nitrate, but even in saturated solution it only causes partial precipitation of *TMV* in a paracrystalline state without splitting off the nucleic acid. $Ba(NO_3)_2$ is much less soluble, and a saturated solution makes *TMV* opalescent, but causes little precipitation and less fission. $Ca(NO_3)_2$, on the other hand, precipitates *TMV*. The suspension has a similar appearance to suspensions made by the action of the strontium salt, but the protein precipitate carries down with it most of the P. Various modifications in technique have been tried but a satisfactory yield of nucleic acid has not yet been got after treatment with $Ca(NO_3)_2$.

DISCUSSION

One point stands out clearly from these experiments as it did from earlier experiments (Bawden & Pirie, 1940*a*, *b*) with urea and some other agents—plant viruses differ greatly in the ease with which their nucleic acid can be separated from their protein. In some viruses the nucleic acid may not be connected at all, but held in a protein cage as Markham (1953) suggests with turnip yellow mosaic virus, though this suggestion does not seem to be really necessary (Pirie, 1953). It is conceivable that in other viruses the link is salt-like; the mutual precipitation of proteins and nucleic acids is well known. Greenstein (1944) has suggested that viruses are constructed in this way, but such a structure is hardly compatible with the behaviour of viruses such as *TMV* and tomato bushy stunt, which can be sedimented intact on the ultracentrifuge over a wide pH range and in environments of varied ionic composition. Although alike in this respect, these two viruses differ in the ease with which protein and nucleic acid can be separated. Thus, boiling, or exposure to such agents as strong acetic acid, urea, phenol, etc., is sufficient with *TMV*, but not with the bushy stunt virus, although some of these treatments cause a solution of it to coagulate. The same difference appears in the experiments described in this paper. Tobacco necrosis virus and normal leaf nucleoprotein are intermediate in their behaviour. Several explanations are possible. Some nucleoproteins may give a denatured protein with a physical structure that impedes the diffusing away of the nucleic acid, even although any chemical link between the two has been broken. This explanation would account for the lack of success that sometimes attends attempts to separate nucleic acid from *TMV* precipitates that have been made by adding insufficient $Sr(NO_3)_2$, but it has little else to recommend it. There may, on the other hand, be differences in the affinity of different denatured proteins for nucleic acid, or the link, in different viruses, may be of a chemically different nature. Such evidence as we have so far does not enable a choice to be made between these possibilities.

Among the possible mechanisms of this action, two extremes may be considered. The $Sr(NO_3)_2$ may break the linkage between protein and nucleic acid so that the two well-recognized parts of *TMV* fall apart; any changes there may be in the parts, denaturation of the protein for example, would then be secondary. The action, on the other hand, may be on one or other of the parts so that it is modified in such a way as to be incapable of maintaining the union between them. The observations made so far do not exclude either possibility. The properties of nucleic acids and their breakdown products are well known to be affected by salts; thus Tamm, Shapiro & Chargaff (1952) increased the diffusibility of degraded deoxyribonucleic acid from thymus by exposure to 0·2 M magnesium sulphate and Markham & Smith (1952) got similar results with several ribonucleic acid 'cores' in 2 M sodium chloride. Strontium nitrate, under rather more extreme conditions than those used to cause fission of *TMV*, makes ribonucleic acid from both yeast and *TMV* unprecipitable by hydrochloric acid, and it is reasonable to assume that it has some effect under the conditions used for fission, so that the first step may be a modification in the nucleic acid moiety of the virus. After separation the protein may well be more readily denatured than it was when combined. The protein that Sreenivasaya & Pirie (1938)

separated from *TMV* by treatment with sodium dodecyl sulphate was unstable, and other proteins also are more easily denatured after separation from their prosthetic groups. The presence of undegraded nucleic acid in solution with serum albumin has been found (Greenstein & Hoyer, 1950) to increase the thermal stability of the latter.

Many anomalies appear when the kinetics of the fission of *TMV* by alkali and the subsequent destruction of the liberated nucleic acid are studied. The results were partly systematized by Grégoire (1950), who found that 0·033N sodium hydroxide acting for 4 min. at 18° split *TMV* into at least three products: protein that could be precipitated at pH 5·2 along with a little nucleic acid, nucleic acid which could be precipitated by hydrochloric acid along with a little protein, and a substance, which remained in the acid solution, that could catalyse the conversion of *TMV* nucleic acid into material not precipitated by hydrochloric acid. This action takes place in 0·033N sodium hydroxide during a few hours and the advantage of using 0·5N sodium hydroxide in the conventional methods for making nucleic acid is not that this strength of alkali is needed for the fission but that it is needed to suppress the secondary loss of acid precipitability. The high concentrations of strontium nitrate that are present when it is used to bring about fission would make it difficult to recognize any similar mechanism if it were playing a part here. But the possibility that *TMV* undergoes fission more readily than the other nucleoproteins because of the presence of other catalytic substance in it must be kept in mind in further work.

SUMMARY

1. Tobacco mosaic virus is split at room temperature into denatured protein and free nucleic acid by solutions of strontium nitrate if the concentration of the latter is greater than molar.

2. Other nucleoproteins are less easily split in this way and other related salts are not so efficient as strontium nitrate.

3. Nucleic acid is decomposed by more intense treatment.

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The Action of Some α-Amylases on Amylose

By R. BIRD and R. H. HOPKINS
Department of Brewing and Applied Biochemistry, University of Birmingham

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The action of α-amylase is due to the fission of the α-1:4-glucosidic linkage in amylose and amylopectin. By confining the study to amylose and its fission products, complications arising from the presence of 1:6-glucosidic linkages are avoided. It is well known that the action of this enzyme is at first relatively rapid until the iodine colour disappears (achroic stage), this being usually attained in the case of amylose when about 20% of the linkages have been split. The mixture now consists of short-chain fragments including maltose and glucose (Myrbäck, 1948; Bernfeld, 1951). A much slower reaction, in some cases only one-hundredth as fast, overlaps and succeeds the first one. In this the short-chain fragments are successively split until only maltose and glucose or sometimes maltotriose remain. According to Myrbäck (1948), Bernfeld (1951), Meyer & Bernfeld (1941), Meyer & Gonon (1951), Alfin & Caldwell (1949) and Roberts & Whelan (1951) all except the terminal linkages in