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Nature and specificity of the RNA–protein interaction in the case of the tymoviruses

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[Plate 21]

Dissociation–reassociation experiments performed with turnip yellow mosaic virus in the presence of various RNAs and polynucleotides were used to investigate the degree of specificity and the contribution of the associated RNA moiety to the stability of TYMV. The results emphasize the importance of strategic cytosine residues spread along the RNA chain.

Some insight into the contribution of the protein could be gained from comparison of TYMV and eggplant mosaic virus (EMV), a virus similar to TYMV although its top component contains low molecular mass RNAs able to bind various amino acids. Hydrophobic interactions between protein subunits are less important in EMV than in TYMV, and artificial capsids could be obtained from dissociated EMV coat protein.

Whether the capsid is or is not the precursor of the virion in tymovirus morphogenesis is discussed.

Turnip yellow mosaic virus (TYMV) is a member of the tymovirus group (Harrison *et al.* 1971), composed of viruses having several characters in common such as a $T = 3$ capsid, an RNA with a high content in cytosine (32–38 %), and the presence *in vivo* of capsids stabilized by strong protein–protein interactions. In this group, several types of viruses are more or less serologically related (Koenig & Givord 1974).

Two of these viruses have been extensively studied in our laboratory from the point of view of RNA–protein and protein–protein interactions. We report here the most important results obtained with TYMV and eggplant mosaic virus (EMV): two tymoviruses distantly related serologically.

(*a*) *TYMV*

RNA–protein and protein–protein interactions were studied by means of dissociation–reassociation experiments. Indeed until now no true reconstitution of TYMV from RNA and dissociated-protein subunits has been obtained, either in our laboratory or in other groups. This probably arises from our inability to obtain undenatured small aggregates or monomers of viral protein.

Kaper (1960) had reported earlier that treatment of nucleocapsids of TYMV (bottom component) suspensions at high pH results in formation of empty shells (capsids) having apparently the same features as the native empty shells, but in the conditions used the RNA inside the capsid was degraded into relatively short fragments, the size of which is not so well defined as suggested by Kaper (1965). At relatively high ionic strength and at high pH these small pieces of RNA are able to escape, probably after the capsid has undergone a reversible change in configuration. However, Jonard, Ralijaona & Hirth (1967) and Jonard, Witz & Hirth (1972) were able to demonstrate that whole infectious RNA can escape the capsid in the presence

of 8 M urea at relatively high ionic strength. The mechanism of the release of RNA is not known but the data suggest that at least a part of the RNA is located at the surface of the capsid and that, in TYMV, protein–protein interactions are strong and play a major role in the stabilization of the capsid.

This point was illustrated by further experiments demonstrating that in 8 M urea at low ionic strength the virus collapses and that at very high ionic strength no release of RNA is observed (Jonard 1972). This type of experiment confirms the importance of the hydrophobic forces which are considerably reinforced by ionic strength. On the other hand, electrostatic bonds between RNA and protein seem to have a limited role.

The comparison between the behaviour of the capsids and that of the bottom component at different ionic strengths and pH value in 8 M urea has shown that at pH $\geqslant$ 7 the virus is less stable than the capsids whereas the contrary is observed at low pH. In the latter case, the protonation of a basic amino acid of the capsid, for example, results in the appearance of repulsive forces and in the degradation of the capsid.

The stabilization of the virions at low pH (4.2–4.8) and at relatively high ionic strength (1–1.5) indicates that, in addition to the salt linkage, a new class of interactions between RNA and protein occurs at this pH. It was suggested by Jonard (1972) that at a low pH, interaction between the bases (adenine, guanine, and cytosine) and dicarboxylic amino acids (aspartic and glutamic acid) occurs. This hypothesis was substantiated by several types of experiments reported below.

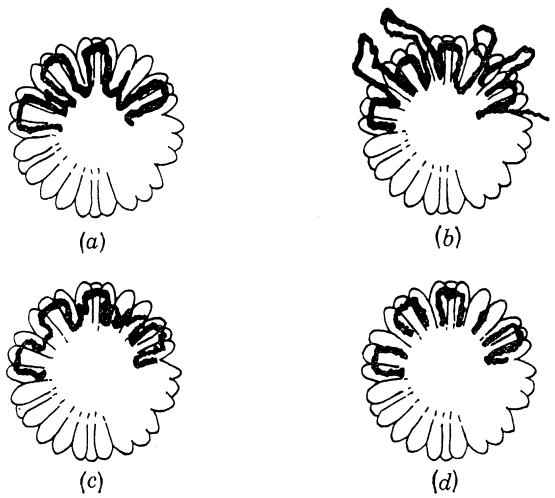

FIGURE 2. A possible model for the localization of RNA in reassociation complexes. (*a*) Model of TYMV, after Klug *et al.* (1966); (*b*) complex 70S; (*c*) complex 105S; (*d*) complex 70S or 105S after RNase treatment.

(*a*) *Dissociation–reassociation experiments*

When bottom component is treated at 25 °C with 8 M urea at pH 7, ionic strength 1, it rapidly dissociates into intact RNA and capsids (artificial top component: or a.t.c.). Upon dialysis of the mixture against acetate buffer at pH 4.2, in the presence of 0.005 M $MgCl_2$ or 0.005 M spermidine, reassociation products were obtained. The products were 70S aggregates and 70S and 105S aggregates in the presence of $MgCl_2$ and spermidine respectively (figure 1, plate 21). Analysis of the aggregates have shown them to be true reassociation products of completely dissociated ATC and RNA, exogenous ^{32}P labelled TYMV RNA being as efficiently incorporated as urea-released RNA.

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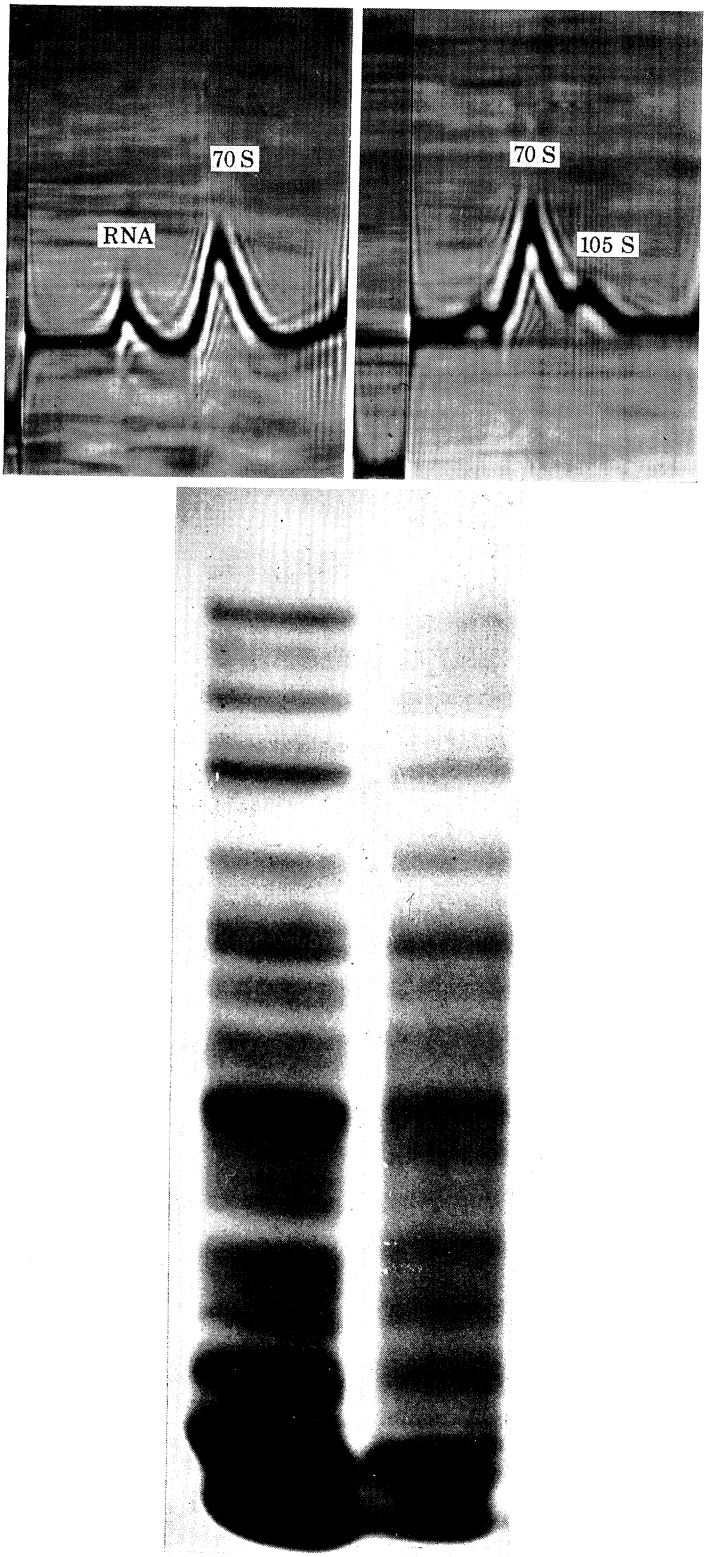

FIGURE 1. Sedimentation patterns of TYMV dissociated by 8 M urea, 1 M NaCl at pH 7 and then dialysed against 0.01 M sodium acetate buffer pH 4.2. (*a*) Experiment performed in presence of 5×10^{-3} M $MgCl_2$ (*b*) Experiment performed in presence of 5×10^{-3} M spermidine.

FIGURE 5. Polyacrylamide slab gel electrophoresis of TYMV partially digested with RNase T1. (*a*) TYMV RNA hydrolystate; (*b*) Fragments reassociated to capsids at low pH.

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On the other hand, heterologous RNAs such as tobacco mosaic virus or alfalfa mosaic virus RNAs are not encapsidated, indicating that the reassociation is a specific process. Analysis by CsCl density gradients shows that both types of aggregates have their full RNA complement. But according to their sedimentation constant and appearance in electron micrographs, 105S particles resemble true virions, whereas in 70S component only part of the RNA is located inside the capsid. Both 70S and 105S particles are partially sensitive to pancreatic RNase, giving rise to 75S aggregates. Furthermore, both species dissociate into capsid and free RNA when the pH is raised from 4.2 to 7. Figure 2 shows hypothetical structures for the 105S and 70S aggregates. It is noteworthy that only minute amounts of fast sedimenting material (or none at all) are formed if separately prepared RNA and ATC are mixed together in 0.01 M phosphate buffer, pH 7, in the presence of $MgCl_2$ or spermidine and dialysed against pH 4.2 buffer, even if the mixture is treated by 8 M area plus 1 M NaCl before dialysis. These observations suggest that carboxyl groups of the protein can interact strongly with nucleotide amino groups via hydrogen bonds at pH close to 4.5 (Jonard 1972; Jonard *et al.* 1972).

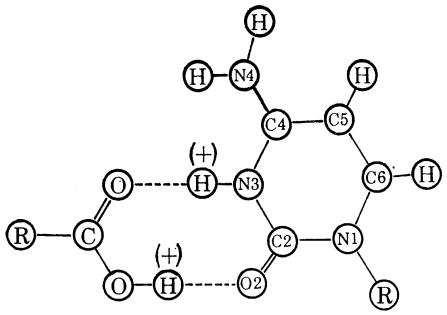

FIGURE 3. A model for the association of cytosine with a carboxyl group at low pH.

The existence of interactions between bases and protein was also supported by the experiments of Dorne, Jonard, Witz & Hirth (1971) who showed that treatment of TYMV with low concentrations of $HgCl_2$ results in the degradation of the virus into RNA and protein aggregates having various molecular masses. This can be explained by the high affinity of mercury for SH groups (4 per subunit in the case of TYMV). Indeed, it is known that SH groups play an important role in the stability of the TYMV capsid as was suggested earlier by the study of the influence of parachloromercurybenzoate on the stability of TYMV (Kaper & Houwing 1962). At high concentrations of mercury, no degradation of the virus occurs but when the virus is first treated with low concentrations of $HgCl_2$ the degradation products reassociate into compact particles as the concentration of mercury is increased. At low concentrations, mercury associates preferentially with SH and gives rise to a base–Hg–base complex which disfavours the base–protein interactions of the native virus. Both types of reactions lead to the collapse of the virus. At high concentrations of Hg, base–Hg–protein complexes are formed which stabilize or favour the reassociation of viral RNA and protein. This confirms the importance of the protein–base interactions in the stability of TYMV.

At low pH adenine, guanine, and cytosine are protonated on the N1 for adenine, N7 for guanine and N3 for cytosine, the pK values being 4.2, 3.3 and 4.6 respectively. It was proposed by Jonard (1972) that cytosine forms a relatively stable complex with the –COOH group of dicarboxylic amino acids. If we remember that TYMV RNA contains about 40% cytosine

it seems plausible that this base may play a central role in the base–protein interactions (figure 3).

To support this point of view, several types of experiments were performed. The results demonstrate that at low pH when poly A, poly C and poly U are added to the reassociation mixture obtained by dissociating TYMV with urea at neutral pH, only poly C is able to associate with the capsids. In contrast with the other polynucleotides poly G hybridizes with TYMV RNA.

The importance of C in the reassociation between TYMV RNA and capsids was further demonstrated by treating ^{32}P labelled TYMV RNA with bisulfite in conditions where cytosine is transformed into uracil. Figure 4 shows that when only 8 % of the cytosine is modified no reassociation occurs although no degradation of viral RNA was observed. In fact, up to 80 % of the cytosine residues are reactive with bisulphite. As the reaction with bisulphite is specific for cytosine that is not involved in double helices, the majority of the cytosine in TYMV RNA must be located in single stranded stretches and the most reactive among them probably form a special class playing a strategic role in the association of viral RNA with capsids at low pH.

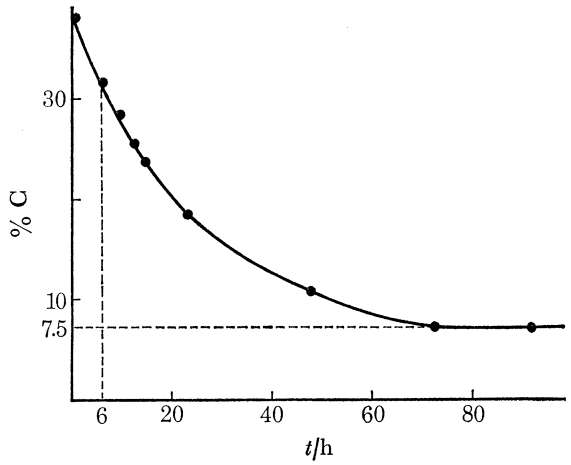

FIGURE 4. Rate of conversion of cytosine into uracil upon treatment of TYMV RNA by 2 M sodium bisulphite at pH 5.8.

From recent unpublished results obtained in our laboratory it is clear that no long stretches of poly C exist in TYMV RNA, but oligo C containing 4–6 cytosine residues are apparently spread along the whole RNA chain. Indeed, as indicated in figure 5, plate 21 most of the fragments arising from a partial T1 digestion of TYMV RNA are able to reassociate efficiently with ATC.

The problem is now: have these observations something to do with TYMV morphogenesis *in vivo*? It is known that in some cases (Fernandez-Tomas & Baltimore 1973) procapsids are formed and the RNA enters these procapsids at a penultimate stage of morphogenesis. There is nothing to prove that something analogous may occur with TYMV, especially as no true virus has been obtained in our reassociation experiments. On the other hand, the conditions under which reassociation occurs are far from physiological. It is worth noting however, that inoculation of various plants, with TYMV nucleocapsids results in the formation of capsids and liberation of viral RNA (Fritsch 1972) demonstrating that some cell structures are able to liberate RNA without disruption of the capsid. On the other hand, it seems that in addition

to salt linkages other interactions between RNA bases and the protein (particularly cytosine-protein interactions) must exist.

(*b*) *Eggplant mosaic virus*

The impossibility of obtaining native protein from TYMV suspensions suggested to us that study of another member of the tymorivus group might be rewarding. Eggplant mosaic virus was chosen as the subject. Its characteristics are reported to be similar to those of TYMV. Before trying to reconstitute this virus, some of its properties were studied in our laboratory in collaboration with the laboratory of Professor Duranton in Strasbourg (Bouley *et al.* to be published).

EMV sediments in a sucrose gradient or in gradients of a caesium chloride as two main peaks with an additional small peak close to the heaviest peak. The origin and significance of the minor component is not yet known. Only the heaviest component is infectious and gives rise to necrotic lesions on *N. glutinosa*. The RNA extracted from heavy component has a molecular mass of 2.3×10^6 and the base composition is C 38%, A 22%, G 17% and U 23% (Bouley 1975). The top component, in contrast with that of TYMV, contains several molecules of short RNA, with molecular masses in the range of 30000. On the average each capsid contains 2–3 molecules of such RNA. The RNA of the infectious particle was tested for its amino acid acceptor property and in contrast with TYMV, which binds only valine, it was found to accept two amino acids: valine and lysine. It has been demonstrated that valine is bound to the high molecular mass RNA whereas the lysine is bound to a small RNA having the characteristics of a tRNA (Pinck, Genevaux & Duranton 1974). In fact, it is possible to separate the lysyl-tRNA (which is a true tRNA) from the viral RNA, as they are associated by hydrogen-bonding only. Several observations suggest that synthesis of the lysyl-tRNA encapsidated with viral RNA is enhanced in the infected plants. At present, however, its origin and significance remains unclear.

The RNAs extracted from top component have been shown to bind several amino acids. Three of them are bound preferentially: lysine, arginine and valine; traces of alanine, histidine, leucine, phenylalanine and tyrosine are also bound. It is not known if these RNAs are authentic tRNAs, but that which accepts lysine seems to be tRNA-like in that it contains abnormal bases. It is worth noting that small RNAs are present in the capsids of Oncorna viruses, such as Rous sarcoma virus and avian myoloblastosis virus; some of them seem to be cellular tRNA free in the capsid, others are associated (probably to hydrogen bonding) with the viral RNA; the choice of the selected RNAs seems to be under the control of the viral genome. Our discovery is the first example of analogous small RNAs in the case of a plant virus. Their role is now under investigation.

Recently, in our laboratory, the sequence of the last 60 nucleotides at the 3′OH end of the infectious RNA of EMV was determined (Briand *et al.* in preparation) and it was discovered that the structure of this piece of RNA has some features in common with valine tRNAs extracted from yeast. The implication of this discovery will be discussed elsewhere.

In any case, EMV has some peculiar features. This is particularly clear when the stability of EMV is compared to that of TYMV (Bouley 1975). From the study of the action of urea at different ionic strengths and pH it appears that bottom component of EMV collapses completely in 4 M urea at low ionic strength, whatever the pH. In 8 M urea it was degraded at any ionic strength and pH. High pH values tend to destabilization of the virion, as at low i.s.

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4 M urea is required to degrade the virion at pH 4.8 or 7, and only 2 M near pH 9. But the special class of very strong stabilizing forces present in TYMV at low pH apparently does not exist in EMV; the behaviour of the latter is very much the same at pH 7 and 4.8. The RNA that is liberated by the collapse of the virion is always degraded, even if experiments are performed in the presence of bentonite. This is probably due to the presence of RNases on the capsid.

We can conclude from this systematic study that in EMV, as in TYMV, hydrophobic bonds stabilize the capsid but that they are apparently much weaker than those stabilizing TYMV. It has not proved possible, however, to relate the amino acid content of each type of virus to the hydrophobic bonds in the architecture of the capsid. None the less, it may be noteworthy that polar amino acids are no more numerous in EMV than in TYMV (Bouley 1975).

Alkaline treatment of bottom component at low ionic strength results in dissociation of the virus and in the formation of protein aggregates of 2.5S. High temperature favours the process of dissociation. If bottom component is incubated for 2 h at pH 10 at 40 °C and at high ionic strength (2 M NaCl), artificial top component is formed, but the liberated RNA is degraded even in the presence of RNase inhibitors. This artificial top component (a.t.c.) always contains a small amount of RNA (approximately 1–2 %). Whatever the conditions we never obtained uncontaminated a.t.c. This is, of course, in contrast with the artificial top component prepared from TYMV bottom component which is essentially free of RNA. But the more surprising feature of this a.t.c. is that the remaining RNA, when extracted and tested for its ability to bind amino acids, can accept phenylalanine, tyrosine, lysine and arginine. Controls have shown that RNA extracted from the bottom component of EMV binds only valine and lysine as described above. No other amino acids were bound whatever the concentration of RNA used. It is likely that these RNAs are present in bottom component and are not liberated during alkaline treatment. The reason for their 'masking' in the bottom component remain to be elucidated. In any case 'transfer RNA' present in ATC are firmly bound to capsid.

The fact that protein–protein interactions are weaker in EMV than in TYMV and also that the polypeptide chain in EMV contains only 2 cysteine residues encouraged us to try to obtain native protein subunits. Protein was extracted from bottom component rendered free of top component by differential ultracentrifugation by treatment with acetic acid at 66 % in the presence of 10^{-2} M DTE. The precipitated RNA was eliminated by centrifugation. After dialysis overnight against 0.02 M sodium acetate pH 3.5, 10^{-3} M DTE buffer, the solution was concentrated by ultrafiltration through Amicon membrane 10 000. Starting from fractionated bottom component, a protein could be obtained, characterized by a u.v. absorption spectrum with a maximum at 276 nm, a minimum at 237 nm and a ratio of the absorbancies at 276 nm and 237 nm of about 2.30. Top component protein, on the other hand, could not be freed completely from contaminating RNA.

Protein extracted from bottom component was soluble at low ionic strength only, especially at pH $\geqslant$ 4. In 0.02 M sodium acetate buffer containing 10^{-3} DTE it sedimented at 12S and 24S at pH values between 3.5 and 4.5, and at 56S at pH = 5.25–5.45. In the electron microscope filaments could be seen in the first case, and empty spherical particles, of some 30 nm in diameter in the second one. Dissociated protein is insoluble at pH 7, whatever the ionic strength. But at least some of the spherical particles, once formed at the latter pH are stable at pH 5.25.

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Conclusion

We can conclude from the reported experiments that the tymovirus group contains viruses which are essentially stabilized by hydrophobic protein–protein interactions. The strength of these interactions varies however from one virus to another. The empty shells, always present in the natural preparations of these viruses, are much more stable than the nucleocapsid: surprisingly the presence of RNA destabilizes the protein–protein interactions. A more or less speculative model has been proposed to explain this situation which contrasts greatly with that observed, for example, in the case of bromoviruses.

The significance of the empty shells in the virus preparation is unclear. It is tempting to imagine that they arise by the loss of their RNA at various phases of virus multiplication: the fact that the *in vivo* uncoating of nucleocapsids produces significant amounts of empty shells supports this point of view. However, the fact that isolated protein from EMV in the absence of infectious viral RNA is able to reaggregate into empty shells very similar to natural ones suggests that they could be 'procapsids', a precursor in the morphogenic process. However, nothing proves that this is the case. Furthermore, till now the conditions necessary and the nature of the material obtained in the reported dissociation–reassociation experiments do not support such a hypothesis. In fact only true *in vitro* reconstitution in conditions as close as possible to the physiological are likely to give an unambiguous answer to this question. In this respect EMV could be a good tool.

Another important point is the relative 'specificity' of the reassociation between the viral RNA and the capsids and the role of the cytosine residues.

We could consider, for example, that the recognition of viral RNA by tymoviruses capsids is more specific than the recognition of TMV RNA by its own protein. Indeed, TMV double disks are able to recognize several unrelated viral RNAs. This could be due to the fact that in the case of TMV probably a relatively short and more or less specific nucleotide sequence is recognized, whereas in TYMV several nucleotide stretches dispersed along the RNA have to interact with the capsid to give rise to a nucleocapsid.

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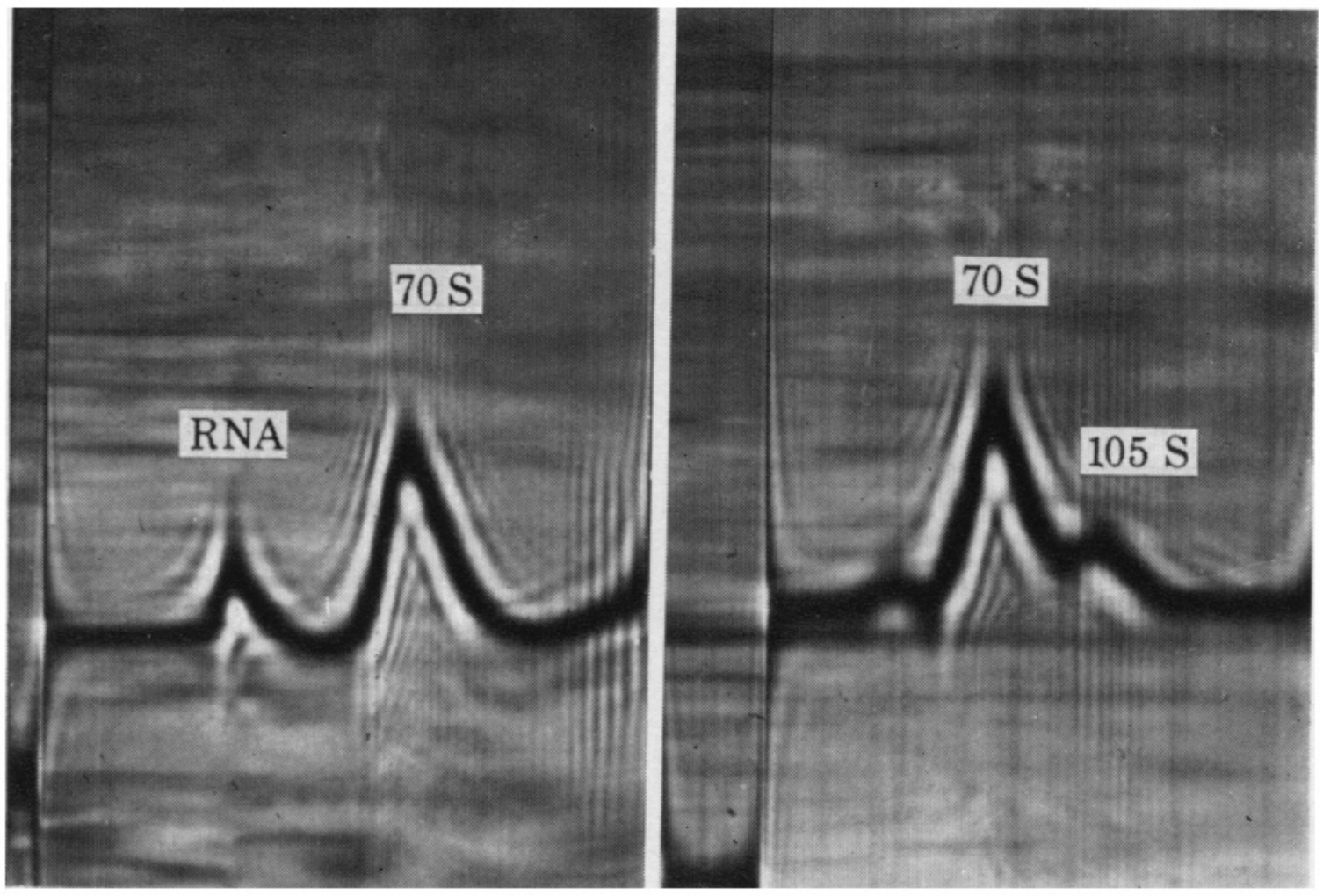

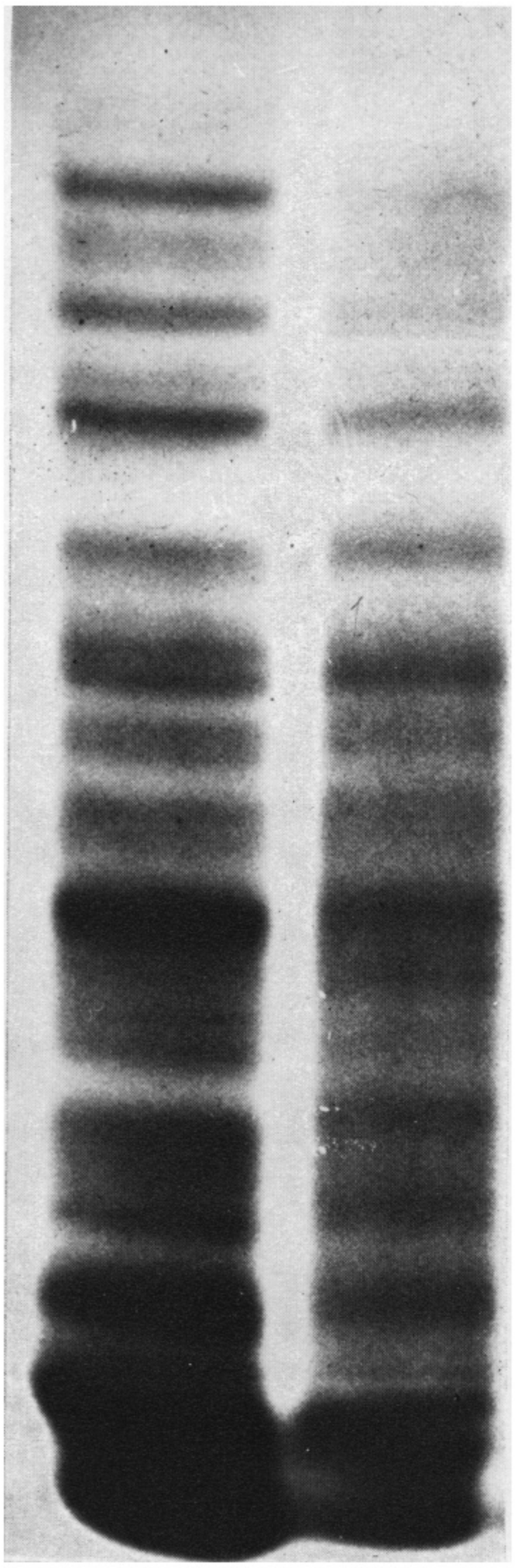

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FIGURE 1. Sedimentation patterns of TYMV dissociated by 8 M urea, 1 M NaCl at pH 7 and then dialysed against 0.01 M sodium acetate buffer pH 4.2. (*a*) Experiment performed in presence of 5×10^{-3} M $MgCl_2$ (*b*) Experiment performed in presence of 5×10^{-3} M spermidine.

FIGURE 5. Polyacrylamide slab gel electrophoresis of TYMV partially digested with RNase T1. (*a*) TYMV RNA hydrolystate; (*b*) Fragments reassociated to capsids at low pH.