Low pH RNA-Protein Interactions in Turnip Yellow Mosaic Virus

II. Binding of Synthetic Polynucleotides of TYMV Capsids and RNA

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It has been previously shown that turnip yellow mosaic virus capsids and RNA obtained by incubation in 8 M urea, 1 M NaCl at pH 7 could be reassociated by dialysis against low ionic strength buffer, in the presence of MgCl₂ or spermidine. The nucleoprotein complexes differed from native virions in stability at neutral pH and in RNase-resistance at low pH, but the association is specific, as TMV-RNA or alfalfa mosaic virus-RNA was found not to interact with capsids under such conditions.

We present here further data concerning the specificity of this interaction. Radioactive poly(A), poly(C), poly(G) and poly(U) were added to the dissociation mixture of TYMV in the presence of 5 x 10⁻³ M MgCl₂. It was found that only poly C reassociated specifically with "nascent capsids." Poly(A) did not interact at all with either capsids or RNA. At either neutral or acidic pH, small amounts of poly U bound to capsids, but not to RNA or virions. Poly(G) forms a hybrid with TYMV-RNA.

The nature of low pH RNA-protein interaction in TYMV is discussed.

INTRODUCTION

Turnip yellow mosaic virus (TYMV) is a small isometric plant virus that belongs to a family of viruses characterized by the high cytosine content of their RNA and the presence of empty capsids in purified preparations. The arrangement of protein subunits and the distribution of nucleic acid in the particle have been investigated by a combination of electron microscopy and X-ray diffraction (Finch and Klug, 1966; Klug et al., 1966): part of the RNA is clustered within the 32 morphological units of the T = 3 protein shell. Physical-chemical studies indicated that a special class of RNA-protein interactions exists in TYMV at low pH (Kaper, 1968, 1969, 1971; Jonard, 1972), and that it is possible to dissociate TYMV into infective RNA and empty capsids at pH 7 in 8 M urea, 1 M NaCl (Jonard et al., 1967).

We have described (Jonard et al., 1972) specific nucleoprotein complexes formed from dissociated TYMV-RNA and capsids by dialysis against a low pH, low ionic strength buffer: 70 S products form in the presence of 0.005 M MgCl₂, and both 70 and 105 S form in the presence of 0.005 M spermidine (TYMV sediments at 115 S and empty shells at about 50 S under the same conditions). These results supported Kaper's second hypothesis that carboxyl groups of the protein in TYMV strongly interact with nucleotide amino groups via hydrogen bonds at a pH close to pH 4.5 (Kaper, 1969, 1972). The present paper reports the results obtained with mixed reassociation experiments performed in the presence of syn-
thetic polyribonucleotides. It will be seen that only poly(C) binds specifically to "nascent capsids", whereas poly(G) forms a hybrid with TYMV-RNA.

MATERIALS AND METHODS

Virus and RNA. TYMV was isolated from frozen Chinese cabbage leaves. Plant proteins were removed from the sap by precipitation at pH 4.8. The virus was precipitated at pH 7 by polyethyleneglycol (5%) in the presence of NaCl (3%). The pellet was resuspended in 0.01 M sodium phosphate buffer, pH 7, and submitted to two cycles of low and high speed centrifugation. Concentrations were estimated using a specific extinction coefficient $E_{260} = 8.4 \text{ cm}^2/\text{mg}$ (Horn et al., 1963).

The RNA was extracted with phenol in the presence of bentonite, precipitated with two volumes of ethanol and stored at $-20\degree$. It was redissolved in 0.01 M sodium phosphate buffer, pH 7, or 0.01 M Tris-HCl buffer, pH 7.4, before use. Concentrations were estimated spectrophotometrically, using an extinction coefficient $E_{260} = 21.8 \text{ cm}^2/\text{mg}$ (Strazielle et al., 1965).

Tritium-labeled polyribonucleotides were purchased from Miles Chemicals (USA) and unlabeled ones from P.L. Biochemicals Inc. (USA). Poly(A), poly(C), and poly(U) were used without further purification. Poly(G) samples were dialysed overnight at 4\degree against large volumes of 0.01 M EDTA, 0.01 M Tris-HCl buffer, pH 7.4, to remove divalent ions (Pochon and Michelson, 1965).

Hybridization experiments. Hybridization experiments of poly(G) and viral RNAs were performed according to Englander et al. (1972). Mixtures of poly(G) and viral RNA in 8 M urea, 1 M NaCl, 0.01 M Tris-HCl buffer, pH 7.4, were heated at $60\degree$ for 5 min and dialysed against a 0.5 M NaCl, 0.01 M Tris-HCl buffer preheated to the same temperature. Hybridization took place during cooling to room temperature. Samples were analyzed in a 5–20% sucrose gradient spun for 14 hr at 20,000 rpm in a SW 27 rotor.

Dissociation-reassociation experiments. Dissociation-reassociation experiments were performed according to the technique described by Jonard et al. (1972). Two milliliters of a solution of TYMV at 5 mg/ml in 0.01 M phosphate buffer, pH 7, were incubated at 40\degree for 3 or 5 min in 8 M urea, 1 M NaCl, 0.005 M MgCl$_2$, 0.01 M phosphate buffer, pH 7, in the presence of bentonite (0.06%). For mixed reassociation experiments $^3$H-labeled polyribonucleotides were added to the TYMV solution after urea treatment. The dissociation mixture was dialysed overnight against either 0.001 M MgCl$_2$, 0.01 M phosphate buffer, pH 7 (buffer A) or 0.001 M MgCl$_2$, 0.01 M acetate buffer, pH 4.2 (buffer B). Dialysis against buffer A was used as a control for dissociation of TYMV. If necessary the sample dialysed against buffer B was divided into three aliquots before further analysis: one was kept as a control of the reassociation, the second was treated with pancreatic RNase (1 \mu g/ml at 20\degree for 30 min) and the third was further dialysed against buffer A to test its stability at neutral pH.

All samples were layered on 5–45% linear sucrose gradients prepared in 0.01 M sodium acetate buffer, pH 4.5, and spun for 4 hr at 27,000 rpm in a Beckman SW 27 rotor. The fractions were collected through the bottom of the tube and their radioactivity and optical density were estimated with an Intertechnique SL 30 liquid scintillator and a Zeiss PMQII spectrometer, respectively. As in mixed reassociation experiments only small amounts of labeled nucleic acid (about 0.2 \mu Ci of polynucleotide having a specific radioactivity of the order of 50 mCi per mmole of phosphorus) were added, the OD profile (at 260 nm) gives the result of the homologous reassociation and the radioactivity that of the heterologous one.

Artificial top component ATC-U. ATC-U was obtained by dissociating TYMV for 5 min at 40\degree in 8 M urea, 1 M NaCl, 0.01 M sodium phosphate buffer, pH 7, in the absence of bentonite. It was separated from fragmented RNA by two cycles of high and low speed centrifugation after dialysis of the dissociation mixture against 0.01 M sodium phosphate buffer.
pH 7. Such a preparation of ATC-U contains less than 0.1% of undissociated TYMV, and its physical properties are the same as those of top component obtained by alkaline treatment of TYMV (Kaper, 1964) and of natural top component (Jonard et al., 1967; Jonard, 1972).

RESULTS

Figure 1 shows the result of a mixed reassociation experiment where $^3$H-labeled poly(C) was added to TYMV dissociated at 40° in 8 M urea, 1 M NaCl, 0.01 M sodium phosphate buffer, pH 7, in the presence of 0.005 M MgCl$_2$. After dialysis against buffer A, the radioactivity was located at the top of the gradient, and neither homologous nor heterologous reassociation occurred (Fig. 1A). After dialysis against 0.001 M MgCl$_2$, 0.01 M sodium acetate buffer, pH 4.2 (buffer B); an important fraction of TYMV-RNA reassociated with

\[ \text{FIG. 1. Sucrose density gradient (5-45%) centrifugation of the products obtained in a mixed reassociation experiment where }^{3} \text{H labeled poly(C) was added to TYMV dissociated for 5 min at 40° in 8 M urea, 1 M NaCl, 0.01 M sodium phosphate buffer, pH 7, containing 0.005 M MgCl}_2. \text{ (A) Sample dialysed against 0.001 M MgCl}_2, 0.01 M sodium phosphate buffer, pH 7 (buffer A). (B) Sample dialysed against 0.001 M MgCl}_2, 0.01 M sodium acetate buffer, pH 4.2, (buffer B). (C) Sample B after 30 min treatment at 25° with 1 μg/ml pancreatic RNase. (D) Sample B after dialysis against buffer A. ●—●, optical density at 260 nm; ★—★, $^3$H radioactivity. For the sake of clarity, only one experimental point out of two or three has been plotted. The curve however goes through all points. Centrifugation was for 4 hr at 27,000 rpm in a SW 27 rotor. Sedimentation is from right to left. The arrows indicate the position of TYMV (left) and empty capsids (right) in such a gradient. \]
capsids and sedimented at 70 S (Fig. 1B) whereas another part remained free, sedimenting at about 24 S (fractions 34-35 in Figs. 1A and 1B). About 40% of the initially added radioactive poly(C) also sedimented at 70 S (Fig. 1B). This sample was incubated for 30 min at 20° with pancreatic RNase (1 μg/ml). Figure 1C shows that about one-third of the radioactivity still sedimented to the middle of the gradient after such a treatment. Two peaks may be seen in Fig. 1C, in the region of fractions 25–30: they were separated by two fractions of significantly lower radioactivity. In another experiment they were separated by only one fraction. Thus the bimodal distribution of radioactivity in the middle of the gradient may be significant. Another aliquot of sample B was further dialysed against buffer A: Fig. 1D shows that it dissociated completely into capsids, RNA and poly C at neutral pH.

We have also tested if at variance with TYMV-RNA (Jonard et al., 1972), poly(C) could associate with empty capsids. Two milliliters of ATC-U at 10 mg/ml were incubated with 3H-labeled poly(C) (15 × 10^4 cpm) for 3 min at 40° in 8 M urea, 1 M NaCl, 0.02 M sodium phosphate buffer, pH 7, containing 0.005 M MgCl₂. Aliquots of this mixture were dialysed against buffers A and B and analyzed by sucrose density gradient. We found that at pH 7 the radioactivity sedimented at the top of the gradient. After dialysis against buffer B, no fraction of the gradient was radioactive: all the poly(C) precipitated together with a large proportion of the capsids.

Figure 2 shows that in mixed reassociation experiments performed in the presence of labeled poly(A), no association between poly(A) and capsids took place at either pH.

If 3H-labeled poly(U) was added to the dissociation mixture of TYMV, a small amount (4–5%) of the radioactivity was found in the middle of the gradient after dialysis against either pH 7 or pH 4.2 buffer (Figs. 3A and 3B). The complex obtained at pH 7 was however less resistant to a 30 min treatment at 25° with 1 μg/ml of pancreatic RNase than that obtained at

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**Fig. 2.** Sucrose density gradient (5-45%) centrifugation of the products obtained in a mixed reassociation experiment in the presence of 3H labeled poly A. Experimental conditions and symbols are the same as in Figs. 1A and 1B.
Fig. 3. Sucrose density gradient (5–45%) centrifugation of the products obtained in a mixed reassociation experiment in the presence of H-labeled poly(U). (A) After dialysis against buffer A. (B) After dialysis against buffer B. (C) Association products obtained by mixing H labeled poly U with TYMV and TYMV capsids in 0.01 M sodium phosphate buffer, pH 7. —O—, optical density at 260 nm; —k—, H radioactivity. Centrifugation was for 4 hr at 27,000 rpm in a SW 27 rotor.

pH 4.2. Poly(U) was also added to a mixture of TYMV and ACT-U in 0.01 M sodium phosphate buffer, pH 7; Fig. 3C shows that some radioactivity was found at the capsid level, but not at that of the virus. A similar result was obtained in 0.01 M sodium acetate buffer, pH 4.2.

Figure 4 shows the result of a mixed reassociation experiment performed in the presence of H-labeled poly(G). At both pH 7 and 4.2 a significant fraction of the radioactivity followed very closely the profile of absorbancy at 260 nm in the middle of the gradient. This leads us to think that in fact poly(G) interacted strongly with TYMV-RNA.

The possibility of hybridization of H-labeled poly(G) with RNAs extracted from TYMV led us to examine the closely related eggplant mosaic virus (EMV) as well as tobacco mosaic virus (TMV). These were investigated as described in Materials and Methods. Figures 5B and 5D show that indeed the radioactivity profile followed closely that of optical density for both TYMV- and EMV-RNA, whereas no hybridization occurred between poly(G) and TMV-RNA (Fig. 5C). All three RNAs were
slightly degraded after urea treatment, as described by Boedtker (1968).

**DISCUSSION**

Mixed reassociation experiments reported here show that among the four homopolyribonucleotides tested, only poly(C) binds specifically at low pH to "nascent capsids" of TYMV, with a yield of some 40%, comparable to that of a homologous reassociation (Jonard, 1972; Jonard et al., 1972). Several models may be put forward to explain this preferential interaction of cytosines with TYMV capsids at low pH. As it is strong at low pH only, it may involve double-stranded helices of half protonated poly(C) (Akinrimsi et al., 1963; Langridge and Rich, 1963). But the formation of such structures is a highly cooperative process which occurs over a very narrow range of pH (less than 0.2 units). The sedimentation coefficients of our reassociation products, on the other hand, decrease progressively as pH rises from pH 4.2 to 5.1 (Jonard, 1972), and dissociation occurs at pH 6. The pH dependent RNA release, obtained by heating TYMV at 40°, also occurs at 80% within a pH interval of about one pH unit (see Fig. 4 of Kaper, 1972). This is good evidence for the absence of double-stranded poly(C) at the specific interaction sites. But further experiments are necessary to substantiate any detailed model.

After R.Nase treatment of the reassociation products obtained in presence of poly(C), two peaks of radioactivity, or at least a peak with a shoulder, are found in the middle of the gradient, the smaller peak (or the shoulder) sedimenting more slowly than the major one. This may imply that once enough poly(C) molecules are associated with a capsid which had not already interacted with TYMV-RNA they will prevent its further reassociation with TYMV-RNA.

A small amount of poly(U) associated at acidic as well as neutral pH with "nascent" or purified capsids obtained from TYMV by urea treatment, but not with native TYMV. It is not known whether this difference is due to a slight irreversible change of conformation of the protein shell induced by high urea molarities, or to a difference in the surface properties of the protein shell induced by the absence of RNA. The observation may be related to the differential adsorption by bentonite of natural top...
Fig. 5. Sucrose density gradient (5-20%) centrifugation of the products obtained in a hybridization experiment of poly(G) and viral RNAs. $^3$H-labeled poly(G) was mixed with viral RNA in 8 M urea, 1 M NaCl, 0.01 M Tris-HCl buffer, pH 7.4, at 60°, dialysed against 0.5 M NaCl, 0.01 M Tris-HCl buffer, pH 7.4, preheated at 60° and allowed to cool during dialysis. (A) Poly(G) (control sample). (B) Poly(G) and TYMV-RNA. (C) Poly(G) and TMV-RNA. (D) Poly G and EMV-RNA. -○-○-, optical density at 260 nm; -★-★-, $^3$H radioactivity. Centrifugation was for 24 hr at 27,000 rpm in a SW 27 rotor.

Component and virions of wild cucumber mosaic virus, a virus somewhat similar to TYMV (Hitchborn and Dunn, 1965). In any case, as the interaction of poly(U) with TYMV protein occurs at both neutral and acidic pH, and at a very low yield, it is different from that involving poly(C), which is strong only at low pH.

Reassociation and hybridization experiments have shown that poly(G) associates with TYMV-RNA as well as with RNA from the related EMV, but not with TMV-RNA. The formation of such a hybrid is certainly related to the high cytosine content of TYMV-RNA (38%) and EMV-RNA (38%; Bouley et al. [1975]) compared with TMV-RNA (18%). Matus et al. (1964) and Gigot (1968) also described stable complexes of TYMV-RNA with plant ribosomal RNAs, which very likely are due to the base composition of the aggregating RNAs.

In our experiments with poly(U), we did not detect a hybridization between poly(U)
and TYMV-RNA (see, for example, Fig. 4B). Thus, in contrast to poliovirus RNA or eastern equine encephalitis virus RNA (Armstrong et al., 1972), TYMV-RNA does not seem to contain poly(A) or even oligo(A) segments.

REFERENCES


