

ISOLATION OF REPLICATIVE RNA FROM ALFALFA MOSAIC VIRUS-
INFECTED PLANTS BY CHROMATOGRAPHY ON HYDROXYAPATITE COLUMNS

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Bacterial and animal cells infected with RNA viruses contain a "replicative intermediate" (RI) of the viral RNA (Erikson, Fenwick and Franklin, 1964). Mild pancreatic RNase treatment of RI transforms it into a "replicative form" (RF) which is thought to be a double-stranded RNA differing from the RI in that the dangling parts of the growing "plus" strands have been hydrolyzed.

The isolation of RI from infected bacterial and animal cells has been reported (Franklin, 1966 ; Baltimore, 1968) ; in the case of infected plant cells the RF has been isolated (Burdon et al., 1964 ; Mandel et al., 1964), but not the RI. Having been unsuccessful in isolating the RI of plant viruses on the CF₁₁ cellulose columns used by Franklin (1966) for the RI of phage R 17 RNA, we have used a method developed in Strasbourg for the fractionation of nucleic acids having different secondary structures (Bernardi, 1962 ; 1965 ; 1968) : chromatography on hydroxyapatite (HA) columns. By this method, we have been able to isolate, without prior RNase treatment, the replicative RNA's of turnip yellow mosaic virus (TYMV) and of alfalfa mosaic virus (AMV). We describe here the isolation of the replicative RNA of AMV, a complex virus having four components with different sedimentation coefficients.

MATERIALS AND METHODS

Tobacco plants, N. tabacum, var. Xanthi n.c., were infec-

ted with AMV. After 5-11 days, plants were transferred for 36 hours on a medium containing P^{32} . Total RNA was then extracted with phenol at pH 9.5 in the presence of bentonite (Weissmann *et al.*, 1964), precipitated several times with alcohol and digested with 10 μ g/ml of RNase-free pancreatic DNase (Worthington, Freehold, N.J.) for 15 min at 37°, the solvent being 0.025 M Tris buffer - 0.001 M $MgCl_2$, pH 7.4. Digestion was stopped by adding 2 volumes of cold ethanol. RNA was dissolved in 0.001 M potassium phosphate buffer pH 6.8 (KP), and loaded on columns of HA prepared according to Tiselius, Hjerten and Levin (1956). Chromatographic experiments were carried out at room temperature.

RESULTS

Figure 1 shows the chromatographic behavior of soluble and ribosomal RNA prepared from uninfected plants and separated by precipitation with 2 M LiCl (Baltimore, 1966). Soluble RNA was not retained by the column equilibrated with 0.15 M KP, in

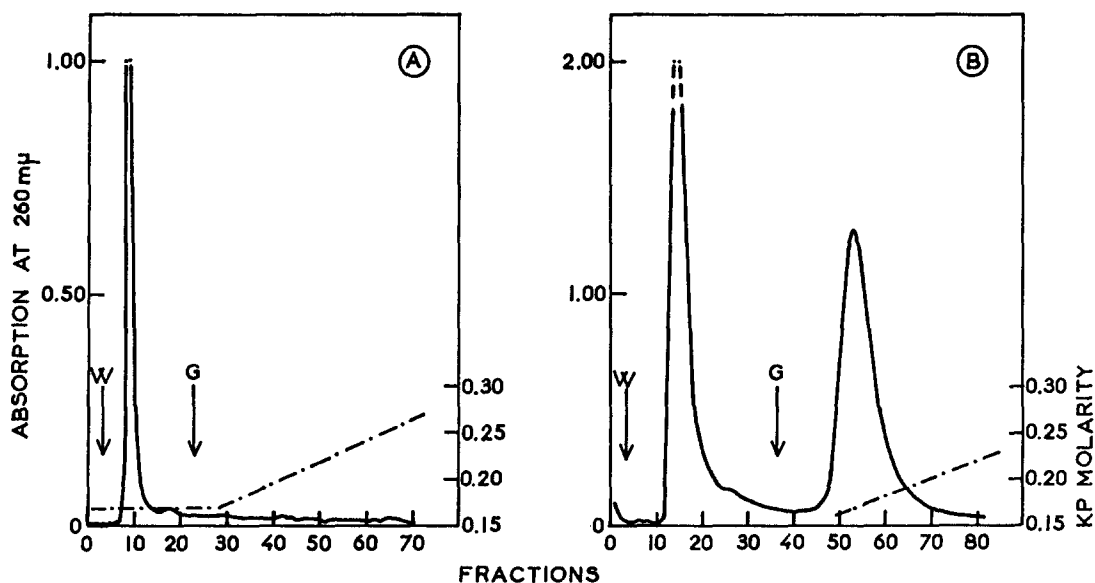


Figure 1A. Chromatography of uninfected Tobacco leaves s-RNA on HA. 1.6 mg were loaded on a 1 x 10 cm column equilibrated with 0.001 M KP. The column was then washed with 0.15 M KP (arrow W). A molarity gradient (0.15 - 0.30 M KP) was applied to the column at the fraction indicated by arrow G.

B. Chromatography of uninfected Tobacco leaves ribosomal RNA on HA. 6.8 mg were loaded on a 2 x 15 cm column equilibrated with 0.001 M KP. Other indications as in A.

agreement with the fact that its elution molarity is close to 0.13 M (Bernardi, 1965) ; ribosomal RNA was in part not retained by the column equilibrated with 0.15 M KP and in part eluted by a molarity gradient (0.15 - 0.30 M) of KP, also in agreement with results already described (Bernardi and Timasheff, 1961 ; Bernardi, 1965 ; 1968).

Figure 2 shows the chromatographic behavior of total RNA from infected leaves. The main difference with the results obtained with RNA from uninfected plants (fig. 1) is that now a significant amount of material is eluted at molarities higher than 0.20 M KP. Fractions eluted at molarities comprised between 0.19

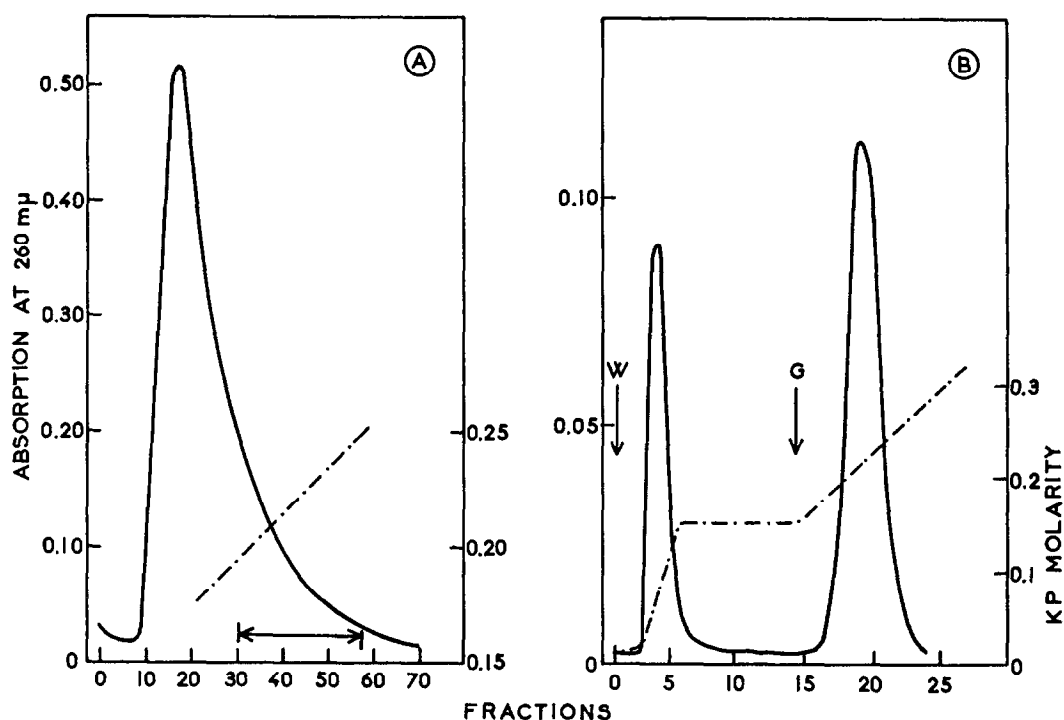


Figure 2A. Chromatography of total RNA from Tobacco leaves harvested 11 days after infection with AMV on HA. 20 mg were loaded on a 2 x 15 cm column equilibrated with 0.001 M KP. The column was then washed with 0.15 M KP until the ultraviolet absorption of the eluent was negligible. A molarity gradient (0.15 - 0.30 M KP) was applied to the column at fraction n°0. Five ml fractions were collected. Fractions indicated by the arrow were rechromatographed.

B. Re-chromatography of fractions eluted by 0.20 - 0.25 M KP of total RNA from Tobacco leaves harvested 8 days infection. 1.4 A_{260} units were loaded on a 1 x 10 cm column, equilibrated with 0.001 M KP. The column was then washed with 0.15 M KP (arrow W) until the ultraviolet absorption of the eluent was negligible. A molarity gradient (0.15 - 0.50 M KP) was then applied to the column (arrow G). 2 ml fractions were collected.

TABLE I

Resistance of chromatographic fractions to pancreatic RNase.

| Fractions | 0.2 M | | | | 0.15 M |
|-----------------------|-------|----|----|----|--------|
| Days after infection | 5 | 7 | 8 | 11 | |
| % Undigested material | 76 | 76 | 72 | 69 | 6 |

RNA chromatographic fractions were dialyzed against SSC (0.015 M Na citrate - 0.15 M NaCl). 100 μ l samples, containing 0.2 - 0.4 μ g RNA, were digested with pancreatic RNase, final concentration 1 μ g/ml, for 10 min at 37°. Digestion was stopped by adding 1 volume of cold 10 % trichloroacetic acid (TCA). 50 μ g of bovine serum albumin in 5 μ l were added and the precipitate was filtered on a 0.45 μ Millipore filter. After washing with 5 % cold TCA, the radioactivity on the filter was measured using a Nuclear Chicago instrument. Less than 0.5 % radioactivity was acid-precipitable after 20 hours hydrolysis with 0.3 N KOH. at 37°.

TABLE II

Reannealing experiments with the 0.2 M fraction of AMV RNA.

| RNA "plus" strands | AMV 200 μ g | AMV 400 μ g | AMV 600 μ g | TYMV 210 μ g |
|-----------------------|--------------------|--------------------|--------------------|---------------------|
| Reannealed RNA, % | 25 % | 31 % | 44 % | 2 % |

2 μ g (14,500 c.p.m.) of the 0.2 M fraction of AMV RNA in 50 μ l of 2.5 x SSC were added to cold AMV RNA ("plus" strand), or TYMV RNA, in the same solvent and heated up in sealed tubes for 3 minutes at 120° and 30 minutes at 85°. The incubation mixtures were made up to 1 ml, the final buffer concentration being 1 x SSC, and digested with pancreatic RNase as described in the footnote to Table I.

and 0.24 M KP (figure 2A) were pooled, dialyzed against water to decrease the KP concentration and rechromatographed. The results obtained are shown in fig. 2B : part of the loaded RNA was washed through the column by 0.15 M KP, the rest being eluted between 0.20 and 0.24 M KP by a molarity gradient.

The properties of this chromatographic fraction (0.2 M fraction) were investigated. Table I shows that the 0.2 M fraction is highly, yet not totally, resistant to pancreatic RNase under conditions where the 0.15 M fraction was almost completely degraded.

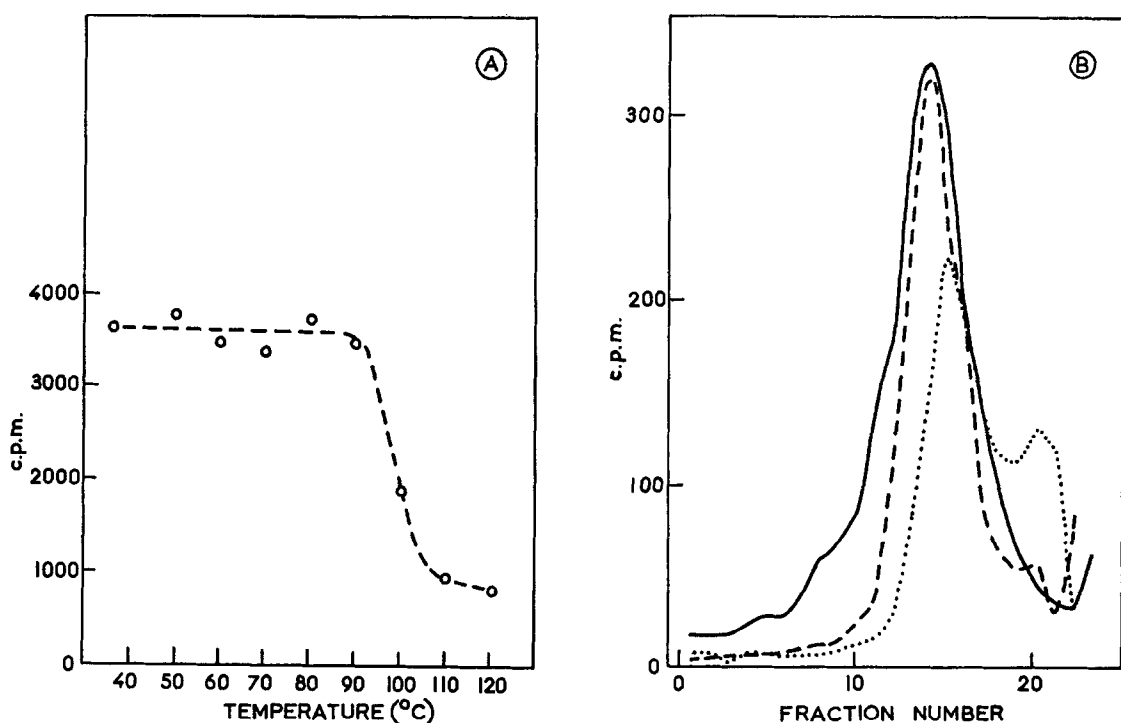


Figure 3A. Acid-insoluble radioactivity of the 0.2 M fraction of AMV-RNA after heating for 5 minutes in sealed tubes at the temperatures indicated in the abscissa, rapid cooling, and digestion with pancreatic RNase. 0.4 µg samples in 0.1 ml SSC were used.

B. Sedimentation of the 0.2 M fraction of AMV-RNA in a sucrose density gradient (5 % to 20 % in SSC). Centrifugation was performed for 17 hours at 25,000 r.p.m. at 5° in a SW-25 rotor in a Spinco model L2 centrifuge. Fractions were precipitated by adding 10 % cold TCA (1.2 ml) and 50 µg bovine serum albumin. Radioactivity was measured after collecting the precipitates on Millipore filters.

The solid line, the dashed line and the dotted line indicate the sedimentation patterns of the 0.2 M fraction before and after treatment with 0.1 µg/ml or 50 µg/ml of RNase, respectively.

Table II shows reannealing experiments carried out with different amounts of AMV RNA ("plus" strand) and with TYMV RNA. The results obtained indicate that up to 44 % of the 0.2 M fraction reannealed with the "plus" strand of AMV RNA, but no reannealing was obtained with heterologous RNA.

Figure 3A shows the melting curve of the 0.2 M fraction as determined by pancreatic RNase digestion, after heating at several temperatures.

When centrifuged in a sucrose density gradient, the 0.2 M RNA fraction sedimented as a band having a sedimentation coefficient close to 15 S (fig. 3B) and showing a heavy shoulder. DNase treatment did not affect this sedimentation pattern.

DISCUSSION

The chromatographic results show that HA columns can easily separate an RNA fraction representing about 0.4 % of total RNA from infected plants and endowed with a different secondary structure.

This RNA fraction can be identified as the replicative RNA of AMV since : a) it can hybridize with homologous "plus" strands, but not with heterologous RNA ; b) it has a double stranded structure, as shown by its melting curve, electron microscopy (work to be published) and elution molarity from HA columns (this is the same as that of double stranded DNA) ; c) it is highly, yet not totally, resistant to pancreatic RNase ; d) it shows a heterogeneous sedimentation peak with a heavy shoulder containing material very sensitive to a mild RNase treatment.

These results suggest that the replicative RNA of AMV are formed by double stranded RNA ; a fraction of these molecules have parts sensitive to mild RNase treatment, possibly single-stranded chains. This is compatible with the HA results, since when double and single-stranded structures coexist in the same molecules, the elution molarity is that of the double stranded structures (Bernardi, 1968 ; Chevalier and Bernardi, 1968).

The heterogeneous nature of the replicative RNA of AMV is not surprising if one considers the extensive labeling of RNA and the asynchronous nature of viral infection in plant cells.

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