REPLICATIVE FORM OF PLANT VIRAL RNA

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The replication of animal and bacterial viruses which contain RNA does not depend on DNA but appears to involve a double-stranded RNA (Montagnier and Saunders 1963, Weissmann and Borst 1963, Baltimore et al 1963, Weissmann et al 1964, Kaener and Hoffmann-Berling 1964). There is evidence to suggest that DNA is not involved in the synthesis of tobacco mosaic virus (TMV) RNA (Ralph and Matthews 1963; Sänger and Knight, 1963). We report evidence for the existence of double-stranded RNA in plants infected with TMV and turnip yellow mosaic virus (TYMV).

In a preliminary examination of the RNA species formed during TYMV multiplication, floated leaf discs from infected chinese cabbage were labelled with cytidine-2-^{14}C for 45 mins. prior to isolation of the RNA by the method of Ralph and Bellamy (1964). RNA was fractionated on linear 5-20% sucrose gradients, at 35,000 revs/min for 5 hours at 4° in a Spinco SW39 rotor. After 4-6 days infection, increased radioactivity compared with RNA from healthy leaf appeared in the 8-16S region. Similar results were obtained using ^{32}P as label. RNA from infected plants showed ribonuclease resistant material in the 8-16S region that was absent in RNA from healthy leaf. A small fraction of the radioactivity in this

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region resisted alkaline digestion. (Fig. 1).

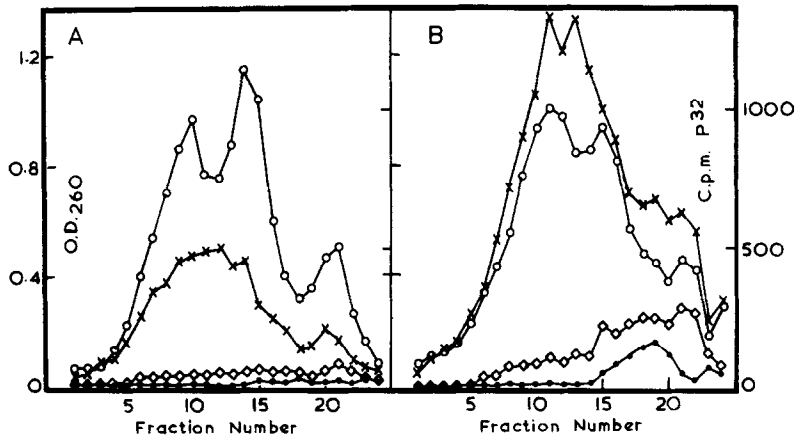

Fig. 1. Sucrose density gradient fractionation of RNA from healthy chinese cabbage leaf (A) and from systemically infected leaf from plants infected for 13 days with TYMV (B). Leaf discs labelled for 45 mins with ^{32}phosphate. 0 —— 0 = OD_{260}; X —— X = total cts/min ^{32}P; ◇ —— ◇ = cts/min ^{32}P resistant to ribonuclease; ● —— ● = cts/min ^{32}P stable to 1N KOH.

To obtain more effective separation of these fractions methylated bovine serum albumin (MAK) columns were employed (Sueoka and Cheng 1962). Linear salt gradients from 0.4-1.0M NaCl containing 0.01M tris-HCl pH 8.5 buffer were used, with a total eluent volume of 220 ml.(columns 7.0 x 1.5 cm diam.). Fig. 2A shows a typical fractionation of RNA from ^{32}P-labelled TYMV infected leaves. The peak of optical density and radioactivity eluted at about 0.75M NaCl contains TYMV RNA (Matus et al 1964). The sharp peak of radioactivity eluting at 0.62M NaCl was resistant to ribonuclease action. (Pancreatic ribonuclease at 5 µg/ml in 0.14M NaCl, 0.014M sodium citrate pH 7.0, and 0.01M $MgCl_2$ at 26° for 30 mins.).

(Fig. 2B). Some radioactivity was also present in the DNA peak eluted at 0.58M salt. The DNA was identified by its susceptibility to deoxyribonuclease and by its banding in Cs_2SO_4 gradients at a density of 1.42 gms/ml. In nucleic acids from healthy leaf there was no radioactive ribonuclease-

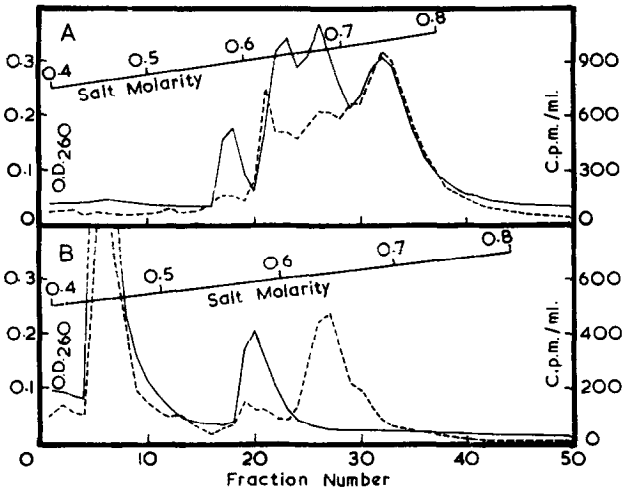

Fig. 2. Fractionation on MAK columns of RNA from leaves systemically infected with TYMV, harvested 17 days after inoculation, and labelled through the roots with P^{32} for 90 minutes. A. Untreated nucleic acids. B. Treated with ribonuclease. —— = OD_{260}; --- = cts/min.

resistant component eluting at 0.62M NaCl and negligible labelling in the DNA.

Samples of the ribonuclease-resistant material eluting at 0.62M NaCl, together with 4.0 μg of marker RNA were centrifuged in Cs_2SO_4 for 72 hrs at 30,000 rpm in a Spinco SW39 rotor at 20°. The ribonuclease-resistant material banded with a peak at a density = 1.64 gms/ml. Marker TYMV-RNA formed a visible layer of precipitated material at a density = 1.65 gms/ml.

Base analyses (Markham 1955) were made on alkaline

digests of the ribonuclease-resistant material eluting at 0.62M NaCl. (Table 1). About 95% of the radioactivity was recovered in the region of the four mononucleotides.

The base composition after 30 mins. labelling was like TYMV RNA. After 4 days it was close to that calculated for a TYMV duplex, while at 90 mins. an intermediate composition was found. A similar trend in the observed base ratio with time of labelling was found using inoculated leaves. The low uridylic acid and high guanylic acid figures are probably due to trailing during electrophoresis (Matthews 1960).

Table 1. Base ratios of ribonuclease-resistant RNA from systemically infected chinese cabbage leaves in which TYMV was increasing rapidly. Base ratios determined from radioactivity in nucleotides.

	^{32}P labelling time			TYMV RNA*	Calculated for TYMV duplex
	30 mins.	90 mins.	4 days		
C	37.7	33.4	28.2	38.0	27.5
G	21.2	23.7	29.5	17.0	27.5
A	23.3	21.0	21.1	23.0	22.5
U	18.1	22.5	21.1	22.0	22.5

* From Markham and Smith (1951)

The following facts rule out the possibility that the ribonuclease-resistant material is a DNA-RNA hybrid:- (i) 95% of the radioactivity after digestion with alkali has the chromatographic and electrophoretic properties of ribomononucleotides, (ii) at longer times of labelling the base ratios are close to those expected for a duplex containing one TYMV-RNA strand and its base-paired complement, (iii) the intact material bands at 1.64 gms/ml in Cs_2SO_4. We therefore conclude that we are dealing with the replicative form of TYMV-RNA.

The unusually high cytidylic acid content of TYMV-RNA offers an opportunity to study the kinetics of viral RNA replication. At short times of labelling the apparent base composition of the duplex, based on radioactivity is that of TYMV (Table 1). This suggests the TYMV-RNA strand is undergoing replication much more rapidly than its complement. This also implies that as a new viral RNA strand is formed it displaces the pre-existing viral RNA from the duplex. This mechanism would be consistent with the assymetric, semi-conservative mechanism suggested by Weissmann et al (1964) for MS2 virus RNA.

Our data on the bouyant density of double-stranded RNA from TYMV together with that published for EMC virus, (Montagnier and Saunders 1963) fr virus (Kaener and Hoffmann-Berling 1964) and MS2 virus (Weissmann et al 1964) show a near linear relationship between density and G + C content. They also show that the bouyant density of double-stranded RNA in Cs_2SO_4 is much more markedly dependent on G + C content than is DNA. The data for the double-stranded RNA of polio virus (Baltimore et al 1963) do not fit this relationship, suggesting either that the polio replicative form has unusual properties or that some artifact was involved in the density determination. (Konrad and Stent 1964).

When nucleic acids from ^{32}P labelled TMV-infected tobacco leaves were fractionated on MAK columns a peak of radioactivity was eluted at about 0.62M NaCl. This material was also resistant to RNAase, and was absent from healthy leaf, suggesting that a double-stranded replicative RNA may be general for plant viruses. With this system however no

consistent difference in DNA labelling was found between healthy and infected leaf.

The significance of the increased labelling of DNA during TYMV infection remains to be determined. It may merely be an indirect effect on DNA synthesis, through stimulation of polyploidy or through some pathological effect on chloroplasts leading to replication of chloroplast DNA.

Tests on the infectivity of the double-stranded RNA from both TMV and TYMV infected plants have so far given equivocal results because of the small amounts of material available.

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References

Baltimore, D., Becker, Y., and Darnell, J.E., Science 143 1034 (1964).
Kaener, H.C., and Hoffmann-Berling, H., Nature 202, 1012 (1964).
Konrad, M.W. and Stent, G.S., Proc. Natl. Acad. Sci. 51, 647 (1964).
Markham, R., In "Modern Methods of Plant Analysis" (K. Paech and M.V. Tracey, Eds.) IV, 246, Springer, Berlin (1955).
Markham, R., and Smith, J.D., Biochem. J., 49, 401 (1951)
Matthews, R.E.F., Virology 12, 521, (1960).
Matus, A., Ralph, R.K., and Mandel, H.G. In preparation.
Montagnier, L., and Saunders, F.K., Nature 199, 664 (1963).
Ralph, R.K., and Bellamy, A.R., Biochem. Biophys. Acta 87, 19 (1964).
Ralph, R.K., and Matthews, R.E.F., Biochem. Biophys. Res. Comm. 12, 287 (1963).
Sänger, H.L., and Knight, C.A., Biochem. Biophys. Res. Comm. 13, 455 (1963).
Sueoka, N., and Cheng, T., J. Mol. Biol., 4, 161 (1962).
Weissmann, C., and Borst, P., Science 142, 1188 (1963).
Weissmann, C., Borst, P., Burdon, R.H., Billeter, M.A., and Ochoa, S., Proc. Natl. Acad. Sci. 51, 682 (1964).