Electron Microscopy of Replicative Form and Single-stranded RNA of Alfalfa Mosaic Virus

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SUMMARY

Double stranded RNAs obtained from plants infected with alfalfa mosaic virus have been studied by electron microscopy using the Kleinschmidt technique. Two characteristic lengths were obtained. A change of length was observed when the two strands were separated with dimethylsulphoxide. This corresponded to a change of configuration from the A form in double-stranded RNA to the B form in single-stranded RNA.

Using dimethylsulphoxide it was possible to visualize single-stranded RNA molecules as elongated filaments.

The electron microscopic results are compared with reported mol. wt values for the different RNAs present in alfalfa mosaic virus.

INTRODUCTION

In previous work, we have used the protein monolayer technique to determine the length in the electron microscope of double-stranded RNA of the replicative form of tobacco mosaic virus (TMV) (Nicolaieff et al. 1970). Since the number of nucleotides in the RNA molecules of TMV is known (Klug & Caspar, 1960), it was possible to determine the distance between nucleotides and therefore the mol. wt of the double stranded (DS) RNA molecules. The percentage of complete DS RNA molecules observed with TMV was not high enough to give a clear-cut determination of the length of the complete molecule. In the present communication, we have studied the DS RNA induced by alfalfa mosaic virus (AMV). This DS RNA shows two characteristic lengths in the electron microscope which are present in greater amounts than with TMV DS RNA (Nicolaieff, Pinck & Hirth, 1969).

In the present work, we used a new approach to confirm that DS RNA has a compact configuration when examined in the electron microscope by the protein monolayer technique. The change in length obtained after separation of the two strands of DS RNA and the increase in length observed when decreasing the molarity of the hypophase were studied in more detail.

A study of the DS RNA induced by AMV is also of interest in view of the different RNAs present in this virus. The values of the mol. wt obtained by different authors are shown in Table 1. We attempt to correlate the lengths of the DS RNA molecules with those of the virus RNA species.

Another aspect of the electron microscopic studies reported in this communication concerns single-stranded (SS) RNA molecules. We describe a method, using dimethylsulphoxide
(DMSO), which permits the visualization of the SS RNA molecules extracted from AMV. These molecules are obtained as elongated filaments when the protein monolayer spreading technique is used. The contrast of the molecules on electron micrographs is sufficiently high to allow a histogram of the length distribution to be made.

**METHODS**

Details of the techniques used have already been described (Pinck, Hirth & Bernadi, 1968; Pinck, 1969; Nicolaïeff, 1969; Nicolaïeff et al. 1969; Pinck, 1970; Nicolaïeff et al. 1970).

**Virus infection.** AMV (the strain used is described in Pinck, 1969) was inoculated on *Nicotiana tabacum*, var. Xanthin. The plants were grown in a greenhouse under artificial illumination of 1500 lux., mean temperature of 22°C.

**RNA extraction.** Total RNA was obtained after phenol extraction of fresh leaf material in the presence of bentonite and in 0.1 M-glycine buffer, 0.01 M-EDTA, 0.1 M-NaCl, and 1% sodium dodecyl sulphate at pH 9.5. The extraction was made after 5 to 7 days of infection with AMV. After ethanol precipitation, this RNA was dissolved in 0.02 M-Tris, 0.005 M-MgCl₂ at pH 7.4 and treated with DNase free of RNase (Worthington) at a concentration of 20 μg./ml. for 30 min. at 37°C (Pinck, 1969).

**Isolation of the DS RNA.** DS RNA was separated from SS RNA without the use of RNase on hydroxylapatite columns with a phosphate buffer (Pinck et al. 1968; Pinck, 1970). Several types of control were done to check that no DNA was present in our preparations; it was found that total RNA from healthy plants contained no DS material when eluted in 0.2 M-phosphate buffer from the hydroxylapatite column (Pinck, 1970) and that the profile of [³²P]-labelled samples obtained by sedimentation on a linear sucrose gradient was not modified when this sample was treated with DNase before centrifuging (Pinck et al. 1968).

**Preparation of DS RNA for electron microscopy.** The technique used has been described in detail elsewhere (Nicolaïeff, 1969; Nicolaïeff et al. 1969, 1970). Briefly, 0.3 ml. of a solution containing the replicative form at 1 μg./ml. in 1 M-ammonium acetate and cytochrome C at 100 μg./ml. was spread with a microsyringe along a glass rod on a 0.2 M-ammonium acetate hypophase following the method of Kleinschmidt & Zahn (1959). The film of protein was compressed and parts of it were picked up on platinum grids covered with a formvar carbon film. The grids were rotated during shadowing with platinum.

**Preparation of dissociated DS RNA samples for electron microscopy.** The DS RNA solution in 0.2 M-phosphate buffer was diluted ten times with double distilled water containing 0.001 M-EDTA to a final concentration of 5 to 10 μg./ml. To 0.1 ml. of the diluted solution, 0.9 ml. of DMSO was added and, before spreading, 100 μg. of cytochrome C in 0.05 ml. of water was added to the DMSO solution of the DS RNA. The protein-RNA film was treated as with DS RNA.

**Preparation of RNAs from AMV.** AMV RNAs were extracted by the phenol procedure. The virus, at a concentration of about 10 mg./ml. in 0.01 M-phosphate buffer pH 7.0 was mixed with bentonite (1/10th the weight of virus), 0.5% SDS at final concentration, and two vol. saturated phenol. The resulting aqueous layer was again treated twice with phenol. The aqueous layer was then washed twice with ether before precipitation of the RNA with two vol. of cold ethanol.

**Preparation of SS RNA for electron microscopy.** DMSO was used to obtain SS RNA molecules as single elongated chains and EDTA was added to prevent aggregation of SS RNA molecules with divalent ions.
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Fig. 1. Histogram of the length distribution of the DS RNA induced by AMV. The characteristic lengths of 0.55 and 0.95 μm are evident.

The RNA of AMV was diluted in a solution containing phosphate buffer 0.01 M-pH 7 and 0.001 M-EDTA to a final concentration of 5 to 10 μg./ml. To 0.1 ml. of this solution, 0.9 ml. of DMSO was added. 0.5 ml. of the DMSO solution containing the virus RNA was mixed with 0.5 ml. of a solution containing 100 μg./ml. of cytochrome C and 90% DMSO.

The presence of 90% DMSO in the solution of cytochrome C makes it possible to obtain a film, on the air-hypophase interface, corresponding to a completely denatured protein (1 m.²/mg) (Trurnit, 1960). If the RNA solution in DMSO is diluted with cytochrome C in water, the film of denatured protein may be somewhat smaller than expected. The extent of denaturation of cytochrome C on the interface air-hypophase was also found to vary with the batch of cytochrome used.

The spreading of the solution containing RNA and cytochrome in 90% DMSO was made on an hypophase of distilled water. The protein film was picked up on formvar coated grids and treated in the same way as the DS RNA protein film.

RESULTS

Characteristic lengths of replicative RNA

The presence of two characteristic lengths was reported in a preliminary publication (Nicolaieff et al. 1969). Fig. 1 gives the histogram of lengths in a preparation which contained about 13% of molecules in the 0.95 μm. peak, the longest replicative form molecules. The presence of three main lengths is clearly seen. A statistical analysis indicated that these peaks differed significantly at the 95% level. By analogy with previous results (Nicolaieff et al. 1969), the peak observed around 0.2 μm. probably corresponds to degraded material. The two small peaks at 0.32 and 0.7 μm. have been found in different experiments and may be real.

The presence of these characteristic lengths is more clearly seen when studying a fraction
obtained by sucrose gradient sedimentation. The profile of the sucrose gradient of the preparation used is given in Fig. 2 and is similar to the one of DS RNA induced by TMV (Nicolaieff et al. 1970). The arrow points to the fraction studied by electron microscopy. This fraction contained the largest amount of DS RNA (15% by weight of the sample). Since it is in the middle of the profile, one would expect that short molecules as well as long ones would have been eliminated. In fact, we found a polydisperse length distribution containing all the lengths present in the initial sample but only with less short molecules and more long molecules (Fig. 3a) than in the original sample (Fig. 3b).

**Presence of circular DS RNA molecules**

Fig. 4 shows some of the circular molecules which were observed in preparations of DS RNA from AMV infected plants. Since the purification technique destroys all DS DNA (Pinck et al. 1968; Pinck, 1970), these molecules represent DS RNA. The percentage of these molecules is small, about 1 circle being observed for every 100 molecules, and they are not present in all preparations of DS RNA. The circles are of uniform thickness and appearance. They are not twisted which indicates that one or more breaks probably occur along their lengths. Moreover, the length of the circles is not random but corresponds to the values of the different peaks in the histogram of the linear molecules (see legend of Fig. 4). We tentatively regard these molecules as representing a configuration necessary for the replication of the virus RNAs of AMV, although it cannot be excluded that circularization is the result of cohesive ends due to repetitive sequences in the RNA molecules.

**Effect of DMSO on the stability of DS RNA induced by AMV**

DMSO can separate the two strands of DS RNA of AMV (Nicolaieff et al. 1970). However, we subsequently observed that the dissociation of the two strands is not always
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complete. Fig. 5 shows an electron micrograph of DS RNA and SS RNA obtained after action of DMSO in which some DS RNA molecules are easily recognized by their thickness. Other samples have been found to be more resistant to the action of DMSO. In some instances even when the solution of DS RNA in 90% DMSO was heated to 100° for 10 min., some DS molecules remained and formed aggregates as shown in Fig. 6a and b. Thus, some DS RNA appears to have a stable configuration after extraction; it is also possible that an impurity prevents the complete separation of the two strands. The same observation was made by Pinck using polyacrylamide gel electrophoresis (L. Pinck, unpublished results) and by Romanova et al. (1970) on the RF of encephalomyocarditis virus.

Changes in the distance between the nucleotides along the DS RNA induced by AMV

Fig. 7a shows the histogram of the length distribution of another sample of DS RNA induced by AMV. The 0.95 μm. peak corresponds to 7 % of the measured sample. The same sample, when spread on a double distilled water hypophase instead of the usual 0.2 M-ammonium acetate hypophase, had the length distribution shown in Fig. 7b. There is a shift of the 0.95 μm. peak to a length of 1.1 μm. but the percentage of this peak remains the same: 7.5 % of the measured sample. We observed an analogous shift of the length of the 0.5 μm.
peak (see Fig. 7a, b) in which the peak was displaced by 10%. The 0·5 μm. peak represents 51% in Fig. 7a and 45·5% in Fig. 7b.

When the same sample was heated with DMSO, the length distribution of SS and DS molecules was that shown in Fig. 7c and d, respectively. Fig. 7c shows a significant decrease of the 0·5 μm. population and a further elongation of the 0·95 μm. class (10% of the measured population) to a mean length of 1·25 μm. The overall length diminution suggests that there are some breaks in the two strands which are revealed when the two strands are separated. Fig. 7d gives the histogram of the length distribution of the DS RNA molecules not dissociated after treatment with DMSO. The profile of the histogram is similar to that of the initial sample with a first maximum corresponding to fragments of the DS molecules, a second peak at 0·5 μm. and long molecules up to a length of 1·3 μm. It is unlikely that the
FIG. 5. Electron micrograph of a DS RNA induced by AMV and diluted to a final concentration of 90% of DMSO and kept at room temperature. SS RNA molecules are clearly distinguished from DS RNA molecules.

observed elongation of the DS molecules is caused by end-to-end aggregation, since DMSO prevents the formation of hydrogen bonds and the presence of EDTA complexes divalent cations.

Length distribution of the single-stranded virus RNA of AMV

The use of EDTA and DMSO which prevents the action of divalent ions at appropriate pH and breaks hydrogen bonds respectively gives solutions of SS RNA molecules without secondary structure. The spreading of these solutions using the protein monolayer technique permits the visualization under the electron microscope of elongated filaments. Fig. 8 shows a typical contrast electron micrograph of the SS RNA of AMV. The molecules are seen as thin filaments of uniform thickness, most of them are well separated and easy to measure,
Fig. 6. (a) Electron micrograph of DS RNA induced by AMV, diluted to a final concentration of 90% of DMSO and heated at 60°C for 10 min. DS RNA molecules are still present after this treatment although some of them are aggregated. (b) Electron micrograph of the same sample but heated at 100°C for 10 min. Some DS RNA molecules are still present.
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Fig. 7. Histograms of (a) Length distribution of a sample of DS RNA induced by AMV before action of DMSO, spread on 0.2 M-ammonium acetate. (b) Length distribution of DS RNA spread on distilled water. There is a displacement of the maximum length of 0.8-1 µm. to 1.1 µm. (c) Length distribution of the SS RNA molecules obtained after separation of the two strands with DMSO. The maximum length is displaced from 0.8-1 µm. to 1.1-1.3 µm. (d) Length distribution of the DS RNA molecules which were not separated by DMSO treatment.

but a few are tangled and sometimes cannot be measured as also mentioned by Freifelder & Kleinschmidt (1965) for the filaments of SS DNA of coliphage T7.

Fig. 9 shows the histogram of the length distribution of a typical sample of RNA of AMV. The profile of the histogram decreases down to 1.4 µm. after a maximum at 0.2 to 0.3 µm. The arrows refer to calculated lengths expected for SS RNA on a basis of DS RNA lengths (see Discussion).

DISCUSSION

The DS RNA induced by TMV in plants has a maximal length of 1.75 µm. (Nicolaieff et al. 1970). Since a complete RNA molecule of TMV has 6600 nucleotides (Klug & Caspar, 1969) the distance between nucleotides was calculated as 2.65 Å corresponding to the A form of nucleic acid established crystallographically (Arnott et al. 1968). On the other hand, our corresponding measurements of the length of SS RNA molecules of TMV indicate that these
Fig. 8. Electron micrograph of SS RNA molecules extracted from AMV particles. The contrast is sufficient to allow length measurements.
molecules have a mean length of 2.13 μm. It follows that the distance between nucleotides along the SS RNA molecules is 3.3 Å. This value is in good agreement with base stacking along the nucleotide chains occurring perpendicular to the axis of the chain; i.e. the crystallographic structure of type B nucleic acids. The approach used in the experiments reported here was based on the change of length observed when DS molecules become SS ones and the distance between nucleotides was related to the change of structure of the DS RNA. The length measured after the DS RNA of AMV has been dissociated to SS molecules showed an increase from 0.95 μm. to 1.24 μm., in good agreement with a change of configuration from type A to type B structure.

The change of length observed in DS RNA molecules when they are spread on distilled water instead of 0.2 M-ammonium acetate (Fig. 7a, b) is in agreement with a change in the A configuration, from 11 base pairs per turn of the helix with a pitch of 29 Å, to 12 base pairs per turn and a pitch of the helix of 36 Å; this configuration corresponds to the A' form of DS RNA proposed by Arnott et al. (1968). This intermediate configuration corresponding to a distance between nucleotides of the DS RNA of 3 Å was also observed in other
electron microscopic studies of DS RNA (Granboulan & Franklin, 1968; Wolstenholme & Bockstahler, 1967).

A precise knowledge of the configuration of the DS RNA and derived SS RNA as obtained under our experimental conditions permits a direct determination of the mol. wt of both SS and DS RNA molecules.

The 0.95 μm. class of DS RNA molecules induced by AMV and the corresponding SS RNA molecule of 1.24 μm. represent a polynucleotide chain of about 3750 nucleotides and mol. wt of 1.24 x 10^6. This mol. wt is in good agreement with experimental values for that of the virus RNA of AMV corresponding to the bottom component (see Table 1).

The other characteristic length of DS RNA corresponding to 0.50 to 0.55 μm. corresponds to a virus RNA of about 2000 nucleotides and mol. wt of 0.7 x 10^6, in agreement with the values of the virus RNA of the top component of AMV (see Table 1).

The use of DMSO permits the study of SS RNA as a population of elongated filaments. The length distribution of the SS RNA is clearly different from that of DS RNA of AMV. The DS RNA length distribution shows different characteristic lengths but in the virus RNA length distribution (compare Fig. 1 and 9) the characteristic SS RNA species are not clearly evident. From the change of length observed when going from the DS to the SS form, one would expect the two peaks at 0.95 μm. and 0.5 μm. in Fig. 1 to have corresponding peaks at the positions indicated by arrows in Fig. 9. Thus, the shoulder observed in Fig. 9 at a length of 0.6 to 0.7 μm. and the plateau observed at 1.2 to 1.4 μm., which represents 16/659 molecules or 7% by weight of the measured sample, may be significant. Some long molecules escape measurement because of tangling: the 7% is a lower limit.

Fragmentation of the SS RNA molecules during spreading by the protein monolayer technique is unlikely: with SS RNA molecules extracted from nuclei of eukaryotes, we have observed molecules longer than 5 μm. with the same technique. We have also shown that the percentage of complete TMV particles (3000 Å) in a virus preparation and the percentage of non-fragmented RNA molecules obtained therefrom were identical (Oudet, Lebeurier & Nicolaëff, 1970). Furthermore, enzymic fragmentation of the SS RNA is unlikely because DMSO inhibits RNase. Appelbaum, Epstein & Wyatt (1966) assayed the action of bovine pancreatic RNase on yeast RNA at different DMSO concentrations and reported that under conditions in which the enzyme was highly active (in aqueous buffer), 40% DMSO gave 90% inhibition and 60% DMSO apparently gave complete inhibition. Strauss, Kelly & Sinsheimer (1968) also indicated that RNase is inactive in DMSO as do our unpublished data.

The histogram of the length distribution of the SS RNA from AMV particles suggests the presence of hidden breaks in the SS RNA molecules probably resulting from damage incurred during encapsidation. This idea is reasonable because AMV loses specific infectivity even while the virus is multiplying rapidly (Kuhn & Bancroft, 1961). Hidden breaks in superficially intact RNA was first clearly demonstrated for turnip yellow mosaic virus RNA (Haselkorn, 1962) and confirmed later by Hirth, Horn & Strazielle (1965); they have recently been observed by various means by Boedtker (1968; 1971) for TMV RNA.

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REFERENCES


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