

STUDIES ON THE STRUCTURE OF AVIAN MYELOBLASTOSIS VIRUS (AMV) RNA. I. FACTORS AFFECTING ELECTRON MICROSCOPIC VISUALIZATION OF AMV RNA

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Summary. — Factors affecting the visualization of single-stranded (ss) avian myeloblastosis virus (AMV) RNA by the basic protein film technique under strong denaturation conditions were studied. The basic parameters of the electron microscopic picture of ss RNA, involving the visibility, concentration and contour lengths of molecules, were found to be significantly dependent on the (1) quality of denaturing components used for full extension of AMV RNA molecules and (2) spreading and formation of the surface film. Under the conditions used, the presence of artificial secondary structures on AMV RNA molecules was dependent on batches of formamide and urea in the denaturing mixture. The spreading and formation of the surface film were affected by hydrophilicity of glass spreading ramps, speed of spreading, amount of spreading solution, amount of protein in the hyperphase, type of cytochrome c and the presence of substances influencing surface tension. The optimal conditions and spreading arrangement for visualization of ss AMV RNA are described.

Key words: avian myeloblastosis virus RNA; electron microscopy basic protein spreading technique; strong denaturation conditions

Introduction

Electron microscopy represents a suitable method for determining the contour lengths, molecular weights and strandedness of nucleic acids. It is independent of the hydrodynamic properties of the nucleic acids, but many factors can affect linear densities, structural features and the contrast of nucleic acid molecules during the preparation of samples for electron microscopy (Brack and Delius, 1980; Dubochet and Groom, 1981). In some cases, the degradation, particularly of single-stranded (ss) nucleic acids, can occur. The length variations in double-stranded (ds) molecules, depending on spreading conditions, were observed with some bacteriophage DNAs (Inman, 1967; Lang *et al.*, 1967). From this point of view, the ss nucleic acids are much more sensitive to the spreading conditions used than the ds ones. The

dependence of linear densities of ss nucleic acids on different denaturing and spreading conditions was reported elsewhere (Korb *et al.*, 1981).

Electron microscopy of ss RNA from retroviruses has been described in many reports (Granboulan *et al.*, 1966; Kakefuda and Bader, 1969; Sarkar and Moore, 1970; Whalley, 1973; Weber *et al.*, 1974, 1975; Chi and Bassel, 1975; Heine *et al.*, 1975; Jacobson and Bromley, 1975; Kung *et al.*, 1975, 1976, Bender *et al.*, 1978; Murti *et al.*, 1981), but the contour lengths reported for viral RNA vary from 1.6 to 14 μm . Substantial length variations were observed even under the strongly denaturing conditions (Weber *et al.*, 1974; Chi and Bassel, 1975; Heine *et al.*, 1975; Jacobson and Bromley, 1975). In addition, the RNA of avian retroviruses reveals a heterogeneous population of molecules with preferential size classes when analyzed by electron microscopy or sedimentation (Chi and Bassel, 1975; Heine *et al.*, 1975; Korb and Štokrová, 1980).

We are reporting the investigations of factors affecting the contour lengths and structural features of AMV RNA during the preparation of samples for electron microscopy by the protein monolayer technique. We selected the protein technique under denaturing conditions (Robberson *et al.*, 1971) which yields full-extended ss molecules under an optimal arrangement. The presence of cytochrome c protects the fragile ss RNA from mechanical degradation (Naora and Fry, 1977) better than the nonprotein techniques (Vollenweider, 1975).

As mentioned above, various factors can influence the contrast, degree of degradation and extension of ss RNA molecules in the protein film. With some experience, using defined RNA preparations and method of visualization, the relatively small variations in contrast and degradation can be excluded. The most important factors are those which affect the contour length and structural features of ss RNA, i. e. reproducible extension of molecules resulting in a constant base-repeat distance. We showed that the contour length and structural behaviour of AMV RNA under the strongly denaturing conditions and protein monolayer spreading depend on the batch of the denaturing components, the arrangement of spreading and the presence of surface-active substances.

Materials and Methods

Reagents. Formamide 99% from Matheson, Coleman and Bell with a conductivity of 210 μS and 99.5% formamide (Loba-Chemie, Wien) with a conductivity of 170 μS were used. Preparations of cytochrome c were purchased from Calbiochem (lyophilized, crystallized twice from *Candida krusei*), Sigma (Type VI and II A from horse heart) and Boehringer (from horse heart). Urea (ultra pure) was obtained from Schwarz) Mann.

Virus and RNA preparations. The avian myeloblastosis virus (AMV) was purified and pelleted from 150 ml of blood plasma of leukaemic chickens (White Leghorn) as described (Korb and Heine, 1978). The virus pellet was resuspended in TN buffer (0.1 mol/l NaCl, 0.05 mol/l TRIS, pH 8.0), and sodium dodecyl sulfate (SDS) was added to a final concentration of 1–2% together with about 10 μl of diethyl-pyrocabonate (DEPC). This solution was extracted three times with equal volumes of phenol saturated with TN buffer, pH 8. The total RNA was precipitated from the combined aqueous phases with 2.5 volumes of ethanol. Phenol-extracted RNA was fractionated in 10–30% sucrose gradient made in TNE buffer (0.1 mol/l TRIS and

0.001 mol/l EDTA, pH 7.5) in the presence of 0.05% SDS. Gradient centrifugation was carried out in a Spinco SW 41 rotor at 38 000 rev/min for 130 min at 20° C. Fractions were analyzed for A₂₆₀ and the material sedimenting at 60-70 S was pooled, precipitated with ethanol, resuspended in TN buffer, pH 8.0, and prepared for electron microscopy. Φ X 174 DNA was kindly supplied by Dr. U. Heine National Institutes of Health, Bethesda, Maryland, U. S. A.

Electron microscopy. High-molecular-weight AMV RNA was prepared for electron microscopy by the basic protein film technique (Kleinschmidt and Zahn, 1959) modified for strongly denaturing conditions (Robberson *et al.*, 1971). One microliter of RNA (20 µg/ml) in TN buffer, pH 8.0, was added to 100 µl of the 3.9 mol/l urea — 78% formamide mixture and heated at 53° C for 30 sec. The solution was chilled on ice and then mixed with 2 µl of cytochrome c solution (1.5 mg/ml) in 1.5 mol/l TRIS and 50 mmol/l EDTA, pH 8.5. This hyperphase was spread on to a hypophase containing deionized or distilled water (conductivity from 0.36 to 1.00 µS) or 50 % formamide in TE buffer, pH 8.5 (0.01 mol/l TRIS, 0.001 mol/l EDTA). The cytochrome films were picked up on grids coated with parlodion and stained with uranyl acetate (Davis and Davidson, 1968; Davis *et al.*, 1971) and rotary-shadowed at an angle of 7° with Pt/Pd. Preparations of AMV RNA were examined in a Jeol 100 B electron microscope at a magnification of 10 000 using a high voltage setting of 60 kV. Length measurements were made on enlarged (8-fold) photographs using a Hewlett-Packard 9864 A Digitizer equipped with a Hewlett-Packard 9830 calculator.

Results

Effect of the denaturing components on visualization of AMV RNA

The various denaturing agents can affect the hypophase surface tension and therefore the spreading of the protein film. The changes in AMV RNA length distribution, depending on different batches of formamide and urea, were first examined.

Formamide. The length distributions of viral RNA varied to a great extent with different batches of formamide. The length distributions of AMV RNA of following the use of MCB or Loba-Chemie formamides are compared shown in Fig. 1. Under optimal conditions, in the presence of cytochrome c Type VI from Sigma in MCB formamide spreading (Fig. 1-I), the distribution profile of AMV RNA revealed five size classes with the mean lengths of 0.46 ± 0.074 , 0.75 ± 0.076 , 1.03 ± 0.066 , 1.38 ± 0.091 and 1.76 ± 0.105 µm, into which fell 15, 22, 20, 29, and 11 % of the molecules, respectively. The ss RNA molecules were fully extended without visible secondary structures (Fig. 2-I). In the presence of Loba-Chemie formamide nonspecifically localized secondary structures were seen (Fig. 2-II) and the mean length of molecules in the highest size class was only 1.39 ± 0.069 µm involving only 5 % of the molecules. Over 50 % of the molecules fell into size classes from 0.2 to 0.7 µm (Fig. 1-II).

Urea as a component of the denaturing mixture affected not only the extension of RNA molecules but also their visibility. Best results were obtained with ultra pure urea from Schwartz/Mann (USA). The use of another type of urea, even purified by crystallization, led to bad visibility and formation of artificial secondary structures on RNA molecules in the protein film (not shown).

Spreading and formation of surface film

Glass slides. Appropriate and reproducible spreading was markedly affected by the hydrophilicity of the glass slides used as ramps. We used diffe-

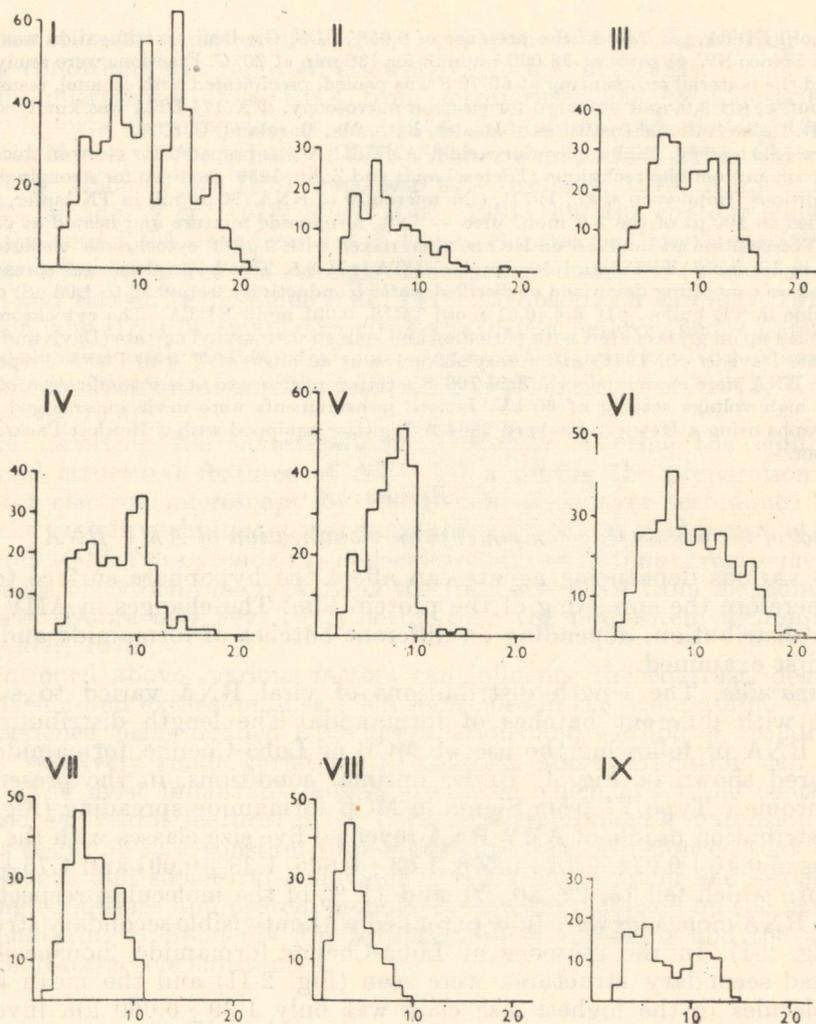


Fig. 1.

Histogram showing examples of the 60-70 S AMV RNA length distributions observed with various arrangements of strong denaturation spreading conditions

I — Optimal conditions:

Reagents: ultra pure urea (Schwartz/Mann), formamide (MCB), cytochrome c Type VI from horse heart (Sigma)

Spreading of 50 μ l hyperphase was carried out by an Eppendorf pipette over a glass slide cleaned with HCl-HNO₃, onto an area of 30 cm² of deionized water as a hypophase

II — Loba-Chemie formamide

III — glass slide with rough surface

IV — glass pipette with 0.65 outlet diameter

V — glass pipette with 0.40 mm outlet diameter

VI — cytochrome c from *Candida krusei* (Calbiochem)

rent techniques of surface arrangement. The best spreading was obtained with glass slides treated by a HCl-HNO₃ (3 : 1) mixture. The distribution profile of RNA spread in this way is shown in Fig. 1-I and was described above. In our hands the glass slides treated with HNO₃, chromium-sulfuric acid, detergents, or combinations of these method, gave worse and unreproducible results: they became quickly hydrophobic.

When glass slides with rough surface (quite different source and quality) were used, only three size classes on the lengths of 0.58 ± 0.145 , 0.98 ± 0.089 and 1.35 ± 0.092 μm and 45, 36 and 19 % the molecules were found. The length of molecules in the highest size class was from 1.2 to 1.59 μm and only 19 % of them were present in this length category. Many of the RNA molecules contained short ds regions (Fig. 2-III) which were localized nonspecifically.

Speed of spreading. The dependence of surface film formation on the spreading speed was studied using pipettes with different outlet diameters. We compared glass pipettes with an outlet diameter of 0.4 and 0.65 mm and a 50- μl Eppendorf pipette. We obtained fully extended AMV RNA molecules under rather high speed of spreading with the Eppendorf pipette. The length distribution profile was described above and shown in Fig. 1-I. With a glass pipette with an outlet diameter of 0.65 mm four peaks on the length distribution profile were found (Fig. 1-IV) with mean lengths of 0.45 ± 0.091 , 0.75 ± 0.080 , 1.03 ± 0.096 and 1.46 ± 0.093 μm and 30, 24, 40 and 5% of the molecules, respectively. Glass pipettes with an outlet diameter of 0.4 mm (lowest speed of spreading) yielded only three peaks on the length distribution profile (Fig. 1-V) with the mean lengths of 0.37 ± 0.069 , 0.78 ± 0.139 and 1.35 ± 0.100 μm and 16, 82 and 2% of the molecules, respectively. In this case, as little as 5% of the molecules fell into the highest class with a mean length of only 1.35 μm . When these glass pipettes were used, short base-paired regions on the RNA molecules were observed (Fig. 2-IV, V). With lower speeds of spreading, the formation of artificial secondary structures was more intensive.

Amount of spreading solution. We investigated its effect on the quality of protein surface film by spreading 30, 50, 70 and 100 μl of the hyperphase on to the same area of the hypophase (about 30 cm^2). We achieved the most uniform film and RNA extension by spreading 50 μl of the hyperphase (Fig. 1-I, 2-I). By contrast, with 30 μl of spreading solution some parts of a given hypophase area remained uncovered by the film. Higher amounts of hyperphase (70, 100 μl) led to the formation of artificial secondary and kinky structures (not shown).

The amount of protein in the spreading solution varies depending on the type of electron microscopic analysis and the type of nucleic acid. For

- VII — cytochrome c Type IIA from horse heart (Sigma)
 VIII — cytochrome c from horse heart (Boehringer)
 IX — 50% formamide, 0.01 mol/l TRIS pH 8.5, 0.001 mol/l EDTA in hypophase
 Abscissae: length of RNA molecules in μm ; ordinates: number of molecules

visualization of ds DNA by the aqueous technique or for heteroduplex analysis, the use of cytochrome c at a concentration of as much as 100 $\mu\text{g/ml}$ (Mickel *et al.*, 1977; Carré and Attardi, 1978; Nešvera *et al.*, 1978) and, in some cases, even 250 $\mu\text{g/ml}$ has been reported (Virrankoski-Castrodeza and Parish, 1980). Lower concentration of protein, approximately from 30 to 50 $\mu\text{g/ml}$, have been used for electron microscopic analysis of ss DNA and RNA (Chi and Bassel, 1975; Heine *et al.*, 1975; Jacobson, 1976).

We compared the lengths and structural features of AMV RNA prepared under strongly denaturing conditions using three concentrations of cytochrome c, namely 30, 100 and 300 $\mu\text{g/ml}$, in the hyperphase. A smooth background and secondary structure-free spreading of RNA molecules was obtained only with 30 $\mu\text{g/ml}$ of cytochrome c in the hyperphase. Higher concentrations of cytochrome c formed a rough background with poor contrast of the molecules (not shown).

Type of cytochrome c. It is known that some types of cytochrome c contain nucleases and that every type and batch of cytochrome gives different results in the spreading technique (Brack and Delius, 1980). We therefore tested several types of cytochrome c (Calbiochem, Sigma Type VI and IIA, and Boehringer). The best extension of AMV RNA molecules was obtained with cytochromes from Sigma (Type VI) and Calbiochem (Fig. 2-I, VI). The length distribution profile of AMV RNA spread in the presence of cytochrome c from Sigma (Type VI) is shown in Fig. 1-I and its character with five size classes was described above. With Calbiochem cytochrome c the length distribution profile (Fig. 1-VI) also revealed five size classes with the mean lengths of 0.46 ± 0.089 , 0.75 ± 0.076 , 1.05 ± 0.076 , 1.05 ± 0.079 , 1.29 ± 0.050 and $1.59 \pm 139 \mu\text{m}$ and 20, 29, 21, 13 and 17% of the molecules, respectively. However, we found two size classes (Fig. 1-VII) with the mean lengths of 0.50 ± 0.151 and $0.92 \pm 0.078 \mu\text{m}$ using the Sigma Type IIA cytochrome c and only one size class (Fig. 1-VIII) with a mean length of $0.42 \pm 0.181 \mu\text{m}$ when the RNA was spread with cytochrome c from Boehringer. The RNA molecules were not only shorter, but also thicker with a high background in both cases (Fig. 2-VII, VIII). No substantial differences between AMV RNA spreading were found with filtered or centrifuged cytochrome c solution.

Surface tension at the hypophase-air boundary. An optimal extension and reproducible length of ss RNA are given by surface tension of the hypophase, on which the protein film is formed. This tension is affected by many factors, especially by the presence of surface active substances, type of denaturing agents, amounts of salts both in hypophase and spreading solution, and temperature. Different types of water used as a hypophase were tested because its quality (presence of trace amounts of contaminants) could affect the surface tension. We tested distilled or deionized waters of various conductivity and used ΦX 174 ss circular DNA as a length standard for this purpose. We obtained similar results with both distilled (conductivity of 1.00 μS) and deionized (conductivity of 0.36 μS) water, the length of ΦX 174 DNA having been 1.69 ± 0.101 and $1.66 \pm 0.134 \mu\text{m}$, respectively. On

the other hand, water specially prepared for tissue cultures (conductivity of $0.66 \mu\text{S}$ resulted in $\Phi X 174$ DNA molecules with a length of $1.31 \pm 0.129 \mu\text{m}$.

After addition of 50% formamide, 0.01 mol/l TRIS, pH 8.5, and 0.001 mol/l EDTA to the hypophase, we observed shortening of AMV RNA molecules and a rough background appeared (Fig. 2-IX). Only three peaks occurred on the length distribution profile with mean lengths of 0.38 ± 0.067 , 0.62 ± 0.083 and $1.11 \pm 0.155 \mu\text{m}$, and 28, 29 and 42 % of the molecules, respectively (Fig. 1-IX). The two highest size classes disappeared when compared with spreading where only distilled water was used as a hypophase (Fig. 1-I).

In our experiments, the formation of surface protein film was also affected by the presence of DEPC in the hypophase. DEPC is generally used as a nuclease inhibitor (4 μl added to 200 ml of the hypophase) even for the visualization of rRNA (Robberson *et al.*, 1971). But, in our hands, the presence of DEPC in the hypophase resulted in a low concentration of RNA molecules in the protein film and in poor contrast. The presence of SDS in both the hypophase and the hyperphase had the same adverse effect.

Discussion

The visualization of linear, fully extended ss RNA requires strongly denaturing conditions, where most of the base paired interactions are considerably diminished (Robberson *et al.*, 1971; Mangel *et al.*, 1974; Chi and Bassel, 1975; Heine *et al.*, 1975). The main conclusion to be drawn from our investigations is simply an experimental one, namely that, even under strongly denaturing conditions, a number of factors affect the surface protein film formation and so determine the basic parameters of electron microscopic pictures.

The formation of the surface film in the protein spreading technique depends on the property of the hyperphase and hypophase, the technique and geometrical arrangement of the spreading.

The stability and uniformity of the surface protein film depended on the presence of substances affecting surface tension (e. g., SDS, DEPC) both in the hyperphase and the hypophase. The surface active substances can be present, even in trace amounts, in water, formamide or salts used. DEPC, a nuclease inhibitor, sometimes used in visualization of rRNA (Robberson *et al.*, 1971), under our experimental conditions exerted negative effects by decreasing the concentration of RNA molecules in the film and diminishing their visibility. The quality of the surface film was strongly dependent on the quality of water in the hypophase, which affected the concentration of molecules in the protein film.

Formamide at different concentrations in the hypophase, used to prevent renaturation in heteroduplex analysis (Davis *et al.*, 1971), in our hands led to considerable shortening of the molecules and to the appearance of high background. The front of the film moved slowly from the starting position

and the film covered a smaller area than on the water only. Under these conditions the film formation can favour random base-base interactions resulting in short ds regions. When these regions are shorter than 100 base pairs, they are not visible in the electron microscope and the molecules appear seemingly shorter (Davidson, 1978). For this reason, we preferred using deionized water in the hypophase because the effect of renaturation on the shortening of retrovirus RNA molecules is almost negligible (Kung *et al.*, 1975). The quality of formamide in the hyperphase also affected the length and visibility of AMV RNA molecules. We obtained best results with MCB formamide, its purification (distillation, crystallization) had no effect on the length of RNA molecules. This was in agreement with the findings of Brack and Delius (1980) that formamide alone formed the surface film, even without the addition of cytochrome *c*, irrespective of different brands and purification.

The stability of the surface film and the concentration of RNA molecules in it was found to depend on the amount of cytochrome *c*. Though a concentration of cytochrome *c* as high as 250 $\mu\text{g}/\text{ml}$ (Virrankoski-Castrodeza and Parish, 1980) was used for the visualization of ds DNA, we found that the optimal concentration for ss RNA was 30 $\mu\text{g}/\text{ml}$. Lower concentrations prevented stable film formation, resulting in a loss of most of the RNA molecules. Higher concentrations of cytochrome *c* led to the formation of higher background.

The quality and source of cytochrome *c* play an important role in affecting the length of AMV RNA. Some batches of cytochrome *c* contain nucleases which can degrade RNA. They also differ in their ability to bound to the RNA molecules under strongly denaturing conditions and different amounts protein particles can be aggregated around them. The different amount of aggregated protein particles could also result in irregular surface film formation with an uneven spreading of RNA molecules.

Surface tension is known to depend on the temperature. Therefore our finding that the formation of a protein surface film was affected by temperature of the hypophase was not surprising. We prefer the temperature of 10° C, at which a stable and smooth surface film is obtained.

The hyperphase can be spread on to the hypophase in different ways. For example, touching the pipette containing the hyperphase with the hypophase (Virrankoski-Castrodeza and Parish, 1980), spreading by means of capillary action (Brack and Delius, 1980), or the use of glass slides as spreading ramps (Davis *et al.*, 1971) were described. We used glass slides as spreading ramps and this method was found to be affected by hydrophilicity of the glass surface and speed of spreading. Several methods of hydrophilization of a glass slide were described (Kleinschmidt, 1968; Robberson *et al.*, 1971). They involve the treatment with various acids and detergents. In our experiments we preferred treatment with a 3 : 1 mixture of HCl—HNO₃ which provided a suitable hydrophilic surface of the slides. This resulted in an even spreading, and a smooth, stable film with well extended molecules was observed. Other types of treatment were not satisfactory in our experiments as the slides became quickly hydrophobic. In this case, the surface film

was deformed and RNA molecules were not well stretched, resulting in shortening of the length of RNA molecules, which had a kinky appearance. Such conditions may facilitate the formation of random base-base interactions giving rise to an artificial secondary structure.

For regular film formation an optimal speed of spreading of the hyperphase should be used. When the film was spread slowly, the hyperphase convected intensively and the resulting protein film was deformed. In such an unstable film the different strengths act on the RNA molecules, forming random base-base paired artificial regions.

The geometric arrangement was also found to play an important role. The best results were obtained when 50 μ l of hyperphase on to the surface of 30 cm² of hypophase were applied over a glass slide inserted at an angle of 45°. When the limited area of the hypophase was smaller, the surface film was overcrowded with cytochrome particles and a larger area was covered with the film only partially. The slope of the glass slide affects particularly the falling of hyperphase into the hypophase. When the amount of applied hyperphase exceeded 50 μ l, short ds regions on rough background were observed.

Our results support the idea that ss RNA is more sensitive to the spreading conditions during electron microscopic visualization than ds DNA (Kleinschmidt, 1968; Dubochet and Groom, 1981). Optimal conditions and arrangement of the spreading are necessary to obtain good results. Different batches of denaturing agents, cytochrome c, salts and water have to be checked to obtain well extended RNA molecules with reproducible lengths. This is especially true when molecular weights of various ss RNAs are to be determined precisely as RNAs show different sensitivities to denaturing and spreading conditions resulting in different linear densities (Korb *et al.*, 1981). Therefore the optimal conditions should be strictly kept in order to obtain accurate data.

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Explanation of Electron Micrographs (Plate I):

Fig. 2. Electron micrographs of 60-70 S AMV RNA prepared under various spreading conditions.

I - IX correspond to the conditions as described in Fig. 1. The bar represents 0.5 μm .