Hydrodynamic Properties of RNA: Effect of Multivalent Cations on the Sedimentation Behavior of Turnip Yellow Mosaic Virus RNA

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ABSTRACT: Turnip yellow mosaic virus (TYMV) RNA freshly isolated from the virion with phenol (native RNA) shows a high variability in sedimentation values at low concentrations compared with TYMV RNA devoid of divalent cations and polyamines (naked RNA). An incubation period of 24 h at room temperature reduces this variability and causes a relatively large decrease in s values for both RNAs. It also results in a curvilinear dependence of s on RNA concentration for native RNA, whereas a linear dependence was found for naked RNA. The presence of the multivalent cations in native TYMV RNA samples is supposed to be a major cause for both the variability and the curvilinear dependence. Dialysis and subsequent dilution of the RNA with dialysate instead of buffer lead to the disappearance of this curvilinearity. The curvilinear behavior, therefore, cannot be explained by a rapid monomer–dimer equilibrium of the RNA. A more probable model is release of cations from the RNA and swelling of the RNA at lower concentrations due to the decrease of free divalent cations and polyamines. This was also supported by the results obtained from viscometric measurements. In order to get reproducible results from hydrodynamic measurements on natural RNAs, it is recommended that the RNA sample be incubated for at least several hours up to 1 day at the desired temperature and that the counterion concentration be kept at a constant value during dilution of RNA solutions.

A detailed knowledge of the three-dimensional structure of nucleic acids and of its dynamics is essential for the understanding of their function in biological processes. Hydrodynamic measurements on nucleic acids can be used as a first step to obtain information about general features like size, shape, and hydration and also the interconversion of different conformational states (Bloomfield et al., 1974; Cantor & Schimmel, 1980). For some reason, however, RNA appears to have been less well studied than DNA, which might be due to the greater sensitivity of the former to degradation (by contaminating nucleases or elevated temperature). An interesting feature of most RNA molecules is their great flexibility as compared to double-stranded DNA. Sedimentation coefficients of large viral or ribosomal RNAs can vary an order of magnitude depending on the solvent conditions (Boedtker, 1960; Nisbet & Slayter, 1975). This raises the question whether the conformation of RNA in solution has any relevance to that found in situ, like in viruses, ribosomes, or messenger ribonucleoprotein (mRNP)[1] particles. An obvious example is TMV RNA which adopts an extended helical conformation in situ without any base pairing, whereas it has a more compact conformation with about 60% base pairing when free in solution. On the other hand, RNA (and also DNA) can occur in a very compact form in some spherical viruses (e.g., TYMV). It is therefore of great interest to know how RNA converts from one structural form to another.

A prominent factor leading to conformational changes in RNA is the shielding of the negative phosphate charges by monovalent cations like Na^+ and K^+ or multivalent cations like Mg^{2+} or polyamines. The concentration, type, and binding constant of these cations directly influence the degree of base pairing and/or base stacking. This phenomenon is particularly well studied in the case of the small tRNAs (Crothers & Cole, 1978). RNA preparations isolated from various sources may contain an appreciable amount of multivalent cations. Large quantities of polyamines were shown to be associated with RNAs from ribosomes, plant viruses, and bacteriophages (Bachrach, 1973; Cohen, 1974). For instance, TYMV contains about 500 molecules of spermidine and 2000 Mg^{2+} and Ca^{2+} ions per virion, which corresponds to an almost complete neutralization of the RNA phosphates (Johnson, 1964; Beer & Kosuge, 1970; Cohen & Greenberg, 1981; our observations). These cations usually remain bound to the RNA to a large extent upon isolation of the latter (Cohen & Greenberg, 1981).

Early physicochemical studies on TYMV RNA pointed to a considerable variation in the molecular weight and dimensions as obtained from sedimentation and viscometry measurements (Haselkorn, 1962; Mitra & Kaesberg, 1965). Also, time effects on the hydrodynamic properties were reported (Mitra & Kaesberg, 1965). These features were confirmed later in our laboratory during a study of a compact form of TYMV RNA, the so-called TY-A RNA (Pleij, 1973; Pleij et al., 1977). It was suggested that a high, though variable, content of Mg^{2+} and polyamines associated with the RNA was responsible for this irreproducible behavior. Both our own results and the occurrence of peculiar hydrodynamic phenomena in other studies (Stanley & Bock, 1970; Pearce et al., 1975) have prompted us to study more thoroughly the effect of multivalent cations on the hydrodynamic behavior of TYMV RNA. Moreover, we recently obtained indications for the importance of ionic conditions on the biological properties of some plant viral RNAs. The 3′ terminus of TYMV RNA and BMV RNA adopts a compact tertiary folding in the presence of Mg^{2+}, which explains their tRNA-like properties (Rietveld et al., 1982, 1983). From the results

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[1] Abbreviations: TYMV, turnip yellow mosaic virus; TMV, tobacco mosaic virus; BMV, brome mosaic virus; EDTA, ethylenediaminetetraacetic acid; DEPC, diethyl pyrocarbonate; RNP, ribonucleoprotein.

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obtained in this study, it is concluded that the hydrodynamic parameters, like the sedimentation coefficient, are strongly influenced by multivalent cations which, after isolation, remain bound to the RNA. A change in the composition and temperature of the RNA solution leads to the establishment of a new equilibrium between RNA and bound multivalent cations, which in turn gives rise to a conformational change in the RNA chain (e.g., swelling). The need for dialyzing RNA solutions in order to get reliable results will be valid for all RNAs which have bound large quantities of these multivalent cations. When proper precautions are taken like dilution with dialysate or complete removal of the multivalent cations, then still appreciable time effects on the hydrodynamic properties have to be considered if the RNA conformation in situ deviates considerably from its equilibrium state in solution.

Materials and Methods

Isolation of TYMV and TYMV RNA. TYMV (type strain) was grown on Chinese cabbage (*Brassica pekinensis*, var. Witkrop). Infected leaves were harvested 3–4 weeks after inoculation. The virus was isolated according to the method of Dunn & Hitchborn (1965) and was finally suspended in 0.01 M sodium acetate buffer, pH 6.0, and 1 mM sodium azide and stored at 4 °C. The isolation of RNA occurred according to Gierer & Schramm (1956) by means of two phenol and five ether extractions. After removal of ether with N_2, the RNA solution was stored at −20 °C in plastic vials.

Special Precautions. Glassware was heated at 160 °C overnight or treated with 0.5% DEPC overnight at room temperature when heating had to be avoided, e.g., for viscometer or cells for optical melting experiments. Pipet tips and vials were treated by boiling in 0.5% DEPC for 1 h or overnight at room temperature. Buffers were heated for 1 h at 120 °C. RNA samples were kept in plastic vials to prevent contamination with cations from glass. Centrifuge cells were treated with a sodium bentonite suspension. Dialysis tubing was boiled subsequently in diluted $NaHCO_3$ (4×), H_2O, 1 mM EDTA, and H_2O (2×), and thereafter sterilized for 1 h at 120 °C.

Velocity Sedimentation. Sedimentation was carried out with a Spinco Model E analytical ultracentrifuge (Beckman) and double sector cells with alumina-filled Epon centerpieces. The running speed was 40 000 rpm unless mentioned otherwise. The run temperature was 20 °C. The rotor was cooled to slightly below 20 °C in a cold room just before running. Schlieren optics were used for RNA concentrations of 3 mg/mL. UV optics were used for concentrations from 0.025 to 1 mg/mL with adjustment of the monochromator to higher wavelengths for the higher RNA concentrations. The sedimentation coefficient was obtained from the middle of the leading boundary. No correction was made for the density and viscosity of the buffer, or for radial dilution.

Concentration Determination of RNA. The extinction coefficient $A^{0.1\%}_{260nm} = 23.3\ cm^{-1}$ was used for both native and naked TYMV RNA (Haselkorn, 1962).

Viscometry. An Ubbelohde microviscometer was used with automatic recording of flow times (AVS/G, Schott). The flow time for H_2O was 200 s. The temperature was 20.00 (±0.01) °C. Solvents and solutions were filtered through Millipore filters (0.45 μm) prior to viscometry. The viscometer was cleaned subsequently with ethanolic alkali, diluted HCl, and distilled water and was treated overnight with 0.5% DEPC. RNA samples containing 1 mM sodium azide were equilibrated at room temperature for 24 h prior to measurement. After viscometry, the RNA concentrations were determined, and the degradation of the RNA was checked in some cases by polyacrylamide gel electrophoresis.

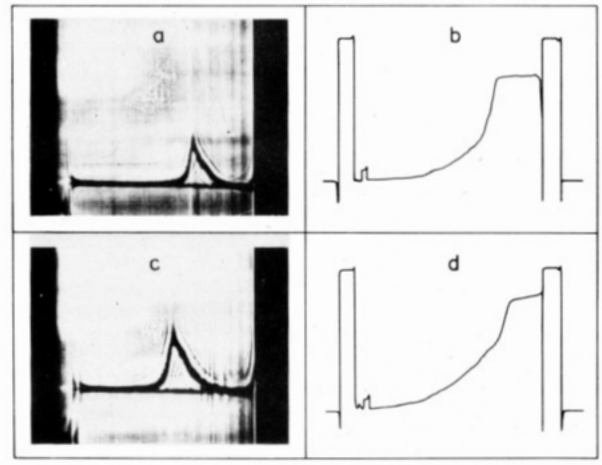

FIGURE 1: Sedimentation patterns of TYMV RNA in 0.01 M sodium acetate, pH 6.0 at 20 °C. (a) Schlieren pattern of unheated RNA (3 mg/mL); (b) UV pattern of unheated RNA (35 μg/mL); (c) schlieren pattern of RNA (3 mg/mL) heated for 10 min at 65 °C; (d) UV pattern of RNA (35 μg/mL) heated for 10 min at 65 °C. The schlieren patterns were obtained 70 (a) and 62 min (c) after attaining a speed of 52 000 rpm. The UV scans were made 47 (b) and 54 min (d) after reaching a speed of 40 000 rpm.

Polyacrylamide Gel Electrophoresis in 98% Formamide. Gel electrophoresis was essentially according to Lehrach et al. (1977). Precipitated RNA samples (20 μg) were dissolved in 0.1 mL of 98% formamide, 6 mM NaH_2PO_4, and 6 mM Na_2HPO_4 and heated for 5 min at 55 °C. Electrophoresis was performed in 3.5% polyacrylamide tube gels in phosphate-buffered formamide. The electrode compartments contained 0.02 M sodium phosphate, pH 7.5. After electrophoresis, the gels were rinsed with water in order to remove most of the formamide and monitored for UV absorbance at 260 nm.

Detection and Removal of Polyamines and Divalent Cations. Polyamines were extracted from the RNA sample with 2% perchloric acid and detected and quantified with high-voltage paper or thin-layer electrophoresis followed by ninhydrin staining according to Inoue & Mizutani (1973), or as dansyl derivatives with thin-layer chromatography according to Herbst & Dion (1970). Mg^{2+} was extracted with 1% perchloric acid and quantified with atomic absorption spectrophotometry as described by Rodgers (1964). Polyamines and divalent cations were removed by precipitation of the RNA in 0.3 M sodium acetate, pH 6, and 66% ethanol. The pellet was dissolved in 0.01 M sodium acetate, pH 6, and an aliquot was withdrawn for polyamine and Mg^{2+} determination. The precipitation procedure and sampling were repeated several times.

Optical Melting Experiments. The optical density of RNA samples was 0.5 OD unit at 260 nm. Solutions and solvent in the cells were degassed under vacuum, and the cells were covered with paraffin oil in order to prevent evaporation. Optical melting was determined with a Unicam SP 1800 UV spectrophotometer. Cells were placed in a heating block regulated by an SP 876 temperature controller. The heating rate was 0.2 °C/min. No correction in optical density was made for thermal expansion.

Results

Figure 1 shows sedimentation patterns of TYMV RNA in 0.01 M sodium acetate buffer, pH 6.0, as determined in the analytical ultracentrifuge at both high (Figure 1a) and low (Figure 1b) RNA concentration. Although sedimentation of polyelectrolytes, like RNA, in buffers of low ionic strength

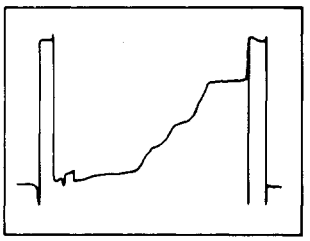

FIGURE 2: UV sedimentation pattern of unheated TYMV RNA (35 μg/mL) in 0.01 M sodium acetate, pH 6.0, when the temperature control (set at 20 °C) during the run was poor. The scan was made 45 min after attaining a speed of 40 000 rpm.

can give rise to serious problems like the Johnston–Ogston effect or the primary charge effect, we have used this buffer of 0.01 M sodium acetate, pH 6.0, in the majority of the measurements reported in this paper, for the following reasons: (1) high salt concentrations induce RNA aggregation, especially in the case of TYMV RNA (Pleij et al., 1976); (2) multivalent cations like Mg^{2+} and polyamines present in TYMV RNA samples are more firmly bound to the RNA at low ionic strength, thereby compensating for a low Na^+ ion concentration in the buffer; (3) degradation of TYMV RNA appeared to be minimal at pH 6.0. The pattern obtained at higher RNA concentration points to the presence of one major RNA component (M_r 2.0 × 10^6) which corresponds to the genome of TYMV (Noort et al., 1982). The asymmetry of the boundary suggests the presence of small amounts of faster sedimenting material that we ascribe to aggregated RNA. No such aggregated material was observed at the leading edge of the main boundary at the lower RNA concentrations mainly studied in this paper. At low concentrations, however, a more polydisperse pattern was obtained in which the major boundary still sediments with the velocity expected for the genomic TYMV RNA and which consists of 50–80 wt% of the total RNA present. The slower sedimenting RNA is thought to originate from genomic RNA molecules which have so-called hidden breaks and fall apart upon lowering of the RNA concentration (see Discussion). RNase activity could be excluded, since no further increase in slowly sedimenting material was observed after incubation of the RNA sample for 1 or 2 days at room temperature (H. G. Eecen, unpublished results). TYMV RNA heated for 10 min at 65 °C yielded a pattern identical with that of unheated RNA in the case of high RNA concentration (Figure 1c), whereas at low concentration (Figure 1d) more slowly sedimenting material became apparent. Time and temperature during the heating are too moderate to cause thermal cleavage of phosphodiester bonds (H. G. Eecen, unpublished results). The 20–50% of RNA in the fast sedimenting boundary obtained upon heating of the RNA is therefore thought to represent intact genomic RNA originally present in the TYMV preparation. This percentage in turn depends on the condition of the virus growth and the time of virus harvest (Dunn & Hitchborn, 1966; Lightfoot et al., 1980). The apparent stability of TYMV RNA against deaggregation at high RNA concentration can be ascribed to the fact that more multivalent cations remain bound to the RNA at high than at low RNA concentration (also see Discussion). These multivalent cations effectively shield the negative phosphates, thereby increasing the melting temperature of the double-stranded regions in RNA.

Occasionally, sedimentation patterns at low RNA concentrations showed one or more sharp boundaries besides the main fast sedimenting one (Figure 2). This phenomenon was also reported by Mitra & Kaesberg (1965) and Pleij (1973). These extra boundaries were found in this study to occur only when

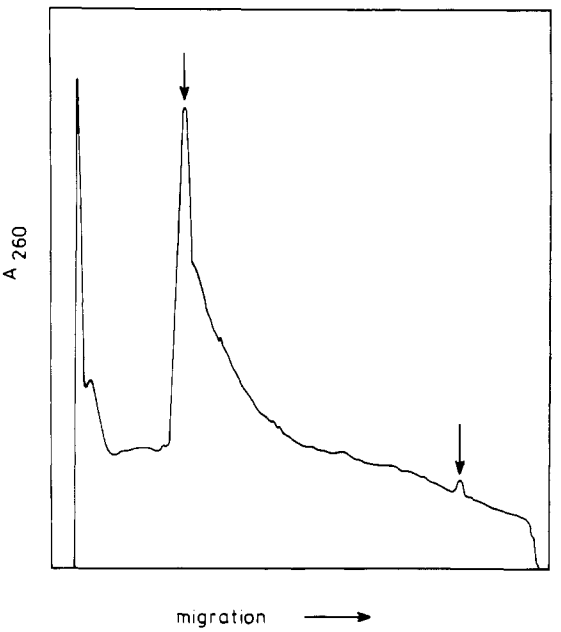

FIGURE 3: Polyacrylamide gel electrophoresis of TYMV RNA in 98% formamide. The arrows give the positions of genomic RNA (M_r 2.0 × 10^6) and coat protein messenger RNA (M_r 0.24 × 10^6), respectively.

the temperature control during the run was poor, when the RNA concentration was low, or when the temperature was relatively high (20 °C). We therefore assume that these slower sedimenting boundaries are due to convection in the cell, caused by temperature gradients in the rotor. This is especially a serious problem at low absolute concentrations of solute and/or shallow solute concentration gradients (Schachman, 1959; Hill et al., 1977). In schlieren patterns, these artifacts can be discerned as spikes in the leading or trailing edge of the peak [compare Figure 1c and Schachman (1959)]. Indications for the artificial nature of the extra boundaries observed in the analytical ultracentrifuge were obtained from polyacrylamide gel electrophoresis of TYMV RNA under denaturing conditions in the presence of formamide (Figure 3). No other components besides the intact genomic RNA (M_r 2.0 × 10^6) and the subgenomic messenger RNA for the coat protein (cpmRNA, M_r 0.24 × 10^6) were found (Pleij et al., 1976; Noort et al., 1982). On the other hand, sucrose gradient centrifugation of TYMV RNA under nondenaturing conditions gives rise to a very reproducible pattern in which clearly five RNA size classes could be observed (Pleij et al., 1976; Lightfoot et al., 1980). The origin and nature of the four slower sedimenting size classes remain obscure as yet.

Sedimentation coefficients of the main boundary corresponding to genomic TYMV RNA were determined. This RNA consists either of intact RNA chains or of aggregates of fragments. Both types of RNA particles have the same molecular weight of 2.0 × 10^6 and are here defined as 2M RNA. The sedimentation properties of TYMV RNA described in this paper were determined on samples having a size distribution similar to that shown in Figure 1b. This means that the sedimentation coefficient of 2M RNA is influenced by the smaller RNA fragments. Extrapolation of the sedimentation coefficient to zero concentration, however, is assumed to give the correct s value for 2M RNA.

A plot of the sedimentation coefficient of 2M RNA in 0.01 M sodium acetate buffer as a function of the total RNA concentration is given in Figure 4. The various RNA samples were prepared by dilution of an RNA stock solution with acetate buffer. This stock solution was stored frozen after isolation from the virus with phenol (see Materials and

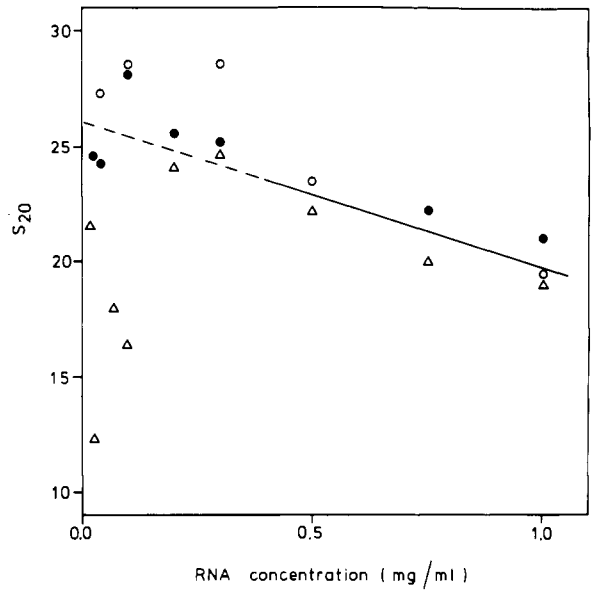

FIGURE 4: Dependence of the sedimentation coefficient of native TYMV RNA on RNA concentration. RNA samples were incubated for 16 h at 4 °C and subsequently for 2 h at room temperature prior to centrifugation. The three symbols (●, ○, △) represent separate experiments starting from one single RNA preparation. Solvent was 0.01 M sodium acetate, pH 6.0. Centrifugation was at 20 °C.

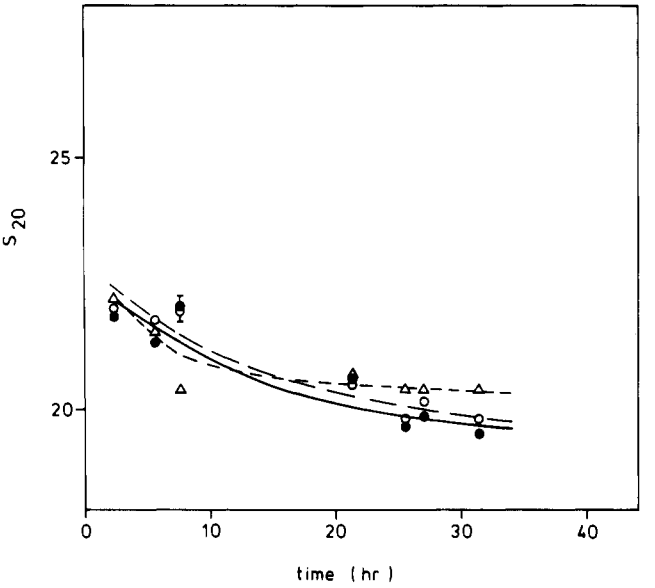

FIGURE 5: Dependence of the sedimentation coefficient of native TYMV RNA on time of incubation at room temperature. RNA samples with three different concentrations [(●) 25 μg/mL; (○) 40 μg/mL; (△) 75 μg/mL] were prepared and incubated at room temperature for various periods of time prior to centrifugation. Solvent was 0.01 M sodium acetate, pH 6.0. Centrifugation was at 20 °C.

Methods) and is defined here as native TYMV RNA. After preparation, the RNA samples were left at 4 °C for 16 h to dissolve possible aggregates and were subsequently kept at room temperature for 1–2 h prior to centrifugation. From Figure 4, it is clear that the *s* value of native TYMV RNA is not very reproducible and varies erratically at concentrations below 0.5 mg of RNA/mL, preventing any meaningful extrapolation to zero concentration. An extrapolation of the *s* values obtained above 0.3 mg of RNA/mL gives a sedimentation coefficient of 26 S which is the value usually found at low RNA concentrations in solutions with ionic strengths of 0.1 M (Haselkorn, 1962; Pleij, 1973). As an explanation for the irreproducible *s* values at low RNA concentrations, it may be argued that the TYMV RNA molecule had not attained true equilibrium after the isolation, freezing, thawing, and dilution steps. We therefore measured the influence of time after sample preparation on the sedimentation velocity of 2M RNA. As can be seen in Figure 5, the *s* value decreases steadily and significantly at all concentrations measured as a function of time. That this is not due to RNase activity, as has been mentioned above, is indicated by the constant ratio between the amount of rapidly sedimenting RNA and slower sedimenting material. This behavior was also described by Mitra & Kaesberg (1963) and was interpreted as a swelling of the RNA molecule after its isolation from the virion where it occurs in a very compact state. We therefore conclude that for reliable measurements the TYMV RNA has to be left at 20 °C for a considerable period of time. For practical reasons, we assumed that a state of equilibrium was reached after 24 h of incubation.

Figure 6 shows the concentration dependence of the *s* value for native TYMV RNA kept for 24 h at room temperature. There is both less variability and better reproducibility than in the experiments given in Figure 4. (Note the different concentration scales.) It is also striking that the plot shows a downward curvature at low RNA concentrations so that a linear extrapolation is not possible. Furthermore, at about 0.3 mg/mL, a lowering in the *s* value is observed from above 24 S (see Figure 4) to 20 S, in agreement with the result shown

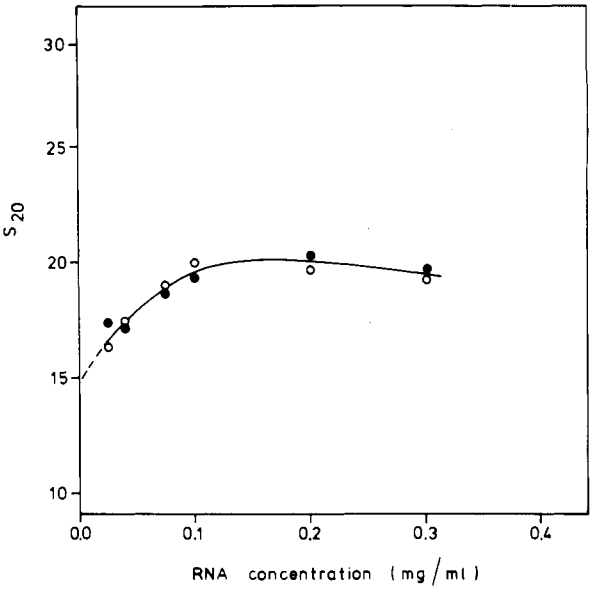

FIGURE 6: Dependence of the sedimentation coefficient of native TYMV RNA on RNA concentration. RNA samples were incubated for 24 h at room temperature prior to centrifugation. The open and closed circles represent two separate experiments. The dashed part of the curve gives a tentative extrapolation to zero concentration. Solvent was 0.01 M sodium acetate, pH 6.0. Centrifugation was at 20 °C.

in Figure 5. A similar downward curvature in the plot at lower RNA concentrations was reported for ribosomal RNA (Stanley & Bock, 1970; Pearce et al., 1975). The latter authors ascribed this behavior to a rapidly equilibrating monomer–dimer system in solution, which leads to an increase of the *s* value of the main boundary at higher RNA concentrations. According to this model, the *s* value for the monomer is obtained at zero concentration.

In our view, an alternative origin for this curvilinear dependence of *s* on RNA concentration could be the presence of large amounts of multivalent cations, like Mg^{2+} or polyamines bound to TYMV RNA and also to ribosomal RNA.

Table I: Removal of Polyvalent Cations from TYMV RNA by means of Precipitation

no. of pptn	no. of cations per molecule of RNA (±estimated error)		
	Mg^{2+}	spermidine	spermine
0	1020 ± 30	463 ± 4	18.6 ± 0.6
1	67 ± 3	72 ± 1	7.9 ± 0.6
2	7.8 ± 0.4	16.5 ± 0.3	5.4 ± 0.3
3	0.9 ± 0.2	1.36 ± 0.04	0.82 ± 0.06
4	0.4 ± 0.1	0.47 ± 0.03	0.28 ± 0.04
5	0.3 ± 0.1	0.34 ± 0.03	0.18 ± 0.03
6	0.4 ± 0.2	0.26 ± 0.05	0.20 ± 0.05

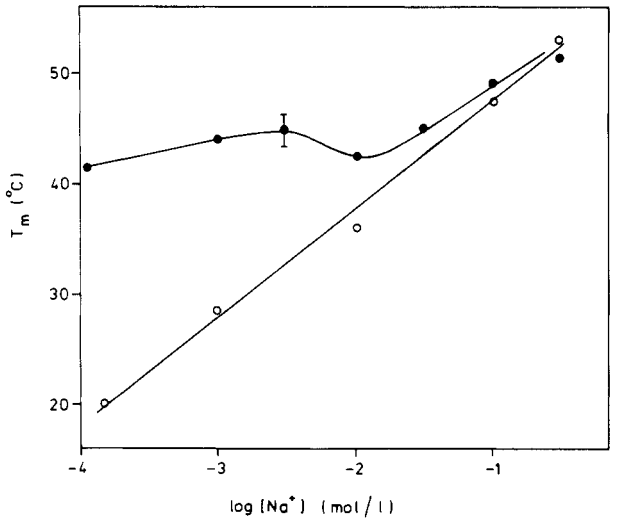

FIGURE 7: Dependence of the midpoint temperature (T_m) for TYMV RNA on sodium acetate concentration: (●) native TYMV RNA; (○) naked TYMV RNA.

We therefore examined the sedimentation properties of TYMV RNA deprived of these multivalent cations and compared the behavior of this so-called naked RNA to that of native RNA. Divalent cations and polyamines were removed by repeated precipitation of the RNA with ethanol in the presence of 1 M sodium acetate buffer, pH 6.0 (see Materials and Methods). After four cycles of preparation, the RNA appeared to have lost almost all of its Mg^{2+} and spermidine (see Table I). This loss of multivalent cations was consistent with data from optical melting curves for both native and naked TYMV RNA determined as a function of the sodium acetate concentration. From Figure 7, it is clear that naked RNA yields a normal linear dependence of T_m on Na^+ concentration as found usually for polynucleotides (Kotin, 1963; Schildkraut & Lifson, 1965; Record, 1967), in contrast to native RNA which showed only a very slight increase in the Na^+ concentration range studied. At high ionic strength, both RNAs yield the same T_m. These features are an indication for bound divalent cations and/or polyamines as is known from studies with double-stranded DNA or RNA (Guschlbauer et al., 1968; Horacek & Cernohorsky, 1968; Burnett et al., 1975). The plots of s against concentration of naked TYMV RNA are given in Figures 8 and 9. Ultracentrifugation was performed 1–2 (Figure 8) and 24 h (Figure 9) after the RNA solution was left at room temperature. Some variability in the s values below 0.1 mg/mL is found after 1–2 h, but to a much lesser extent than for native RNA (compare Figure 4). Extrapolation to zero concentration, considering concentrations of 0.2 mg of RNA/mL and higher, yields a value of about 21 S. The drop in the s value of naked TYMV RNA upon longer incubation at room temperature again is considerable, but even more remarkable is the linear curve obtained for the entire concentration range studied and with a negative slope as expected theoretically (Schachman, 1959). The extrapolated sedimentation coefficient in this case is about 13 S. We also note that a tentative extrapolation of the downward curve in the case of native RNA (Figure 6) gives a value which is nearly identical with that of naked RNA measured under identical conditions.

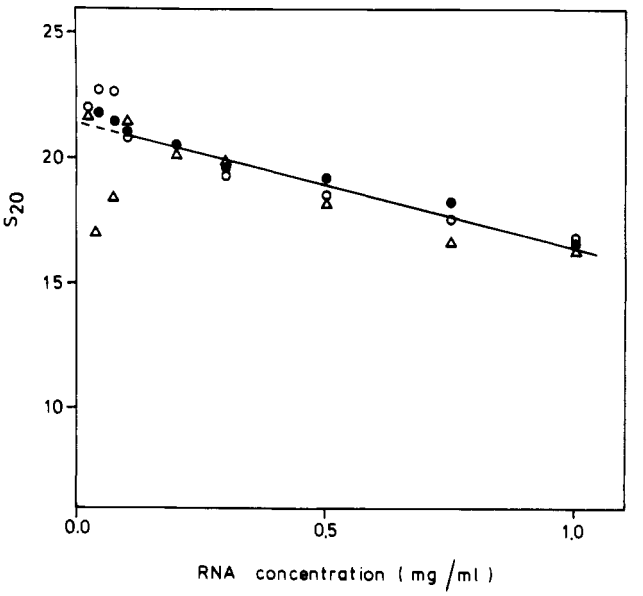

FIGURE 8: Dependence of the sedimentation coefficient of naked TYMV RNA on RNA concentration. RNA samples were incubated for 16 h at 4 °C and subsequently for 2 h at room temperature prior to centrifugation. The three symbols (●, ○, △) represent separate experiments. Solvent was 0.01 M sodium acetate, pH 6.0. Centrifugation was at 20 °C.

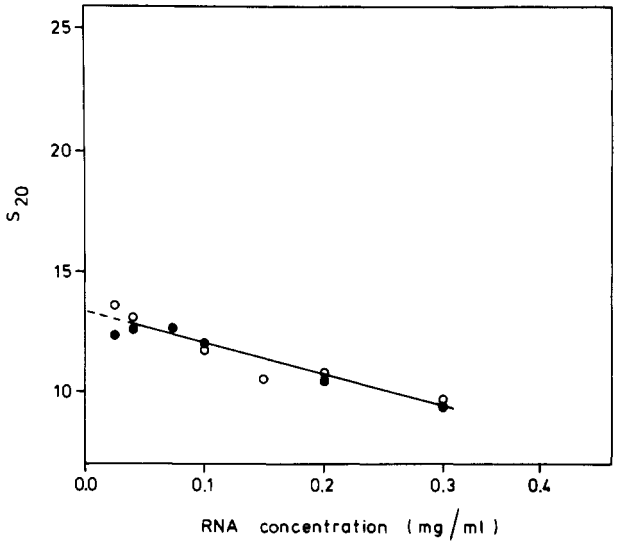

FIGURE 9: Dependence of the sedimentation coefficient of naked TYMV RNA on RNA concentration. RNA samples were incubated for 24 h at room temperature prior to centrifugation. The open and closed circles represent two separate experiments. Solvent was 0.01 M sodium acetate, pH 6.0. Centrifugation was at 20 °C.

To get more information on the influence of multivalent cations on the hydrodynamic properties of TYMV RNA, we have studied native and naked TYMV RNA under various conditions. It appeared that different curvatures can be obtained depending on the conditions used (see Figure 10). Naked RNA was dialyzed against 0.01 M sodium acetate, pH 6.0, to which spermidine was added (10^{-5} M). The dialyzed solution was diluted with either dialysate or sodium acetate buffer without added spermidine and analyzed in the analytical

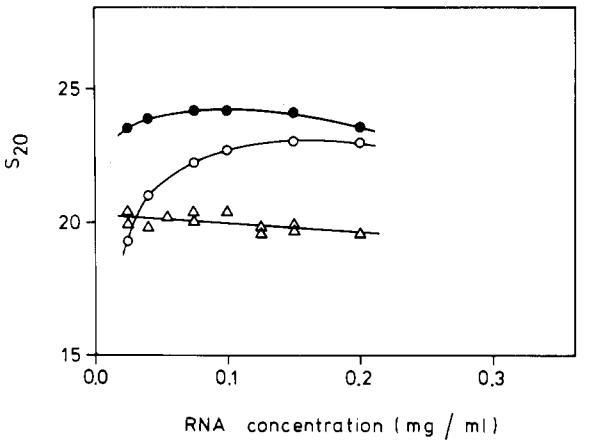

FIGURE 10: Dependence of the sedimentation coefficient of TYMV RNA on RNA concentration as a function of ionic conditions. Naked RNA (3.3 mg/mL) was dialyzed at 4 °C against 0.01 M sodium acetate, pH 6.0, and 0.01 mM spermidine hydrochloride and diluted with 0.01 M sodium acetate, pH 6.0 (○), or with dialysate (●). Native RNA (3.3 mg/mL) was dialyzed at 4 °C against 0.01 M sodium acetate, pH 6.0, for 3 days and subsequently diluted with dialysate (△). Incubation of the RNA samples was for 16 h at room temperature prior to centrifugation. Centrifugation was at 20 °C.

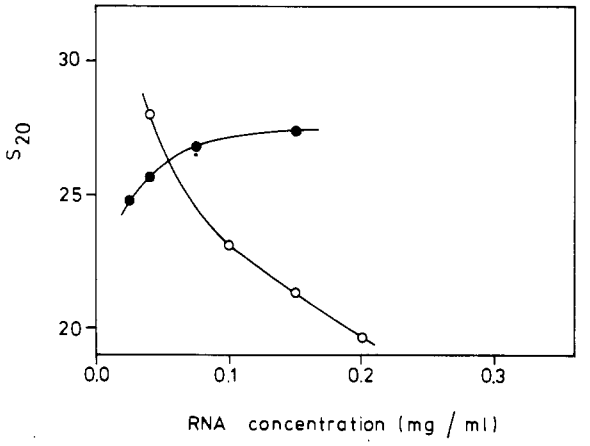

FIGURE 11: Dependence of the sedimentation coefficient of TYMV RNA on RNA concentration as a function of ionic conditions. Naked TYMV RNA samples of different RNA concentrations were prepared from a stock solution by dilution with 0.01 M sodium acetate, pH 6.0, and spermidine hydrochloride until an end concentration of 0.01 M sodium acetate, pH 6.0, and 0.01 mM spermidine hydrochloride was reached (○). Native TYMV RNA was dialyzed at 4 °C against 0.1 M sodium acetate, pH 6.0, and subsequently diluted with a fresh buffer of the same composition (●). Incubation was for 16 h at room temperature prior to centrifugation. Centrifugation was at 20 °C.

ultracentrifuge. If the RNA samples were prepared by dilution with sodium acetate buffer only, a curve for the s values was obtained which was similar to that for native TYMV RNA (compare Figure 6). Dilution with dialysate resulted in a less pronounced curvature of the plot. Interestingly, dialysis of native TYMV RNA against 0.01 M sodium acetate followed by dilution with dialysate now resulted in a linear regression. Under certain conditions, the regression can even be curved upward. This was observed when the sedimentation coefficient of naked RNA was determined on samples diluted with sodium acetate buffer to which spermidine was added (Figure 11). When native TYMV RNA was analyzed in a buffer with a higher ionic strength (0.1 M sodium acetate, pH 6.0), a nonlinear plot was also found (Figure 11) though less curved than that observed for 0.01 M sodium acetate. This curvature was also less pronounced when buffers with higher pH values (7.0–8.0) were used or when the RNA samples were kept and studied at 4 °C instead of 20 °C (results not shown).

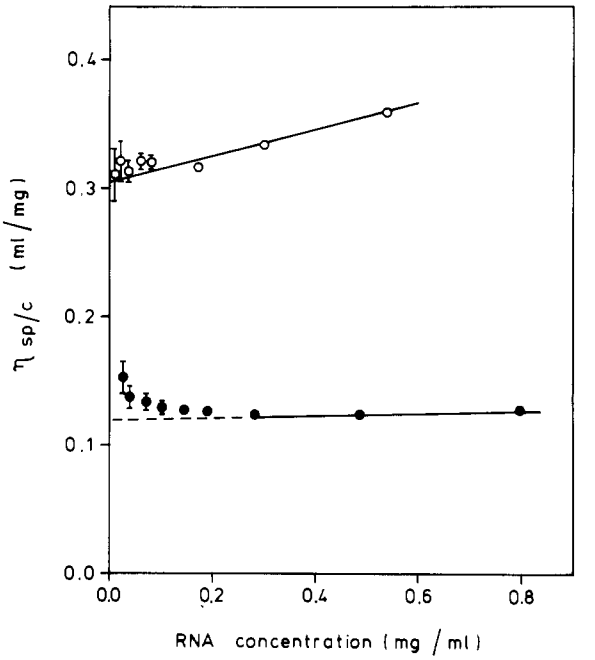

FIGURE 12: Dependence of the reduced viscosity of TYMV RNA on RNA concentration: (●) native TYMV RNA; (○) naked TYMV RNA. RNA samples were prepared by dilution with 0.01 M sodium acetate, pH 6.0. Incubation of the RNA samples was for 16 h at room temperature prior to viscometry. Temperature was 20.00 °C. Error bars for the standard deviation at low concentrations are indicated.

To get more insight on the phenomenon of the downward curvature in the plot of s vs. RNA concentration, we also performed viscometric experiments with native and naked TYMV RNA, respectively. Figure 12 shows the dependence of $\eta_{sp/c}$ on RNA concentration for both types of TYMV RNA in 0.01 M sodium acetate buffer, pH 6.0, after prior incubation of the RNA samples for 24 h at 20 °C. The concentration range studied was from 0.8 mg of RNA/mL down to 25 μg/mL. This implies that the data for the lower RNA concentrations necessarily will have a rather large error. The plot for naked TYMV RNA is seen to be linear with a positive slope, whereas that for native RNA shows a significant upward curvature at RNA concentrations below 0.2 mg/mL. In comparison witth native TYMV RNA, naked TYMV RNA shows a large increase in the $\eta_{sp/c}$ values, which is in agreement with the measured differences in sedimentation values.

Discussion

The major conclusions from the hydrodynamic studies presented in this paper are as follows: (1) The conformation of TYMV RNA shows a strong time-dependent behavior after its isolation and upon a change in the composition or temperature of the solution. This manifests itself in a decrease of the s value with time over the entire concentration range studied. (2) Native TYMV RNA, having bound multivalent cations, exhibits a curvilinear regression of its s value on concentration at low RNA concentrations, whereas a linear dependence was found for naked RNA (devoid of multivalent cations like Mg^{2+} and spermidine) or for native RNA if dilution was performed with dialysate. A nonlinear dependence was also found for native TYMV RNA in viscometry in contrast to naked RNA. (3) The dependence of s on RNA concentration not only may be linear or curved downward but also may even be curved upward dependent on the ionic conditions in the RNA sample and the buffer used as diluent.

The time-dependent decrease in the s value of TYMV RNA may be explained by the very compact conformation of the

RNA in the virion, where the negative phosphate residues are largely neutralized by cations like Mg^{2+}, Ca^{2+}, and polyamines. It is conceivable that the RNA upon isolation from the virion relaxes to a more extended conformation upon losing these multivalent cations (see below) or upon breaking of the interaction with the coat protein.

It is interesting to note that the RNA from TMV shows a rise in its *s* value from 26 to 27 S in 0.1 M sodium phosphate, pH 7, after 2 days of incubation after isolation from the virion (Boedtker, 1960). TMV RNA in situ has no secondary structure with short double-stranded helical regions but shows an extended helical configuration in which the phosphate charges are neutralized by the coat protein. After isolation, TMV RNA will adopt a compact conformation due to the formation of hairpin structures, which explains the time-dependent rise of the *s* value. Such a tendency for both plant viral RNAs to reach a more favorable thermodynamic conformation probably takes place by passing a large number of energy barriers (e.g., melting of double-stranded regions) which are then responsible for the large time effect. This time-dependent behavior is also found for other nucleic acid systems and is frequently coupled with hysteresis phenomena in spectroscopic and potentiometric titration (Cox & Katchalsky, 1972; Revzin et al., 1973a,b; Spodheim & Neumann, 1975; Siano, 1978) or after a melting and cooling cycle (Scheffler et al., 1968). Also, for TYMV RNA, a hysteresis loop is found between pH 4 and 6 in 0.1 M NaCl (Cox, 1966). However, it is uncertain whether the time-dependent conformational changes described here are based on the same mechanisms as those underlying the spectroscopic and potentiometric data.

The fact that native TYMV RNA shows more irreproducible results than naked TYMV RNA demonstrates that multivalent cations are largely responsible for this behavior. It has been shown earlier that when native TYMV RNA is diluted from a stock solution (in 0.01 M sodium acetate, pH 6) to a lower salt strength (0.001 M) a higher irreproducibility in sedimentation and viscometry is observed (Pleij, 1973). We here suggest that this irreproducibility may follow upon a shift in the number of multivalent cations bound to the RNA as caused by a change in the solution conditions like temperature, ionic strength, or RNA concentration. This in turn leads to a new equilibrium conformation for the RNA and thus to changes in its hydrodynamic properties. It has to be noted that the variability of the data as discussed here has to be discerned from the variation in results between different RNA preparations or virus harvests. The latter kind of variation is generally believed to be caused mainly by variable amounts of multivalent cations in the TYMV or TYMV RNA preparation (Mitra & Kaesberg, 1963; Dupont & Horn, 1968).

The curvilinear dependence of the *s* value on RNA concentration in the case of native TYMV RNA is not unique. Such behavior was also found for rRNA from *Escherichia coli* (Stanley & Bock, 1965; Pearce et al., 1975) although other authors report linear behavior (Cammack et al., 1969; Hill et al., 1977). In analogy to the same curvilinearity shown by protein systems, Pearce et al. (1975) suggested the existence of a rapidly changing monomer–dimer system as an explanation for this feature. On dilution of the RNA sample, the equilibrium should shift toward the monomer species, resulting in a concomitant decrease of the *s* value. Such an equilibrium should be fast since no boundaries corresponding to the monomer and dimer were observed in the sedimentation pattern. Sedimentation equilibrium studies appeared to be in favor of this dimerization model. If so, it would be the first description of a rapidly reversible dimerization of RNA in spite of many examples where RNA dimers or higher aggregates were shown to have separate boundaries, especially at higher ionic strengths (Olson et al., 1976; Mezl & Hunt, 1978; Crothers & Cole, 1978; our unpublished observations). On the basis of our results, an alternative explanation for the curvilinear regression seems to be possible. When native TYMV RNA (or rRNA) is diluted simply with buffer, a shift in the equilibrium between free and bound multivalent cations will take place in the direction of the free species. This results in a swelling of the RNA molecule because of the less effective screening of the negatively charged phosphate groups by the Na^+ ions. Support for this model comes from the fact that a linear plot of the *s* values was obtained for RNA which had been dialyzed and diluted with dialysate, or when naked RNA devoid of Mg^{2+} and spermidine ions was studied. Also, the slight upward curvature in the viscometric plot for native TYMV RNA strongly supports this model and is in contradiction with a monomer–dimer system. For a proper comparison of the sedimentation data with those obtained from viscometry, one has to keep in mind, first, that the value of $\eta_{sp/c}$ represents an average for all RNA molecules present and, second, that the 2M RNA deaggregates partially upon dilution to smaller fragments [see Figure 1 and Pleij (1973)]. The latter will cause a lower $\eta_{sp/c}$ value and therefore cannot be responsible for the increase of viscosity at lower RNA concentrations.

In order to obtain meaningful hydrodynamic data for natural RNAs, it is therefore of utmost importance to be aware of a possible presence of multivalent cations like Mg^{2+} or polyamines in the RNA preparations. On dilution of the RNA, it is necessary to maintain a constant activity of all free diffusible components which is achieved by dialyzing the solution and diluting it with dialysate (Casassa & Eisenberg, 1964). The importance of dialysis of polyelectrolyte solutions and the use of dialysate as diluent is stressed in many cases when density, refractive index increments, or light scattering measurements have to be performed (Tanford, 1961; Bloomfield et al., 1974; Cantor & Schimmel, 1980). These principles apparently are ignored or overlooked when hydrodynamic measurements on nucleic acids are performed. The phenomenon of the dissociation of a macromolecule–ligand complex by simply diluting with buffer was described for a DNA–dye complex (Drummond et al., 1966), and it was concluded that the nature of the complex is not well-defined when the chemical potential of the small molecule is not kept constant during dilution [see also Bloomfield et al. (1974)].

The occurrence of large amounts of Mg^{2+} and polyamines in RNP particles like ribosomes, phages, and plant viruses has been reported (Cohen, 1971; Bachrach, 1973). It has been claimed that the RNA phage R17 contains about 1000 molecules of spermidine which are sufficient to neutralize the phosphate groups completely. A certain fraction of these ions remains bound to the RNA after isolation depending on the conditions used (Fukuma & Cohen, 1975). The presence of a chelating agent like EDTA will not be sufficient to overcome the above-mentioned problems, so dialysis of the RNA solutions and dilution with dialysate should be necessary steps in the physicochemical characterization of natural RNAs. Alternatively, bound multivalent cations might be removed from the RNA (e.g., by precipitation), or the RNA preparation is dissolved in buffers with a high ionic strength. However, in these cases, it becomes questionable whether a functionally relevant conformation is retained.

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