*PHYSICAL PROPERTIES OF ROUS SARCOMA VIRUS RNA**

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The RNA's of several RNA tumor viruses have recently been isolated.[1] Two major single-stranded RNA components were obtained, a fast-sedimenting RNA component with a sedimentation coefficient ($s_{20,w}$) of approximately 70*S* and a slowly sedimenting component of approximately 4*S*. Since the reported s_w as well as the corresponding molecular weights of the tumor virus RNA's are much larger than those of most other known viral RNA's, a determination of the structure and molecular weight of tumor virus RNA's is of great interest. Knowledge of the structure of tumor virus RNA's may also help to elucidate their replication, which is interrupted by inhibitors of DNA-dependent RNA synthesis such as actinomycin D;[2] in addition, no virus-specific double-stranded RNA's have been isolated from infected or transformed cells.

In this study the effects of heat and dimethylsulfoxide (DMSO) on the s_w and the electrophoretic mobility of RSV RNA and of other RNA's of similar and smaller sizes were compared. The results indicated that the fast-sedimenting RSV RNA is an aggregate of smaller RNA's rather than a single polynucleotide as assumed earlier.[1]

Materials and Methods.—Standard buffer contained 0.1 *M* NaCl, 0.01 *M* tris(hydroxymethyl)aminomethane(Tris)-Cl, pH 7.4, and 0.001 *M* ethylenediaminetetraacetate (EDTA); and electrophoresis buffer contained 4 m*M* Tris-Ac, pH 7.2, 2 m*M* NaAc, 1 m*M* EDTA, 0.2% SDS, and 5% glycerol.

Viruses: Labeling with 5-H^3-uridine or P^{32} and purification of the Bryan strain of RSV[1, 3] and of Newcastle disease virus (NDV)[4, 5] have been described.

Preparation of the RNA's: RNA's were isolated by the phenol-SDS method.[5] The fast-sedimenting RNA components of RSV and NDV were prepared by glycerol gradient sedimentation (Fig. 1*A*). C^{14}-TMV RNA was a gift of Dr. H. Fraenkel-Conrat.

Melting of RNA's by heat and DMSO: RNA solutions in electrophoresis buffer were heated in sealed ampules and then cooled in melting ice. Ethanol-precipitated RNA was washed twice with 75% ethanol, dissolved at maximum concentrations of 1–5 μg/μl in electrophoresis buffer, and 20 vol of DMSO (Matheson and Coleman, spectroquality) were added at 0°C. The solution was incubated for about 30 min at room temperature and then cooled in an ice bath. For analysis in aqueous systems the RNA's were precipitated with 4 vol of ethanol after the addition of sodium acetate to a final concentration of 0.1 *M* (calculated on the basis of the aqueous volume) and carrier RNA to a total of at least 20 μg. After at least 2 hr at −20°C, the precipitate was collected by centrifugation at 30,000 *g* for 10 min.

Results.—Effects of heat and DMSO on the sedimentation velocity of RSV RNA in aqueous buffers: To test whether the fast-sedimenting RSV RNA represents a single polynucleotide or an aggregate, a melting experiment was carried out. Melting of single-stranded RNA by heat or DMSO destroys its secondary structure.[6] If the RNA is a single polynucleotide, this process is almost completely and instantly reversible,[6] but if the RNA is an aggregate, disaggregation will be observed.

A solution of H^3-RSV RNA, P^{32}-NDV RNA, and 100 μg TMV RNA was

divided into three equal aliquots (A, B, C). A served as a control to test the s_w of the unmelted RNA's. B was incubated at 80°C for 2.5 minutes, and C was incubated in 95 per cent DMSO. All three samples were then analyzed by glycerol gradient sedimentation. The gradients (Fig. 1) indicate that the s_w of H^3-RSV RNA was converted from 62S (Fig. 1A) to 36S (Fig. 1B, C) as a result of the heat or the DMSO treatment, while the s_w of NDV RNA (54S) and TMV RNA (31S) remained unchanged. The s_w of RSV RNA and NDV RNA were calculated by the method of Martin and Ames[7] with TMV RNA as a 31S[6] sedimentation marker. By the use of Spirin's relation, molecular weight (MW) = $1550 \times s^{2.1}$ (ref. 6), an approximate (MW) of 2.9×10^6 can be calculated for 36S RSV RNA. This MW is about four times smaller than that previously reported for RSV RNA.[1] A comparison of the distributions of the RNA's after melting showed that all three viral RNA's formed distinct peaks. However, their shapes were asymmetrical, which indicated that all three RNA's contained heterogeneous, more slowly sedimenting RNA species.

To determine whether RSV RNA was fragmented by shear forces during isolation, the RNA was released from the virus by pronase and SDS and directly sedimented before and after heat denaturation. To 200 μl of pronase at 1 mg/ml (Calbiochem, 2 hr predigested in 5 mM EDTA at 35°C) in 5 mM EDTA, pH 7.5, and 0.4 per cent SDS were added 100 μg TMV RNA and P^{32}-NDV RNA in 50 μl electrophoresis buffer and purified H^3-uridine-labeled RSV, grown during the 24 hours before the experiment, in 150 μl 10 mM NaCl, 2 mM EDTA, pH 7.5. The mixture was incubated for 20 minutes at 36°C and divided in I and II. II was heated to 80°C for 2.5 minutes. The sedimentation pattern of I was almost identical to that shown in Figure 1A, i.e., RSV RNA sedimented faster than NDV RNA; and that of II was almost identical to that shown in Figure 1B, i.e., RSV RNA sedimented slower than NDV RNA and slightly faster than TMV RNA.

Thus it can be concluded that the heat or DMSO-induced conversion of the 62S RSV RNA to a 36S RNA is probably the result of a disaggregation into smaller subunits or possibly of an extreme conformational change.

Hydrodynamic properties of the 62S and 36S RSV RNA compared to those of NDV and TMV RNA: Sedimentation patterns of 62S and of unfractionated RSV RNA directly from RSV consistently showed the following two peculiarities: First, in comparison with the peaks of NDV RNA and TMV RNA (Fig. 1A) the peak of RSV RNA (at concentrations of $< 0.1A_{260}$) was reproducibly broad; and it was asymmetrical, containing more radioactivity in the leading than in the trailing half of the peak, which indicates that RSV RNA is more heterogeneous than TMV and NDV RNA. Second, it was remarkable that the relative reduction of s_w of 62S RSV RNA after sedimentation at low ionic strength was less than that of NDV and TMV RNA (Fig. 2A). With TMV RNA as a 12S[8] sedimentation marker, unmelted RSV RNA can be calculated to sediment at 34S and NDV RNA at 24S, under the conditions of the experiment. Thus at low ionic strength the s_w of TMV RNA was reduced to 39 per cent, that of NDV RNA to 44 per cent, but that of RSV RNA was only reduced to 55 per cent of their values at high ionic strength. In contrast the s_w of 36S RSV RNA was reduced approximately as much at low ionic strength as those of TMV and NDV

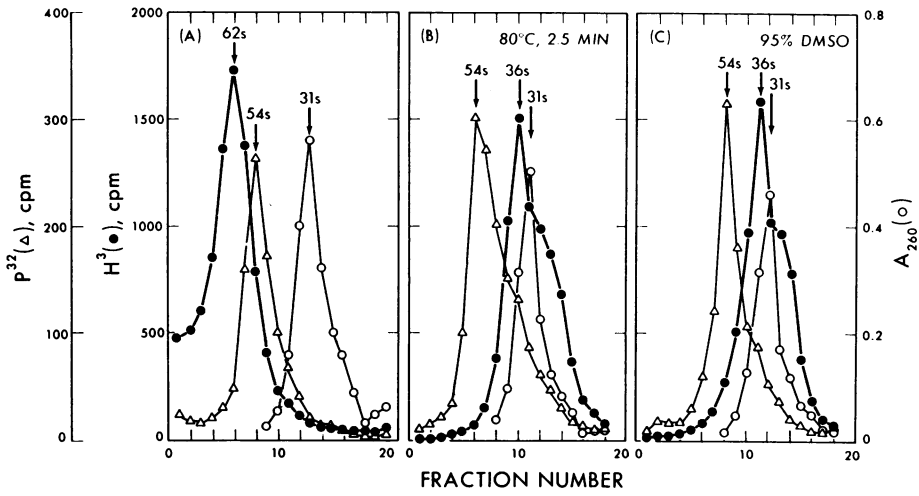

FIG. 1.—Sedimentation of H^3-RSV RNA (●), P^{32}-NDV RNA (Δ), and TMV RNA (○) before (*A*) and after (*B*, *C*) melting by heat or DMSO.

A mixture of H^3-RSV RNA, P^{32}-NDV RNA, and 100 μg TMV RNA in 450 μl electrophoresis buffer (*Methods*) was divided into three equal aliquots (*A*, *B*, and *C*). (*A*) was sedimented directly after the addition of NaCl to 0.1 *M*. (*B*) was incubated at 80°C for 2.5 min, and then NaCl was added to 0.1 *M* prior to sedimentation. (*C*) was incubated for 30 min at room temperature with 20 vol of DMSO; the RNA's were then precipitated (*Methods*) and redissolved in 150 μl standard buffer for sedimentation analysis. Centrifugation was in a glycerol gradient (15–30% w/v) in standard buffer for 60 min at 65,000 rpm in a Spinco SW 65 rotor at 8°C. Four-drop fractions were collected. The absorbance at 260 mμ was measured for each fraction after dilution with 200 μl H_2O. After the addition of 200 μg yeast RNA, TCA-precipitable radioactivity was collected and washed with 5% TCA on Millipore filters. The dried filters were counted in 10 ml toluene-based scintillation fluid in a Tri-Carb scintillation counter.

RNA's. As shown in Figure 2*B*, after melting, the s_w of NDV RNA (17*S*) was 32 per cent; that of TMV RNA (12*S*), 39 per cent; and that of RSV RNA (13.5*S*), 37 per cent of the respective values of these RNA's in standard buffer (Fig. 1*B*, *C*).

Thus the dependence of the s_w on ionic strength of 62*S* RSV RNA is quite different from those of 36*S* RSV, TMV, and NDV RNA's. This could be due to the large size of 62*S* RSV RNA, which would render the RNA more flexible and would allow it to assume a more compact tertiary structure at low ionic strength than smaller RNA's. But it could also be due to a special three-dimensional structure of 62*S* RSV RNA (such as an aggregate structure), which would interfere with the unfolding of the RNA at low ionic strength. This is suggested by the observation that the hydrodynamic properties of RSV RNA after melting are very similar to those of TMV and NDV RNA's.

Sedimentation of RSV RNA in DMSO: To determine the MW of RSV RNA independently of its putative aggregate structure and conformation in aqueous solvents, a sedimentation analysis in 99 per cent DMSO was carried out. The s_{DMSO} of a given RNA in 99 per cent DMSO has been found to be only related to its MW by the equation $s_{25,DMSO} = 0.052\ MW^{0.31}$.[9] As seen in Figure 3*A*, the s_{DMSO} of H^3-RSV RNA is significantly lower than that of P^{32}-NDV RNA and

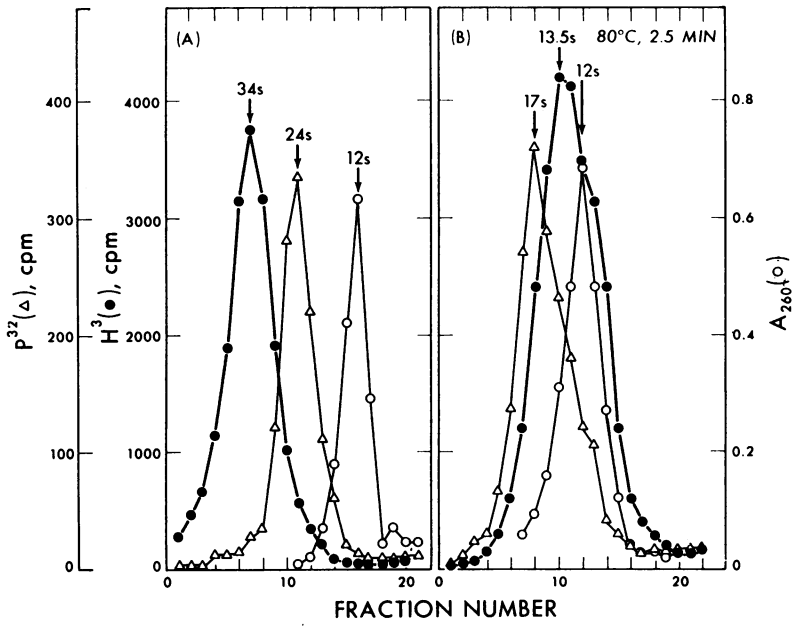

FIG. 2—Sedimentation of H^3-RSV RNA (●), P^{32}-NDV RNA (Δ), and TMV RNA (○) before (*A*) and after (*B*) heating at low ionic strength.

A mixture of H^3-RSV RNA, P^{32}-NDV RNA, and 80 μg TMV RNA in 300 μl electrophoresis buffer was divided into two equal aliquots (*A*, *B*). (*A*) was analyzed directly by sedimentation in a glycerol gradient (15–30%, w/v) containing 1 m*M* EDTA and 1 m*M* Tris HCl, pH 7.4. Centrifugation was for 2 hr as described for Fig. 1. (*B*) was incubated at 80°C for 2.5 min and then sedimented 4 hr as described for (*A*).

slightly higher than that of TMV RNA. A comparable result was obtained when H^3-RSV RNA was sedimented with P^{32}-chick cell RNA's (Fig. 3*B*). With TMV RNA as a reference of 4.9*S* (ref. 9) in 99 per cent DMSO, the s_{DMSO} of RSV RNA was estimated[7] to be 5.35*S*, which corresponds to an MW of 3.1×10^6 and that of NDV RNA to be 6.7*S*, which corresponds to an MW of 6.3×10^6. This MW of RSV RNA is in accord with that estimated above for 36*S*, but not for 62*S* RSV RNA in glycerol gradients. The MW estimates of NDV RNA in aqueous buffers[4] and in DMSO are in good agreement. This suggests that the observed change of the s_w of RSV RNA upon melting is most likely caused by a disaggregation of 62*S* RSV RNA into smaller RNA's. But it cannot be excluded that the 62*S* RSV RNA represents an extremely compact conformation of a single RNA with an MW of only 3.1×10^6.

Electrophoresis in polyacrylamide gels of RSV RNA before and after melting: If the low s_w of RSV RNA after melting were due to an irreversible or very slowly reversible unfolding of a single polynucleotide, its electrophoretic mobility (EM) in polyacrylamide should be lower than that of the 62*S* RNA. If, however, 62*S* RSV RNA is an aggregate, the EM of RSV RNA should increase after disaggregation. This has been observed in the experiments shown in Figure 4. The EM of 62*S* RSV RNA was slightly lower than that of NDV RNA (Fig. 4*B*). But if

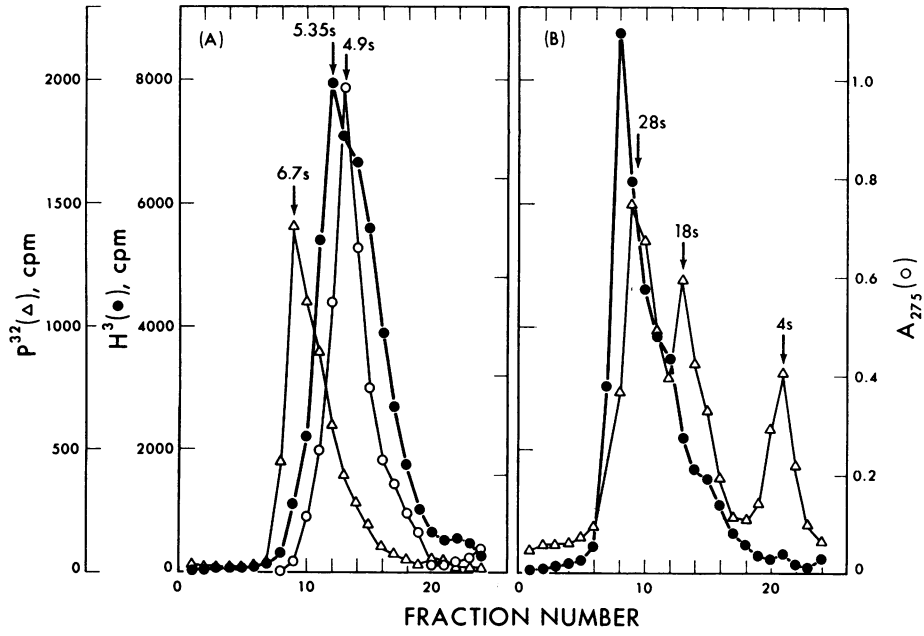

FIG. 3.—Sedimentation of H^3-RSV RNA (●), P^{32}-NDV RNA (△), and TMV RNA (○) (*A*) and of a mixture of H^3-RSV RNA (●) with P^{32}-cell RNA (△) (*B*) through DMSO.

(*A*) H^3-RSV RNA, which sedimented at 62*S* in gradients like that shown in Fig. 1*A*, P^{32}-NDV RNA, and 50 μg TMV RNA in 15 μl electrophoresis buffer was incubated with 200 μl DMSO for 30 min at room temperature. The solution was then layered on a sucrose gradient (5–20%, w/v) in 99% DMSO containing 10 m*M* LiCl and 0.5 m*M* EDTA. Centrifugation was for 7 hr at 65,000 rpm in a Spinco SW 65 rotor at 23°C. Six-drop fractions were collected. The absorbance at 275 mμ was measured for each fraction prior to determination of the radioactivity in 10 ml Bray's scintillation fluid. (*B*) A mixture of H^3-RSV RNA and P^{32}-cell RNA was prepared and sedimented as described for (*A*) but centrifugation was for 10 hr. The *S*-values in (*B*) refer to the $s_{20,w}$ of chick cell RNA's.

the two RNA's or a mixture of H^3-RSV RNA and C^{14}-TMV RNA or P^{32}-cell RNA's were melted prior to electrophoresis, the EM of H^3-RSV RNA was completely changed (Fig. 4*C*, *D*, *E*), whereas those of P^{32}-NDV RNA (Fig. 4*B*, *D*), C^{14}-TMV RNA, and the cell RNA's (not shown prior to melting) were unchanged. The difference between the EM of RSV RNA before and after melting was directly demonstrated by coelectrophoresis of 62*S* and 36*S* RSV RNA's with different radioactive labels (Fig. 4*A*). The largest component of RSV RNA moved as a sharp peak after melting with an EM which was significantly higher than that of NDV RNA and somewhat lower than that of 28*S* cell RNA and 31*S* TMV RNA (Fig. 4*C*, *D*, *E*). The rest had a heterogeneous distribution of higher EM's which were mainly in the mobility range of 28*S* and 18*S* cell RNA's. A linear relationship between the relative EM of an RNA in polyacrylamide gels and the logarithm of its MW has been described recently.[10] With this formula an MW between 2.3 and 2.7 × 10^6 can be estimated for RSV RNA after melting if the EM of RSV RNA is related to those of TMV RNA (MW = 2 × 10^6)[6] or 28*S* cell RNA (MW = 1.8 × 10^6)[6] and NDV RNA (MW about

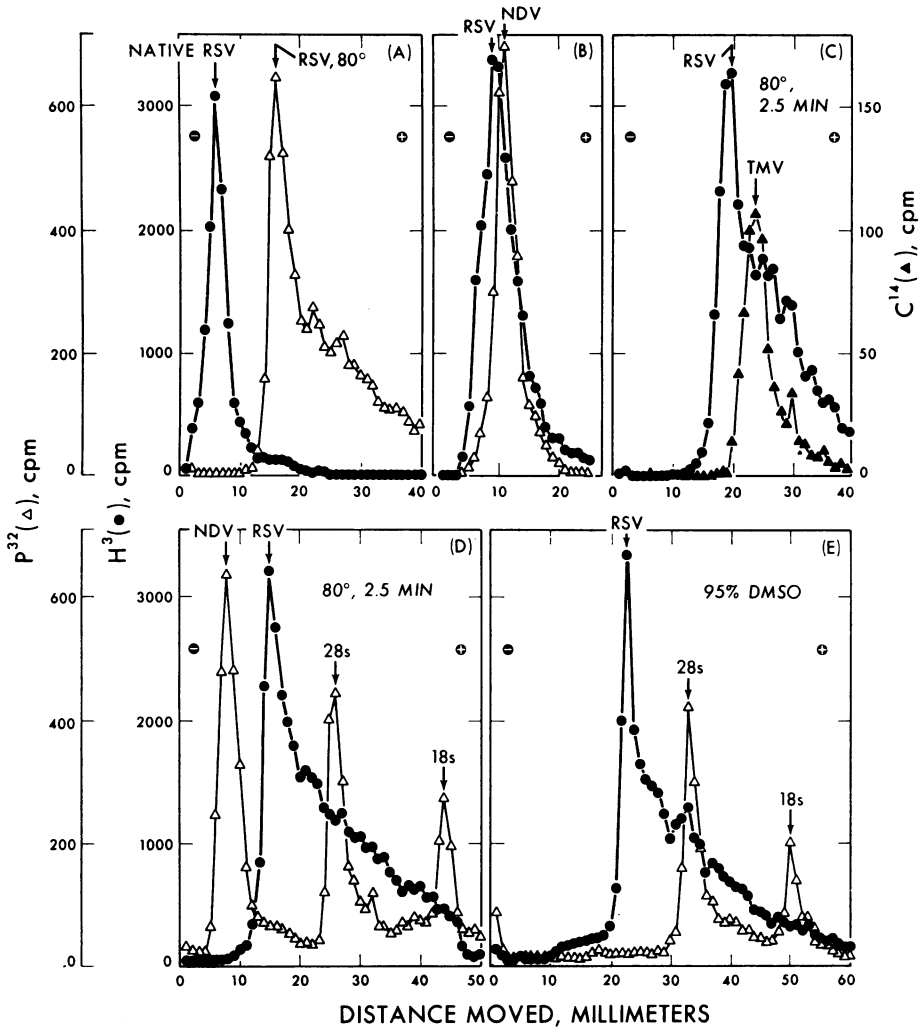

FIG. 4.—Electropherograms of RSV RNA and various marker RNA's before (*A*, *B*) and after (*A*, *C–E*) melting by heat or DMSO. Electrophoresis was in 2.2% polyacrylamide gels as described previously.[5] Samples were applied in electrophoresis buffer (*Methods*).

(*A*) Coelectrophoresis of unmelted H^3-RSV RNA (●) and P^{32}-RSV RNA (△), which had been incubated at 80°C for 2 min in 20 μl electrophoresis buffer. Electrophoresis was for 3.5 hr at 8 v/cm. (*B*) Electrophoresis of unmelted H^3-RSV RNA (●) and P^{32}-NDV RNA (△) for 4 hr at 10 v/cm. (*C*) Electrophoresis of a mixture of H^3-RSV RNA (●) and C^{14}-TMV RNA (▲) after incubation at 80°C for 2.5 min under the conditions described for (*A*). (*D*) Electrophoretic pattern of a mixture of H^3-RSV RNA (●), P^{32}-NDV RNA (●), and P^{32}-cell RNA (△) after incubation at 80°C for 2.5 min in 40 μl electrophoresis buffer. Electrophoresis was as for (*A*). (*E*) Electrophoresis of a mixture of H^3-RSV RNA (●) and P^{32}-cell RNA (△) which was incubated in 95% DMSO (*Methods*) prior to analysis under the conditions described for (*A*).

6.3×10^6), which indicates that melting of 62*S* RSV RNA results in disaggregation.

Dissociation temperature of RSV RNA: The described experiments suggest

that RSV RNA is an aggregate which could be linked by divalent cations or by base-paired overlapping regions of the RNA subunits. Elimination of divalent cations was attempted by dialysis. H^3-RSV RNA was dialyzed in presence of TMV RNA and P^{32}-NDV RNA in the cold for 24 hours each against 1000 volumes of 2.5 *M* LiCl, 0.05 *M* EDTA, 0.01 *M* Tris HCl, pH 7.4, 0.02 per cent SDS; 1000 volumes 2.5 *M* LiCl, 0.002 *M* EDTA, 0.01 *M* Tris HCl, pH 7.4, 0.02 per cent SDS; and finally 1000 volumes of 2 m*M* EDTA. This treatment did not change the s_w of any of the three RNA's. A salt-bonded RNA aggregate of influenza virus could be completely dissociated under similar conditions.[11]

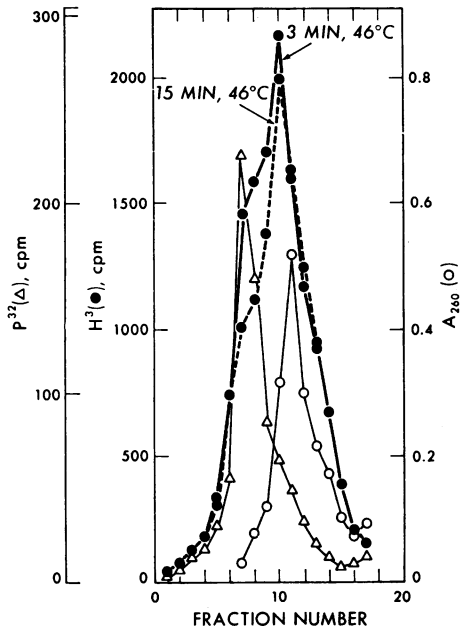

FIG. 5.—Sedimentation of H^3-RSV RNA (●), P^{32}-NDV RNA (△), and TMV RNA (○) after incubation for 3 min, and 15 min at 46°C in 0.01 *M* NaCl, 1 m*M* Tris-Cl, pH 7.4, 1 m*M* EDTA, and 0.1% SDS. NaCl was added to the RNA solutions to a final concentration of 0.1 *M* prior to sedimentation as described for Fig. 1.

Next the stability of the s_w of RSV RNA was studied after exposure to various temperatures. The s_w of RSV RNA remained unchanged after three minutes at 40°C. Between 40 and 52°C a transition of the high s_w to lower s_w was observed. Heating the RNA for three minutes at 46°C results in a partial transition of the s_w from 62 to 36*S*. When heating at 46°C was prolonged from 3 to 15 minutes, the sedimentation profile of RSV RNA slowly approached that of completely disaggregated RNA (Fig. 5). The transition was complete (as in Fig. 1*B*, *C*) after exposure for three minutes to 52°C or higher temperatures up to 100°C. This result is consistent with the idea that RSV RNA is an aggregate held together by weak bonds, for example by base-pairing.

Discussion and Summary.—RSV RNA appears to be an aggregate containing several RNA's, one major 36*S* RNA species and some minor heterogeneous RNA's. The 36*S* RSV RNA has an approximate MW of 3.0×10^6, as determined by sedimentation in aqueous buffers, in 99 per cent DMSO, and by electrophoresis in polyacrylamide gels. The minor RNA's could be essential viral RNA's or they could be fragmented 36*S* RNA's. The latter is suggested by analogy to NDV and TMV RNA, which to a lesser extent also contain smaller RNA's as revealed by melting. Since the aggregate is rather specific and could not be reaggregated (at 36°C in 0.01 *M* NaCl) after disaggregation, it possibly derived from one virus particle. The occurrence of RNA aggregates is not unique among viral and cellular RNA's. An aggregate of influenza virus RNA[11] has

already been discussed. Further, fragmented TMV RNA has been shown to preserve its apparent high MW in aqueous solutions at room temperature.[6] Analogous results were also obtained for an RNA aggregate of turnip yellow mosaic virus.[12, 13] Similarly, hydrodynamically intact ribosomal RNA's, which had been fragmented by RNase, have been described.[14]

Whether biologically active RSV RNA, however, is a continuous polynucleotide or an aggregate cannot be answered directly because no RNA-infectivity has been demonstrated. But by analogy with the RNA's of NDV, influenza virus, TMV, and the RNA's of the animal cell, it would appear that RSV RNA has an aggregate structure for the following two reasons: (1) The same method of (and simultaneous) extraction yields RNA's whose hydrodynamic properties are not changed by melting in the cases of NDV, influenza virus,[5] TMV, and the animal cell, but are changed by melting in the case of RSV. (2) When tests are done on chick cells, the ratio of physical particles to infectious particles for RSV[15] is about the same as those of NDV[16] and influenza virus.[16] Accordingly, it may be expected that the RNA of RSV would show the same degree of chemical integrity after isolation by the same method as the RNA's of NDV and influenza virus. This, however, was not the case for 62*S* RSV RNA. But the chemical integrity of the 36*S* RSV RNA was similar to that of the RNA's of NDV and influenza virus.

Similar experiments indicate that an RSV RNA-like aggregate is also present in mouse leukemia virus, a member of another group of RNA tumor viruses.[17]

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