JOURNAL OF VIROLOGY, Feb. 1974, p. 261-268

Vol. 13, No. 2
Printed in U.S.A.

Molecular Weight Determination of Sendai and Newcastle Disease Virus RNA

DANIEL KOLAKOFSKY, EDWARD BOY DE LA TOUR, AND HAJO DELIUS

Département de Biologie Moléculaire, Université de Genève, Genève, Switzerland, and Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Received for publication 21 September 1973

The molecular weights of Sendai and Newcastle disease virus RNA were estimated by sedimentation in sucrose gradients and by length measurements in the electron microscope under both denaturing and nondenaturing conditions. Sedimentation analyses under denaturing conditions yielded molecular weight estimates of 2.3×10^6 to 2.6×10^6, whereas length measurements yielded estimates of 5.2×10^6 to 5.6×10^6 for both denatured and nondenatured viral RNA. It would appear that the conditions of denaturation used (99% dimethyl sulfoxide at 26 C, and reaction with 1.1 M formaldehyde for 10 min at 60 C) do not equally denature parainfluenza virus RNA and other RNAs, such as cellular rRNA, 45*S* rRNA precursor, and R17 RNA.

The molecular weight of Sendai RNA estimated by sedimentation in sucrose gradients containing 99% dimethyl sulfoxide (DMSO) was found to be approximately half of that estimated under nondenaturing conditions and accounts for only half the RNA estimated to be present in the viral nucleocapsid (13). Three possible explanations of this difference have been investigated.

(i) The Sendai viral genome, like that of the RNA tumor viruses, is composed of a complex of more than one RNA chain which disaggregates upon denaturation (8, 10, 14).

(ii) The apparent molecular weight obtained by sedimentation analysis under nondenaturing conditions is artificially high due to the secondary structure of Sendai RNA, and the estimate obtained under denaturing conditions is more reliable. In this case, the viral nucleocapsid would contain two separate RNA chains of similar length, though not necessarily of identical sequence.

(iii) Conversely, sedimentation analysis in 99% DMSO is unreliable due to different degrees of denaturation of Sendai and the marker RNAs we have used for calibration, and the value obtained under nondenaturing conditions is therefore more reliable. In this case, the viral nucleocapsid would contain one continuous RNA chain of approximately 5×10^6 daltons.

By using mixing experiments in 99% DMSO, sedimentation analysis of formaldehyde-reacted RNAs in sucrose gradients with and without 1.1 M formaldehyde, polyacrylamide gel electrophoresis of formaldehyde-reacted RNAs, and length measurements of DMSO-treated, formaldehyde-treated, and heated Sendai and Newcastle disease virus (NDV) RNA in the electron microscope, we conclude that only the third possibility is correct; that is, Sendai RNA is a single, continuous RNA chain of approximately 5×10^6 daltons.

MATERIALS AND METHODS

Preparation of ^{32}P-48S Sendai RNA. Three-day-old confluent cultures of MDBK cells (obtained from P. Choppin) in 75-cm^2 Falcon tissue culture flasks were infected with 10 mean egg infective doses per cell of Sendai virus (Harris strain). After 45 min at 30 C, the infecting medium was removed and replaced with 8 ml of medium composed of 9 parts of phosphate-free medium 199 (Wellcome Laboratories) and 1 part of Dulbecco modified Eagle medium containing 50 μCi of carrier-free $^{32}PO_4$ per ml. At 18 h post-infection, the medium was removed (and discarded) and replaced with 8 ml of the above medium containing 10 μCi of $^{32}PO_4$ per ml. At 36 h postinfection, the medium was removed and centrifuged for 10 min at $5,000 \times g$ to remove cellular debris, and the virus was pelleted through a cushion of 25% glycerol in TNE (13) for 2 h at 36,000 rpm in the International A-170 rotor (8 C). The virus pellet was dissolved in TNE containing 0.5% sodium dodecyl sulfate (SDS) and 0.2 mg of Proteinase K (Merck) per ml at an approximate concentration of 2 mg/ml and incubated for 15 min at 25 C, and 150-μliter amounts were then centrifuged on 5 to 23% sucrose gradients as previously described (13). Fractions containing the 46 to 50*S* RNA were pooled, recovered by ethanol precipitation, and dissolved in 1 mM sodium acetate (pH 5.3), 1 mM EDTA, and 0.05% SDS (RNA buffer). RNA prepared as above had a specific activity of approximately

400,000 counts per min per μg on isolation.

Fragmentation of 48S Sendai RNA. A 0.4-ml amount of 0.2 M sodium carbonate was added to 1.2 ml of water containing 0.65 mg of Sendai 48S RNA (both solutions having been prewarmed to 50 C), and the solution was incubated for 15 min at 50 C. After addition of sodium acetate (pH 5.3) to a final concentration of 0.2 M, the RNA was precipitated with 2 volumes of ethanol, recovered by centrifugation, dissolved in 0.3 ml of RNA buffer, and sedimented on two 5 to 23% sucrose gradients (a third gradient contained ^{14}C-labeled cell RNA markers) for 3 h at 52,000 rpm (8°) in the SW56 rotor (13). By monitoring absorbance at 260 nm, the RNA was found to sediment as a broad band (3 to 27S) with a peak at 16S. The RNA was divided into 3 to 12S and 12 to 27S portions, and the 12 to 27S RNA was recovered by ethanol precipitation and again treated with sodium carbonate as above for a further 7 min at 50 C. Upon centrifugation in sucrose gradients, the RNA was found to sediment as a broad band (3 to 20S) with a peak at 10S. The 3 to 12S RNA from both gradients was pooled, precipitated with 2 volumes of ethanol, recovered by centrifugation, and dissolved in RNA buffer. Sendai RNA fragments prepared as above represented approximately 65% of the starting RNA.

Polyacrylamide gel electrophoresis. Gels of 2.2% acrylamide, 0.11% bis-methylene acrylamide, and 0.6% agarose were polymerized in 9-cm (0.5-cm internal diameter) quartz tubes in a buffer containing 40 mM triethanolamine (pH 7.8), 20 mM sodium acetate, 2 mM EDTA, and 2.5% glycerol. Electrophoresis was carried out for 2 h at 10 V/cm and 4 C (until bromophenol blue marker dye had just run off the gel) in the above buffer plus 0.2% SDS. After scanning at 260 nm in a Gilford spectrophotometer, the gels were frozen and cut into 2-mm slices. Slices were incubated with 0.4 ml of 0.5% SDS for 6 h at 50 C and were then counted by liquid scintillation after the addition of 3.6 ml of Aquasol (New England Nuclear Corp.).

Other methods and materials. ^{3}H-labeled 48S Sendai RNA, non-radioactive Sendai and NDV 48S RNA, and ^{14}C-labeled RNA from uninfected mouse kidney cells were prepared as previously described (13). Formaldehyde treatment of RNA and its centrifugation in sucrose gradients containing 1.1 M formaldehyde in SPB buffer were performed essentially as described by Boedtker (5).

RESULTS

Does Sendai RNA disaggregate upon denaturation? As previously shown (13), Sendai RNA which sediments at 48S in ordinary sucrose gradients sediments at $32S_{DMSO}$ in sucrose gradients containing 99% DMSO (relative to 18 and 28S rRNA markers arbitrarily set at 18 and $28S_{DMSO}$). Calibration of these DMSO gradients with 18S rRNA, R17 RNA, 28S rRNA, and 45S rRNA precursor yielded an estimated molecular weight of 2.3×10^6 for Sendai RNA under these conditions, whereas calibration of our ordinary sucrose gradients with 18 and 28S rRNA yielded a molecular weight estimate of 5.0×10^6. An estimation based on the chemical composition and length of the viral nucleocapsid relative to tobacco mosaic virus yielded a value of 5.0×10^6 to 5.6×10^6 daltons of RNA per nucleocapsid. The possibility that the Sendai genome like that of avian myeloblastosis virus (AMV) was segmented (8, 10, 14) and that the approximate 5.0×10^6 daltons of RNA that each Sendai nucleocapsid contained was present as a noncovalent complex of more than one RNA chain was also examined. RNA which had sedimented at $32S_{DMSO}$ was recovered from the DMSO gradient by ethanol precipitation and was then centrifuged on an ordinary sucrose gradient with untreated Sendai RNA. The results of this experiment (13) showed that, in contrast to AMV RNA, the DMSO-treated Sendai RNA was indistinguishable in its sedimentation properties from untreated Sendai RNA. This suggested that, if Sendai RNA was disaggregating in 99% DMSO, it was capable of rapid and quantitative reassociation upon removal of the RNA from DMSO (presumably during its concentration by ethanol precipitation and solution in water). To test this possibility further, the following experiments were carried out.

(i) Since brief periods of heating have been shown to disaggregate AMV RNA (8, 10, 14), ^{32}P-48S Sendai RNA in 1 mM EDTA, pH 7.0 (pretreated by passage through a column of Chelex-100) was heated in boiling water for 2 min, quick cooled, and then sedimented in ordinary sucrose gradients. Although some (minimal) thermal degradation did take place, the sedimentation rate of the bulk of the Sendai RNA was unaltered (data not shown). Therefore, if the Sendai genome were segmented, heating for 2 min at 98 C was not sufficient to effect the disaggregation.

(ii) To avoid steps which tend to concentrate RNA and therefore enhance its possible reassociation, ^{32}P-48S Sendai RNA was placed in 99% DMSO at a concentration of 1 μg/ml, diluted 10-fold with water, and directly centrifuged in ordinary sucrose gradients. Sendai RNA so treated was found to sediment unaltered as a homogenous band at 48S (data not shown). Although unlikely, the possibility cannot be excluded that Sendai RNA, even at such low concentrations (0.1 μg/ml), was capable of reassociation.

(iii) If 48S Sendai RNA is composed of a complex of two RNA chains which disaggregate in 99% DMSO to form two 32S RNAs and then reaggregate upon removal of the RNA from the denaturing solvent, mixing of the 32S RNAs should take place during this treatment. Furthermore, if while disaggregated, RNA frag-

ments derived from 48S Sendai RNA are added in excess, 32S Sendai RNA should preferentially reassociate with these fragments rather than with themselves to form a complex which would sediment between 32 and 48S, depending on the size of the fragment. Non-radioactive 48S Sendai RNA was therefore fragmented by limited base hydrolysis to a size of approximately 20,000 to 350,000 daltons (3 to 12S) (see Materials and Methods). ^{32}P-48S Sendai RNA was first sedimented in a 99% DMSO gradient, and the four peak fractions sedimenting at approximately $33S_{DMSO}$ (relative to ^{14}C-labeled cell RNA markers sedimented in a separate tube) were pooled. Two samples were then mixed with a 200- and a 400-fold excess by weight of Sendai RNA fragments (in 90% DMSO), respectively, while a third sample received no addition. After heating for 5 min at 37 C, the RNA was recovered from the DMSO solution by ethanol precipitation and dissolved in water, and ^{14}C-labeled cell RNA was added as sedimentation markers and resedimented in ordinary sucrose gradients. The results (Fig. 1) demonstrate that even a 400-fold excess of fragments does not significantly affect the sedimentation properties of the ^{32}P-Sendai RNA. This suggests that 48S Sendai RNA does not disaggregate into more than one RNA chain in 99% DMSO, but represents a single, continuous RNA chain.

There is, however, a possible objection to the interpretation of this last experiment. The above model assumes that very limited sequences of RNA in a much larger RNA are involved in holding RNA chains together. It is possible that, for the association to take place, the sequences involved must exist in a given structure which is so maintained by the rest of the RNA chain and that fragmentation of the RNA destroys this structure. If this were so, the non-radioactive fragments added in the above experiment might not compete in the reassociation of ^{32}P-Sendai RNA even at a 400-fold excess of RNA chains ranging in size from 20,000 to 350,000 daltons. Although each of the above experiments does not unequivocally exclude the possibility that 48S Sendai RNA disaggregates upon denaturation, taken together they strongly suggest that 48S Sendai RNA represents a single, continuous RNA chain.

Sedimentation of Sendai RNA under conditions of formaldehyde denaturation. In view of the different sedimentation properties of Sendai RNA relative to rRNAs in water and 99% DMSO, we next examined the possibility that the sedimentation value obtained in 99% DMSO was peculiar to the use of the nonaqueous solvent. Reaction of RNA with aqueous formaldehyde (HCHO) is also known to denature RNA (4) and, although formaldehyde does not destroy single-stranded stacking (15) and is therefore not as strong a denaturing agent for RNA as DMSO, Boedtker has shown that this method destroys secondary structure sufficiently to allow the resolution of RNAs by chain length (5, 6). ^{3}H-labeled Sendai RNA and ^{14}C-labeled cell RNA were therefore heated (10 min at 60 C) in the presence of 1.1 M formaldehyde and centrifuged in aqueous sucrose gradi-

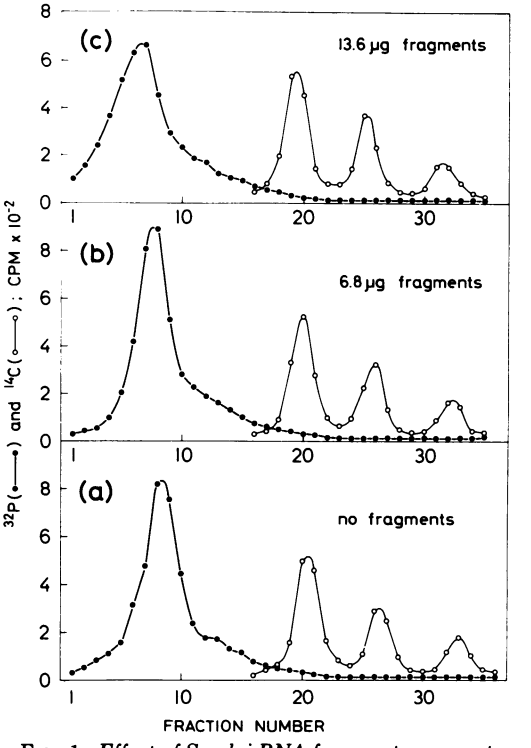

FIG. 1. *Effect of Sendai RNA fragments on renaturation of Sendai RNA from 99% DMSO. ^{32}P-48S Sendai RNA (0.16 µg, 24,000 counts/min) and ^{14}C-labeled cell RNA markers were sedimented separately in 99% DMSO-sucrose gradients as previously described (13). The four peak fractions of the ^{32}P-Sendai RNA, which sedimented at approximately $33S_{DMSO}$ (13), were pooled and divided into three 170-µliter samples containing 0.034 µg of ^{32}P-Sendai RNA (5,200 counts/min) each. Non-radioactive Sendai RNA fragments (1.36 mg/ml in 90% DMSO; see Methods and Materials) were added to two of the samples as indicated (6.8 µg of fragments, representing a 200-fold excess by weight, panel b, and 13.6 µg of fragments, a 400-fold excess, panel c), and the RNA was recovered from the DMSO solution by ethanol precipitation and dissolved in 0.1 ml of RNA buffer. Marker ^{14}C-labeled cell RNA was added to each sample, which was then sedimented in 5 to 23% sucrose gradients as previously described (13).*

ents containing 1.1 M formaldehyde. The results (Fig. 2) show that Sendai RNA sediments as a homogeneous band at $34.5S_{HCHO}$ relative to 18 and 28*S* rRNA markers, a result similar to that found with sucrose gradients containing 99% DMSO. By using *Escherichia coli* 4*S*, 16*S*, and 23*S* RNA as calibration markers, Sendai RNA is found to sediment at $37S_{HCHO}$ (data not shown). Since reaction with formaldehyde is only slowly reversible (4), the absence of formaldehyde in the sucrose gradient does not significantly affect the result. Figure 3 shows the result of a similar experiment in which duplicate samples of ^{3}H-labeled Sendai RNA and ^{14}C-labeled cell RNA were both placed in 1 M formaldehyde, but only one sample (*panel* b) was heated in formaldehyde and then centrifuged on sucrose gradients not containing formaldehyde. Formaldehyde-treated (i.e., heated in formaldehyde) rRNA sediments only slightly slower than untreated rRNA (compare panels a and b), whereas formaldehyde-treated Sendai RNA sediments considerably more slowly then untreated Sendai RNA. Calibration of these gradients with mouse 18 and 28*S* rRNA and *E. coli* 16 and 23*S* rRNA yield molecular weight estimates of 2.6×10^6 to 2.8×10^6 for Sendai RNA. The difference in sedimentation properties of Sendai RNA under denaturing and nondenaturing conditions previously noted with DMSO is therefore not peculiar to the use of this nonaqueous solvent.

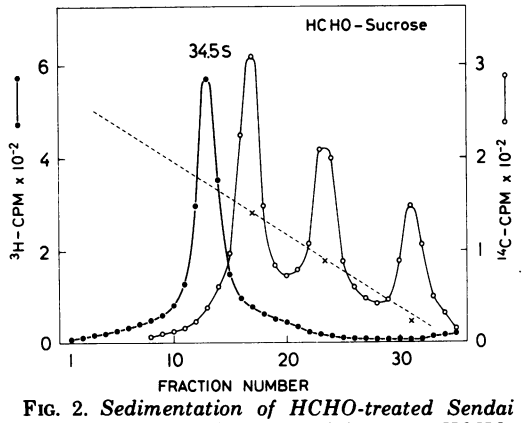

FIG. 2. *Sedimentation of HCHO-treated Sendai RNA in sucrose gradients containing 1 M HCHO. Eleven microliters of 28% HCHO in SPB buffer was added to 3,500 counts/min of ^{3}H-labeled Sendai RNA and 4,000 counts/min of ^{14}C-labeled cell RNA in 90 μliters of SPB buffer, and the sample was heated for 10 min at 60 C. The sample was then centrifuged on 5 to 23% sucrose gradients in SPB buffer containing 1.1 M HCHO (5) for 190 min at 50,000 rpm (8°) in the SW56 rotor. Fractions were collected and processed as for ordinary sucrose gradients (13).*

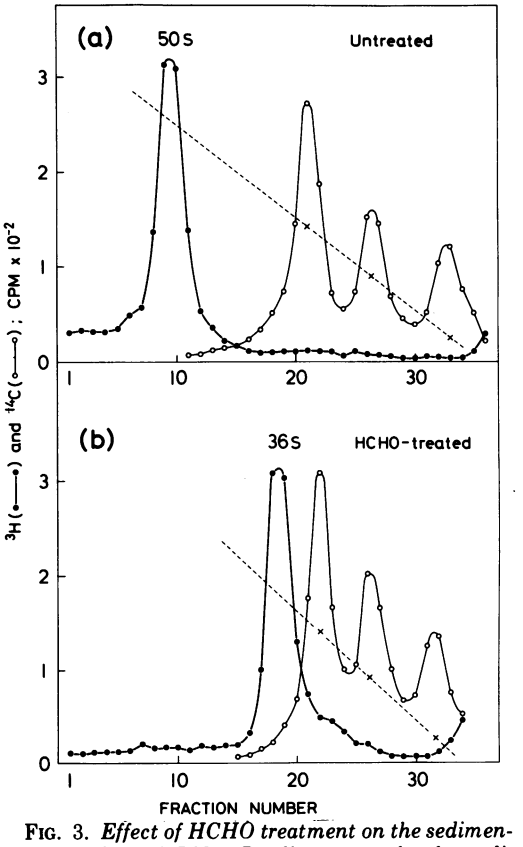

FIG. 3. *Effect of HCHO treatment on the sedimentation of Sendai RNA. Duplicate samples for sedimentation analysis contained 3,500 counts/min of ^{3}H-labeled 48S Sendai RNA, and 4,000 counts/min of ^{14}C-labeled cell RNA in 20 μliters of SPB buffer. Two microliters of 28% HCHO in SPB was added to each sample. One sample (panel a) was kept in ice; the other sample (panel b) was heated for 10 min at 60 C. The samples were then diluted with 80 μliters of TNE buffer and centrifuged on 5 to 23% ordinary sucrose gradients for 105 min at 50,000 rpm (7.5°) and processed as previously described (13).*

Molecular weight determination of Sendai and NDV RNA by electron microscopy. Since molecular weight estimates of Sendai RNA obtained by sedimentation analysis in sucrose gradients varied so greatly depending on whether the sedimentation was carried out under denaturing or nondenaturing conditions (2.3×10^6 to 2.8×10^6 as opposed to 5.0×10^6 daltons), and since this difference was apparently not a result of the disaggregation of the viral genome upon denaturation, this discrepancy would appear to be the result of a marked difference in secondary structure of Sendai RNA relative to the prototype RNAs such as cellular rRNAs used as calibration markers. The sedimentation of RNAs in nondenaturing

sucrose gradients at rates inconsistent with their molecular weights is well known. For example, R17 RNA (27.5S), which is barely distinguishable in sedimentation rate in ordinary sucrose gradients from 28S rRNA, sediments at a rate ($21S_{DMSO}$) consistent with its true molecular weight in gradients containing 99% DMSO (13). It would therefore appear that the molecular weight estimate of 2.3×10^6 to 2.8×10^6 obtained from sedimentation under denaturing conditions is more reliable, and that the estimate of 5.0×10^6 obtained under nondenaturing conditions was due to the fact that the structure of Sendai RNA, like R17 RNA, was considerably more compact than cellular rRNA.

To test this hypothesis, the molecular weight of Sendai RNA was estimated by electron microscopy, a method independent of hydrodynamic properties. Electron microscopy measurements were carried out on both Sendai and NDV RNA, these parainfluenza RNAs being indistinguishable in sedimentation rate in ordinary sucrose gradients (3, 13), sucrose gradients containing 99% DMSO (13), and sucrose gradients containing 1 M formaldehyde (data not shown). By using T4 gene 32 protein to aid in unfolding the RNAs and PM2 DNA as an internal calibration marker, both Sendai and NDV RNA were measured at 5.6×10^6 daltons (Table 1). Moreover, neither heating the parainfluenza RNAs for 2 min in boiling water nor treating Sendai RNA with 99% DMSO significantly altered the length measurements of the longest RNAs (Table 1). An example of these length measurements is shown in Fig. 4.

Electron microscopy measurements were also carried out on untreated and formaldehyde-treated NDV RNA without the aid of T4 gene 32 protein, since it was found not to bind well to formaldehyde-treated RNAs in buffer containing 1.1 M formaldehyde. In these measurements, no internal DNA marker was used for calibration, but R17 RNA, treated identically, was measured on separate grids. The results (Table 2) show that formaldehyde-treated NDV RNA is the same lengths as untreated NDV RNA (within the error of the measurements), yielding molecular weight estimates of 5.2×10^6. An example of these length measurements is shown in Fig. 5.

Under all conditions of length measurement by electron microscopy, Sendai and NDV RNA, whether untreated, treated with formaldehyde, DMSO, or brief exposure to heat, and spread with and without the aid of T4 gene 32 protein, have been found to have molecular weights of 5.14×10^6 to 5.67×10^6. Since this molecular weight estimate is considerably different from that obtained from sedimentation analysis under denaturing conditions (2.3×10^6 to 2.6×10^6), it would appear that Sendai and NDV RNA do not sediment under these denaturing conditions at a rate consistent with their molecular weight.

Are Sendai and rRNA equally denaturable? Since sedimentation under completely denaturing conditions should provide a reliable estimate of RNA chain length, we next examined the possibility that Sendai RNA, under the conditions of denaturation we have used, was not completely denatured but still possessed some structure which would cause it to sediment at a rate slower than that expected from its molecular weight. Structures known to behave accordingly, such as extended regions of double strandedness which increase frictional drag during sedimentation, would also be expected to decrease the electrophoretic mobility of RNA in polyacrylamide gels. However, since electrophoretic mobility of RNAs in polyacrylamide gels is inversely proportional to molecular weight, such structures would cause a decrease in the electrophoretic mobility and hence an overestimation of the molecular weight. Figure 6 shows the results of an experiment

TABLE 1. *Molecular weight determination by electron microscopy*

Sample	RNA in peak (%)	Mean length (μm)	PM2 length	Mean correction[a] for PM2 = 3.45	Daltons ($\times 10^6$)
NDV	88	6.36 ± 0.30	3.39 ± 0.12	6.47	5.63 ± 0.27
NDV heated	53	6.22 ± 0.30	3.50 ± 0.12	6.13	5.33 ± 0.26
Sendai	49	6.01 ± 0.18	3.18 ± 0.11	6.52	5.67 ± 0.17
Sendai DMSO	50	6.36 ± 0.38	3.58 ± 0.11	6.12	5.32 ± 0.32
Sendai heated	47	6.46 ± 0.45	3.59 ± 0.15	6.21	5.40 ± 0.39

[a] PM2 DNA, added as an internal standard, was used to correct for differences in magnification under the assumption that it should have a length of 3.45 μm (6.4×10^6 daltons) when compared to T7 DNA (25.2×10^6 daltons, 13.6 μm), which has been previously used to determine the factor of 870,000 daltons of RNA/μm of RNA-protein complex (7).

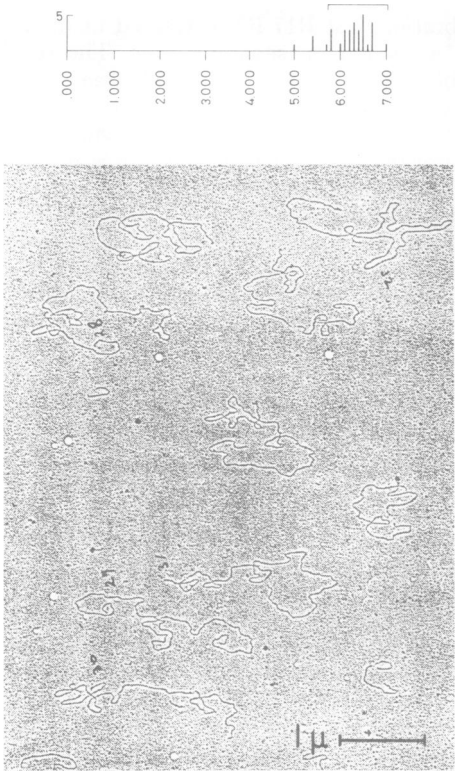

FIG. 4. *Length measurements of NDV RNA by electron microscopy. NDV RNA was reacted with T4 gene 32 protein, fixed with glutaraldehyde, and spread as previously described (7). The graph at the top of the figure shows the frequency distribution of lengths observed (abscissa represents length in micrometers; ordinate represents number of molecules). The horizontal bar in the graph denotes the length distribution of the peak used to determine the mean length. Bar length in photograph (final magnification, 28,800) equals 1 μm.*

designed to test this possibility. Duplicate samples of ^{3}H-labeled Sendai RNA and non-radioactive cell RNA were placed in SPB buffer and made 1.1 M in formaldehyde. One sample (panel b) was heated (10 min at 60 C) while the other (panel a) was left in ice, and then both samples were subjected to electrophoresis in polyacrylamide gels. The arrows in panel a, from left to right, show the positions of formaldehyde-treated Sendai RNA, 28*S* rRNA, and 18*S* rRNA obtained by normalizing their positions so that untreated and formaldehyde-treated 18*S* rRNA coincide. The electrophoretic mobility of Sendai RNA has been decreased proportionately more than that of 28 and 18*S* rRNA by formaldehyde treatment. Assuming that a linear semilogarithmic relationship of

TABLE 2. *Molecular weight determination by electron microscopy*

Sample	RNA in peak (%)	Mean length (μm)	Daltons[a] (× 10^6)
Untreated NDV RNA	49	5.14 ± 0.22	5.14 ± 0.22
HCHO-treated NDV RNA	64	5.24 ± 0.26	5.24 ± 0.26

[a] Bacteriophage R17 RNA, measured under identical conditions on separate grids, was found to have a mean length of 1.2 μm whether or not it had been reacted with formaldehyde. Molecular weight estimates of R17 RNA vary from 1.05 × 10^6 (11) to 1.3 × 10^6 (6). Assuming a molecular weight of 1.2 × 10^6, 1 μm of RNA spread by this technique is equivalent to 1.0 × 10^6 daltons. For details of spreading technique, see legend to Fig. 5.

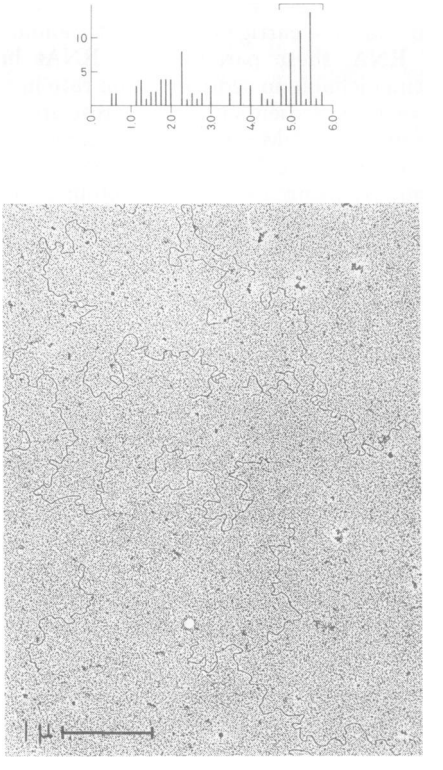

FIG. 5. *Length measurement of HCHO-treated NDV RNA by electron microscopy. RNA was spread without the aid of T4 gene 32 protein essentially as described by Inman and Schnos (12). One microgram of NDV RNA in 50 μliters of SPB buffer was made 1.1 M in formaldehyde and heated for 10 min at 60 C. Five microliters of the RNA solution was then mixed with 20 μliters of buffer as previously described (12) except that the pH was adjusted to 6.4, and 20 μliters of formamide, 4 μliters of 0.1% cytochrome and 5*

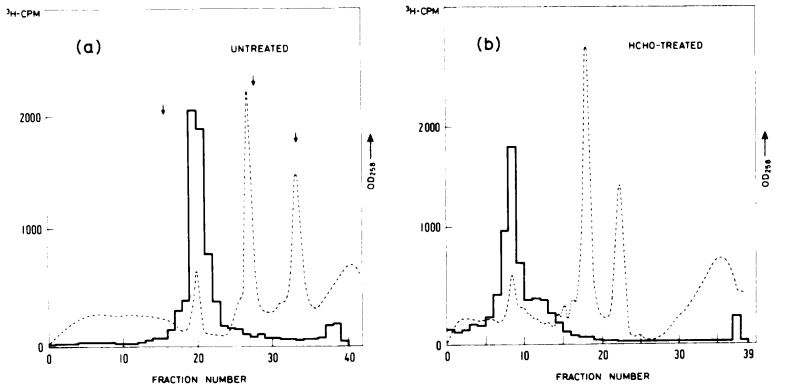

FIG. 6. *Polyacrylamide gel electrophoresis of untreated and HCHO-treated Sendai RNA. Duplicate samples for electrophoresis contained 12,000 counts/min of ³H-labeled 48S Sendai RNA (8,000 counts per min per μg) and 8 μg of cell RNA in 20 μliters of SPB buffer. To one sample (panel B) 2 μliters of 28% HCHO in SPB was added, and the sample was heated for 6 min at 60 C. Each sample was then diluted with an equal volume of 40% glycerol, subjected to electrophoresis in 2.2% polyacrylamide gels containing 0.6% agarose, and processed as described in Materials and Methods.*

mobility versus molecular weight holds true in our gel system as in others (2, 6), the molecular weight of formaldehyde-treated Sendai RNA estimated from the data shown in panel b is approximately 10×10^6, whereas that from panel a (untreated) is 4.7×10^6. Since electron microscopy measurements of the lengths of untreated and formaldehyde-treated NDV RNA show no significant difference, it seems clear that these parainfluenza RNAs under the denaturing conditions we have used still possess sufficient secondary structure which leads to unreliable molecular weight estimations using techniques based on the hydrodynamic properties of RNA.

DISCUSSION

Estimates of the molecular weight of RNAs by sucrose gradient sedimentation or polyacrylamide gel electrophoresis with RNAs of known molecular weight are only reliable when all the polyribonucleotide chains used are true structural homologues, i.e., their Stokes' radius is a monotonic function of chain length. It is clear from the data presented in this paper that the molecular weights obtained under the conditions of denaturation used are not reliable, and that parainfluenza RNAs and cellular rRNA therefore do not have the same hydrodynamic properties in 99% DMSO or after heating in 1.1 M formaldehyde.

Strauss et al. (16) have shown that 99% DMSO at room temperature sufficiently denatures RNAs from 0.55×10^6 to 2.1×10^6 daltons to allow their separation by sedimentation according to chain length. It is unlikely that this method is inapplicable to larger RNAs such as NDV RNA or Sendai RNA simply because of their size, since 45S rRNA precursor (4.1×10^6 [18]) sediments in sucrose gradients containing 99% DMSO at a rate consistent with its molecular weight (13). It seems more likely that the assumption that 99% DMSO at room temperature equally denatures all RNAs is incorrect. The finding of Ariv and Faulkner (1) that Sindbis RNA, another animal virus RNA, sediments in sucrose gradients containing 99% DMSO at a rate considerably slower than that expected from its molecular weight is noteworthy in this respect. Furthermore, high concentrations of formamide, another nonaqueous solvent known to destroy polynucleotide secondary structure (17), has been found by some workers to only partially denature certain RNAs at room temperature (16).

In the use of formaldehyde to denature RNA, Boedtker has shown that reaction for 15 min at 63 C is sufficient to permit molecular weight estimations in sucrose gradients or polyacrylamide gels (5, 6). Although in the experiments reported here the RNAs had been reacted for only 10 min at 60 C to minimize thermal degradation, reaction of the RNAs for 15 min at 63 C did not significantly alter the results presented here. Again, since formaldehyde-

μliters of this mixture was then spread on water processed for electron microscopy as previously described (12). Untreated RNA was processed identically, except that it was not heated. Details of the graph at the top of the figure are given in the legend to Fig. 4. Final magnification of photograph is 33,000.

treated Sendai RNA moves more slowly relative to cellular rRNA on both sucrose gradients and polyacrylamide gels, it seems clear that these RNAs do not have similar hydrodynamic properties and that formaldehyde treatment, like 99% DMSO, does not equally denature all RNAs.

The molecular weight estimates from length measurements in the electron microscope presented here have not been corrected for differences in base compositions between R17 RNA and the parainfluenza RNAs, or for the possible varying degree of stretching of DNA relative to the RNA-protein complex where T4 gene 32 protein has been used. For these reasons the molecular weight estimates have a probable uncertainty of ±10%. Nevertheless, in view of our findings that not all RNAs are equally denatured under such conditions as 99% DMSO or reaction with 1.1 M formaldehyde, we feel that molecular weight estimation methods which are independent of hydrodynamic properties, such as length measurement in the electron microscope, provide a more reliable result.

ACKNOWLEDGMENTS

We thank Pierre-Francçois Spahr for generous advice, encouragement, and support. Andrée Bruschi and Edith Gallay for excellent technical assistance, and Peter Bromley for helpful discussion.

This work was supported by research grant no. 3.816.72 and 3.810.72 from the Fonds National Suisse de la Recherche Scientifique.

ADDENDUM

Duesberg and Vogt have recently estimated the molecular weight of Sendai RNA by electrophoresis in formamide-polyacrylamide gels with 18*S* rRNA, 28*S* rRNA, and tobacco mosaic virus (TMV) RNA as calibration standards (9). By using either 18 and 28*S* rRNAs or 18*S* rRNA and TMV RNA to construct two linear calibration curves, they have obtained by extrapolation molecular weight estimates for Sendai RNA ranging between 2.5×10^6 and 3.8×10^6. However, since they did not observe a linear relationship between the mobilities of the three marker RNAs and the logarithm of their molecular weights, it seems unlikely that linear extrapolation will yield an accurate molecular weight estimate of Sendai RNA. As discussed above, the non-linearity they have observed may be due to unequal denaturation of their marker RNAs.

LITERATURE CITED

1. Arif, B. M., and P. Faulkner. 1972. Genome of Sindbis virus. J. Virol. **9**:102-109.
2. Bishop, D. H. L., J. R. Claybrook, and S. Spiegelman. 1967. Electrophoretic separation of viral nucleic acids on polyacrylamide gels. J. Mol. Biol. **26**:373-387.
3. Blair, C. D., and W. S. Robinson. 1968. Replication of Sendai virus, comparison of viral RNA and virus specific RNA synthesis with Newcastle disease virus. Virology **35**:537-549.
4. Boedtker, H. 1967. The reaction of RNA with formaldehyde. I. Optical absorbance studies. Biochemistry **6**:2718-2727.
5. Boedtker, H. 1968. Dependence of sedimentation coefficient on molecular weight of RNA after reaction with formaldehyde. J. Mol. Biol. **35**:61-70.
6. Boedtker, H. 1971. Conformation independent molecular weight determinations of RNA by gel electrophoresis. Biochem. Biophys. Acta **240**:448-453.
7. Delius, H., H. Westphal, and N. Axelrod. 1973. Length measurements of RNA synthesized *in vitro* by *Escherichia coli* RNA polymerase. J. Mol. Biol. **74**:677-687.
8. Duesberg, P. H. 1968. Physical properties of Rous sarcoma virus RNA. Proc. Nat. Acad. Sci. U.S.A. **60**:1511-1518.
9. Duesberg, P. H., and P. K. Vogt. 1973. Gel electrophoresis of avian leukosis and sarcoma viral RNA in formamide: comparison with other viral and cellular RNA species. J. Virol. **12**:594-599.
10. Erikson, R. L. 1969. Studies on the RNA from avian myeloblastosis virus. Virology **37**:124-131.
11. Gesteland, R. F., and H. Boedtker. 1964. Some physical properties of bacteriophage R17 and its ribonucleic acid. J. Mol. Biol. **8**:496-507.
12. Inman, R. B., and M. Schnos. 1970. Partial denaturation of thymine- and 5-bromouracid-containing λ DNA in alkali. J. Mol. Biol. **49**:93-98.
13. Kolakofsky, D., and A. Bruschi. 1973. Molecular weight determination of Sendai RNA by dimethyl sulfoxide gradient sedimentation. J. Virol. **11**:615-620.
14. Montagnier, L., A. Goldé, and P. Vigier. 1969. A possible subunit structure of Rous sarcoma virus RNA. J. Gen. Virol. **4**:449-452.
15. Stevens, C. L., and A. Rosenfeld. 1966. The secondary structure of polyadenylic acid. Inferences from its reaction with formaldehyde. Biochemistry **5**:2714-2721.
16. Strauss, J. H., Jr., R. B. Kelly, and R. L. Sinsheimer. 1968. Denaturation of RNA with dimethyl sulfoxide. Biopolymers **6**:793-807.
17. Ts'o, P. O. P., G. K. Helmkamp, and C. Sander. 1962. Secondary structures of nucleic acids in organic solvents. II. Optical properties of nucleotides and nucleic acids. Biochem. Biophys. Acta **55**:584-600.
18. Weinberg, R. A., and S. Penman. 1970. Processing of 45S nucleolar RNA. J. Mol. Biol. **47**:169-178.