Replication of Bacteriophage Ribonucleic Acid: Analysis of the Ultrastructure of the Replicative Form and the Replicative Intermediate of Bacteriophage R17

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A detailed qualitative and quantitative comparison was made of the ultrastructure of single-stranded ribonucleic acid (RNA) from bacteriophage R17 and double-stranded replicative form (RF) and replicative intermediate (RI) from cells infected with this bacteriophage. The nucleic acids were prepared for electron microscopy by the protein monolayer spreading technique of Kleinschmidt. Single-stranded RNA aggregated during spreading in the absence of urea, whereas RF and RI did not. On the other hand, RF and RI appeared to be susceptible to shear during spreading, whereas R17 RNA was not. From the maximal length of RF, a base translation of 3.14 Å was calculated. This value favors a 10-fold helix model of double-stranded RNA. The same base translation was found for R17 RNA, indicating a stacked base structure for single-stranded RNA spread in the presence of urea. RI is a branched structure and the branches are removed by ribonuclease treatment. The branches are believed to be nascent single-stranded viral RNA. The contour length of the branch was equal to the contour length of the main chain up to the branch point, as predicted from theoretical analysis of the replication of viral RNA. The structure of RF and the main chain of RI was also analyzed by plotting the log (end-to-end distance squared) versus log (contour length). This demonstrated structures intermediate in stiffness between a random coil and a rigid rod.

The key role of double-stranded ribonucleic acid (RNA) in the replication of the genetic material of RNA bacteriophages has been demonstrated by a wide variety of in vivo and in vitro experiments (9, 26, 27). Two species of double-stranded RNA have been isolated from cells infected with RNA bacteriophage and their properties have been described (1, 10-13). Replicative form (RF) is double-stranded RNA composed of a viral RNA molecule (+ strand) hydrogen-bonded to a complementary RNA molecule (− strand). Replicative intermediate (RI) is double-stranded RNA with nascent single strands. It is presumably derived from a more complex structure in which the RNA is probably associated with RNA polymerase and may sometimes be associated with polysomes (16).

Studies of the in vitro reaction have demonstrated a conversion of parental RNA to RF, subsequent conversion of RF to RI, and finally a transfer of label from RI to newly synthesized single-stranded viral RNA (26). Semiconservative and conservative models for this process have been published (9). Some of the experimental data favor the semiconservative model of replication (7, 8, 24).

In this paper, the ultrastructural characteristics of RF and RI are analyzed. Since RI contains both single- and double-stranded components, it was also necessary to investigate the ultrastructure of single-stranded RNA. Unfortunately, single-stranded and double-stranded RNA molecules had the same width in shadowed preparations, precluding any unequivocal identification of the two components of RI. However, two properties of single-stranded RNA could be exploited in comparative ultrastructural studies. These were its susceptibility to ribonuclease digestion and its intra- and intermolecular aggregation during preparation for electron microscopy.
Some preliminary data have been published on certain of the parameters to be discussed in this paper (14).

MATERIALS AND METHODS

Preparation of RNA. Single-stranded RNA from bacteriophage R17, double-stranded RF, and RI were prepared as previously described (11, 14). RI was denatured by heating to 95 C for 3 min in 10-2 M potassium phosphate buffer (pH 6.6) with 10-2 M ethylenediaminetetraacetic acid (EDTA) or by heating to 37 C for 10 min in phosphate-EDTA containing 85.7% dimethylsulfoxide (DMSO) as described earlier (13). DMSO lowers the melting temperature (Tm) of double-stranded RNA so that it may be denatured under very mild conditions (18).

In some experiments, RI was treated with ribonuclease to digest the single-stranded branches (12). Ribonuclease A (Worthington Biochemical Corp., Freehold, N.J.) was used at a concentration of 0.08 to 0.1 ug/ml, and the RI in 0.1 M NaCl, 0.001 M EDTA, 0.05 M tris(hydroxymethyl)aminomethane-chloride buffer at pH 6.98 (25 C) was treated for 10 min at 37 C (11). After digestion, the ribonuclease was removed by a single phenol extraction (11).

Controls were made by mixing RF and R17 RNA. Some preparations were simply mixed (2.3 optical density units, at 260 mua, for each RNA), and others were mixed and extracted once with phenol after mixing.

Preparation for electron microscopy: dilution of nucleic acids before spreading. The single-stranded RNA extracted from bacteriophage R17 was diluted in urea at a final concentration of either 6 or 8 M, to avoid aggregation of the RNA molecules due to intra- or intermolecular hydrogen-bonding (15; Granboulan, Scherrer, and Franklin, in press; Scherrer and Granboulan, in preparation). The concentration of the single-stranded RNA in urea was about 50 M/ml. RF and RI were diluted to a final concentration of 10 to 30 M/ml, either in (i) 6 or 8 M urea under the same conditions as the viral single-stranded RNA or in (ii) 0.02 M EDTA at neutral pH. Single-stranded RNA preparations spread from 6 to 8 M urea were always made at the same time in order to check the action of urea on the ultrastructure of RI.

RI which had been denatured with DMSO at low temperature or by heating in the absence of DMSO was diluted in the same manner as single-stranded RNA (in 6 or 8 M urea and at final concentrations of 2.5 or 5 M/ml).

In control reconstitution experiments, samples of RF and single-stranded RNA were mixed in equal concentrations and were then diluted in either 8 M urea or 0.02 M EDTA according to the procedure used for RF and RI. The same procedure was also followed on comparable "reconstituted" samples which had been treated with phenol after mixing. The final concentration of these samples was 20 M/ml.

Spreading and shadow-casting. All of the various preparations of nucleic acids described above were spread by the protein monolayer technique of Klein-schmidt and co-workers (19-22). Several proteins were successfully used to form the monolayer: cytochrome c (Fluka, Buch, Switzerland), chymotryptpsinogen (Nutritional Biochemicals Corp., Cleveland, Ohio), diisopropylphosphoryltyrpsin (Worthington Biochemical Corp.), and lysozyme (kindly provided by G. Cohen, Gif sur Yvette). Lysozyme was adopted for routine studies because it seemed to give the best differentiation between RNA molecules and background. It was used at a final concentration of 0.01% in ammonium acetate buffer (1 M, pH 8.0) containing 0.05% isopropanol. The solutions of protein and ammonium acetate buffer were always filtered on membrane filters (average pore diameter, 0.22 M; Millipore Corp., Bedford, Mass.) before use. Depending on the concentration of RNA, 0.1 or 0.2 ml of the RNA solution was added to 1 ml of lysozyme in ammonium acetate buffer; 0.2 ml of this final mixture was gently dropped onto a meticulously cleaned glass slide from which it flowed onto the surface of ammonium acetate buffer (0.015 M, pH 8.0) in a glass dish. The glass slides were cleaned by keeping them in a 1:1 solution of nitric acid in distilled water and then rinsing them with distilled water followed by ammonium acetate buffer (0.015 M, pH 8.0) just before use. They were always handled with forceps.

After spreading, the film was picked up on 300-mesh grids covered with a carbon-coated Formvar film. The specimens were dried in alcohol and isopentane and then shadowed with uranium oxide at an angle of 7° at a vacuum of 5 X 10^-4 mm of Hg. The specimens were rotated at 50 rev/min during shadowing. Some samples of RI were shadowed with platinum-carbon at the same vacuum, but without rotation.

Measurements. The specimens were examined with a Siemens Elmiskop I or a Philips EM 200 electron microscope at nominal magnifications between 15,000 and 30,000. The actual magnifications were determined with the aid of a carbon grating replica.

The lengths of the molecules were measured on prints with a map measurer at final magnifications of 45,000 to 130,000. The end-to-end distance was also determined.

RESULTS

Single-stranded RNA from bacteriophage R17. Although the single-stranded RNA was spread from solutions which were approximately two to five times as concentrated as RF or RI, the frequency of molecules per unit area of grid was far less than for double-stranded RNA. This may be due to the loss of single-stranded molecules in the ammonium acetate solution during the spreading process (14). It is not due to the presence of nucleases in the protein, since ribosomal RNA is not lost during spreading (Granboulan, Scherrer, and Franklin, in press; Scherrer and Granboulan, in preparation).

All of the molecules spread from solutions of 6 to 8 M urea were linear, i.e., noncyclic and nonbranched (Fig. 1). An analysis of 117 molecules...
revealed a modal length between 1.05 and 1.10 μ (Fig. 2) and a mean length of 1.06 ± 0.12 μ.

RF. There was no difference in the shape of RF spread in the presence or absence of urea. In both cases the molecules were linear (Fig. 3); there was no tendency to aggregate in the absence of urea (Fig. 3a) and no branched molecules were found. Besides the prominent population of molecules distributed around a mean length of 1.05 ± 0.03 μ and a modal length between 1.05 and 1.10 μ, there were smaller molecules with lengths corresponding to one-half and one-fourth that of the former population of molecules (Fig. 4).

An analysis of the shape of the spread RF molecules was made by means of a double-logarithmic plot of the mean square end-to-end distance versus the contour length (19, 20, 22). According to this analysis, the molecules spread in the absence of urea were intermediate in structure between a rigid rod and a random coil (Fig. 5).

RI. Whether spread in the presence or absence of urea, all preparations of RI contained linear molecules, some of which were branched and others of which were unbranched (Fig. 6–8). No circular molecules were found in any RI preparations. Branches were attached at all possible positions along the presumed main chain. The length of the branch varied according to the point of attachment to the main chain (Fig. 6). The variation in branch length was very striking in the rare cases of RI with two branches (Fig. 7). When RI was spread in the absence of urea, branches located close to one end of a chain were frequently coiled (Fig. 8c). This configuration was not observed when RI was spread in the presence of urea.

Data on the branches of RI molecules are presented in Table 1. Similar values were found for molecules spread in the presence or absence of urea. Only 50% of the molecules had branches, and almost all of those with branches had only a single branch. Very rarely, molecules with two or three branches were seen, but none with more than three branches. In a few instances, RI molecules appeared to have two branches at one point. Such molecules were examined closely at high magnification; in every case, these were two overlapping molecules. An example of two overlapping molecules is shown in Fig. 9b, a prepara-
tion of ribonuclease-treated R17 spread in the presence of urea.

R1 which had been treated with ribonuclease had few or no branches (Fig. 9). Of a total of 295 molecules spread without urea, only 13 (4.4%) were branched; of a total of 361 molecules spread with urea, only 7 (1.9%) were branched. These preparations behaved like RF, since there was not a striking loss of molecules during the spreading process as there was in the case of single-stranded R17 RNA. Furthermore, the molecules spread in the absence of urea did not form aggregates.

The maximal length of the presumed double-stranded component of R1 was the same as that of RF (Fig. 10, 11). The histogram for 881 molecules spread in the absence of urea had a peak between 1.00 and 1.10 μ (Fig. 10). The mean length calculated for the 333 molecules of length equal to or greater than 1.00 μ was 1.05 ± 0.06 μ. The histogram for 809 molecules spread in the presence of urea also had a peak between 1.00 and 1.10 μ (Fig. 11). The mean length calculated for the 434 molecules of length equal to or greater than 1.00 μ was 1.05 ± 0.04 μ. There were shorter molecules present in both types of preparations and there were peaks corresponding in length to approximately one-half and one-fourth the length of the longest molecules.

The shape or stiffness of the presumed double-stranded component of R1 was analyzed by a double logarithmic plot of end-to-end distance squared versus contour length. When spread in the absence of urea, these molecules were more rigid than a random coil but not as rigid as a stiff rod (Fig. 12). When spread in the presence of urea, the stiffness of the double-stranded component was increased, since the plot continued as
Fig. 3. Replicative form (a) spread in the absence of urea and (b) spread in the presence of urea. Whole molecules (length = 1 μ), as well as one-half and one-quarter molecules, are illustrated. × 60,000.
FIG. 4. Histogram of replicative form spread in the absence of urea; a total of 496 molecules were analyzed.

FIG. 5. Double logarithmic plot of the mean end-to-end distance squared versus the contour length (in µm) of replicative form spread in the absence of urea. In this plot, contour lengths were pooled into groups of 0.05-µ steps; variable sample numbers were available for each group (∅ = more than 50 samples per group; ○ = less than 50 samples per group). The dotted line represents the rigid rod.

a straight line up to the maximal length of RI (Fig. 13). This curve was almost identical to that of RF (Fig. 5).

According to the semiconservative model of replication, the branches of RI are the partially synthesized single strands of viral RNA which are displaced from the double strand as new viral RNA is synthesized (7, 9, 12). Since synthesis and displacement are presumed to occur simultaneously, the length of the branch should be the same as the length of the double strand to the branch point, provided that the mass per unit length of the single-stranded RNA is just one-half that of the double strand (9). This condition seems to be true when one compares the maximal lengths of R17 RNA and RF (see Discussion). Therefore, the model was investigated by measuring the contour length of the presumed double strand up to the branch point.

As already mentioned, it has not been possible to distinguish the single-stranded and double-stranded components of RI by their width. Nevertheless, from evidence presented here (see Discussion) and elsewhere (12), it is clear that one of the branches of the branched molecules is single-stranded. Theoretically, the single-stranded branch could have all possible lengths up to the length of the viral RNA (12). The double-stranded chain should be of constant length equal to the length of RF. Since there seemed to be breakage of the presumed double-strand during
FIG. 6. Replicative intermediate spread in the presence of urea. The arrows point out whole molecules with variation in the branch point and in the length of the branch. Rosette structures such as that seen in 6c were very rarely found in both RI and RF. The nature of these structures is not known. × 60,000.
spreading, the analysis of the relation between length of branch and its position on the main chain was done only on main chains of maximal length.

Despite the problem of identification of the branch, contour lengths of the presumed branch and of the presumed main chain up to the branch point were measured. Often there was a definite

FIG. 7. Replicative intermediate spread in the presence of urea. Whole molecules with two branches (arrows); variation in the branch point and in the length of the branch. × 60,000.
Fig. 8. Replicative intermediate spread in the absence of urea; unidirectional shadowing with PtC; (a, b) whole molecules with single branches; (c) long coiled branch attached to one end of a whole molecule. (a) $\times 105,000$; (b, c) $\times 102,500$. 
FIG. 9. Replicative intermediate treated with ribonuclease A (0.08 μg/ml, 10 min, 37°C) followed by removal of the enzyme by phenol extraction: (a) spread in the absence of urea; (b) spread in the presence of urea. No branches were observed in these preparations. There is a pair of crossed molecules in the upper left-hand corner of 9b. × 60,000.
break in the direction of the molecule at the branch point. Theoretically, the branch contour length ($\lambda$) should equal the length of the double-stranded molecule up to the branch point. Extensive measurements of these two parameters were made on whole RI molecules spread in the absence or presence of urea.

A scatter diagram of the data for RI spread in the presence of urea is shown in Fig. 14. There was a good correlation between $\lambda$ and $y$, the contour length of the main chain to the branch point.

**TABLE 1. Ultrastructure of replicative intermediate**

<table>
<thead>
<tr>
<th>Determination</th>
<th>Spread without urea</th>
<th>Spread with urea$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Total number of whole molecules$^b$</td>
<td>434</td>
<td>50.1</td>
</tr>
<tr>
<td>Total number of branched whole molecules</td>
<td>218</td>
<td>50.1</td>
</tr>
<tr>
<td>Whole molecules with</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No branches</td>
<td>216</td>
<td>49.8</td>
</tr>
<tr>
<td>One branch</td>
<td>191</td>
<td>44.0</td>
</tr>
<tr>
<td>Two branches</td>
<td>22</td>
<td>5.1</td>
</tr>
<tr>
<td>Three branches</td>
<td>5</td>
<td>1.2</td>
</tr>
</tbody>
</table>

$^a$ Spread in the presence of 6 to 8 m urea.

$^b$ Molecules with contour length of 1.0 to 1.1 $\mu$m.

Branches of all possible lengths were found, but most of the branches were 0.2 to 0.4 $\mu$m in length. The maximal length of the branches was 1.05 $\mu$m, approximately equal to the length of the single-stranded R17 RNA.

The apparent correlation between $\lambda$ and $y$ was confirmed by least-squares analysis from the equation $y = b\lambda + c$, where $b$ and $c$ are the constants to be determined (Table 2). This analysis was performed on a LOCI 2a computer with a program which handles a sample of 99 at any one time. Therefore, the samples of 266 molecules (RI spread in the absence of urea) and of 210 molecules (RI spread in the presence of urea) were split into three parts. Since the measurements were made at random, the values of $b$ and $c$ were very similar for each subsample. Whether spread in the absence or presence of urea, the resulting average values of $b$ and $c$ were the same and very close to the expected values of $b = 1$ and $c = 0$.

The distributions of lengths of $\lambda$ from RI spread in the absence and the presence of urea is shown in Fig. 15 and 16, respectively. Neither distribution was uniform, but the distribution was more uniform with RI spread in the absence of urea. When spread in the presence of urea, there was a skewed distribution with a maximum at 0.20 to 0.25 $\mu$m. This can also be seen in the scatter diagram (Fig. 14). In both preparations of RI, there were

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**FIG. 10. Histogram of the presumed main chain of RI spread in the absence of urea; a total of 881 molecules were analyzed.**

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Replicative Intermediate (spread with urea)
809 molecules

Fig. 11. Histogram of the presumed main chain of RI spread in the presence of urea; a total of 809 molecules were analyzed.

Fig. 12. Double-logarithmic plot of $\bar{L}$ versus contour length for the presumed main chain of RI, spread in the absence of urea. At all points the sample was 20 or greater. $\bigcirc =$ individual lengths; for lengths within the ranges indicated, data were pooled to give a sample of 20 or greater. The dotted line represents the rigid rod.

Fig. 13. Double-logarithmic plot of $\bar{L}$ versus contour length for the presumed main chain of RI, spread in the presence of urea. The number of samples per point varied between 26 and 69. The dotted line represents the rigid rod.
branches of all possible lengths up to the length of the single-stranded R17 molecules.

Denatured RI. Samples of RI denatured with DMSO or by heating could be examined only after spreading in the presence of urea. One attempt was made to examine the denatured RI spread in the absence of urea. There was a great deal of inter- and intramolecular aggregation, and therefore the remainder of the preparations were made in the presence of urea. Linear molecules of highly variable length were found in such preparations (Fig. 17). No branched molecules were seen. The length distribution of 860 molecules of RI denatured with DMSO showed a peak corresponding to molecules of R17 RNA (1.05 μ) and a heterogeneous population with lengths between 0.02 and 1.05 μ (Fig. 18). The length distribution of 389 RI molecules denatured by heating showed a similar heterogeneity (Fig. 19).

Mixed populations of RF and R17 RNA. Control experiments were done with 1:1 mixtures of double-stranded RF and single-stranded R17 molecules. In the presence of urea, branched molecules amounted to about 3% of the population (Fig. 20). There was no increase in the percentage of branched molecules in the absence of urea, but aggregates were frequently found. Exactly the same results were obtained with mixtures which had been extracted with phenol before spreading and mixtures which had not been extracted with phenol.

**DISCUSSION**

Since measurements of base translation are available for double-stranded RNA but not for single-stranded RNA, the length of the double-
stranded RF will be considered before that of the single-stranded viral RNA. The histogram of RF has three peaks, the peaks at the shorter lengths corresponding to approximately one-half and one-fourth the longest length. The mean of the cluster around the longest length was 1.05 μ. RF should have 3,342 base pairs, by comparison with R17 (28); therefore, the calculated base translation distance for spread RF was 3.14 A.

According to the original analysis of X-ray diffraction patterns of double-stranded RNA from reovirus, the RNA was thought to be a 10-fold helix with a pitch length of 30.0 A per residue (23, 29). More recent data and theoretical analyses of the structures have revealed an ambiguity, since the diffraction patterns could be accounted for by either a 10-fold or an 11-fold helix (2-4). Since the pitch length is the same for either helix, the base translation may be either 3.00 or 2.73 A per residue. The ambiguity arises from two possible ways of packing molecules in the crystallographic unit cell. Since contour length measurements are made on isolated molecules, this ambiguity does not arise in the present determination. With a base pair translation of 3.14 A and a pitch length of 30.0 A, the number of base pairs per pitch length would be 9.6, thus favoring the original 10-fold helix model of Langridge and Gomatos (23).

The single-stranded R17 RNA distribution has a single peak, and the mean length of R17 RNA is 1.06 μ. The number of nucleotides per molecule is 3,342 (28), and therefore the translation per base residue is 3.17 A for this RNA spread on monolayers from solutions of 6 to 8 M urea. This is the same internucleotide distance determined for RF, implying that the spread single-stranded RNA has a stacked base configuration. Evidence for stacked base structures in single-stranded nucleic acids in solution may be found in recent literature. Only one such example will be cited. The mass per unit length of single-stranded DNA, measured by small-angle X-ray scattering, is just half that of double-stranded DNA, implying stacking and ordering of the base planes in the single-stranded DNA (25).

The length distribution of the presumed main chain of RI is similar to that of RF, with a peak between 1.00 and 1.10 μ and smaller peaks corresponding to one-half and one-fourth this length. Neither RF nor RI had such populations of molecules prior to spreading, according to analyses of the molecular weight distributions by ultracentrifugation (12). The presence of short segments of RF from RNA bacteriophage M12 has also been noted in electron micrographs, but no distributions have been published (1). Populations of "one-half" and "one-fourth" molecules of
bacteriophage T2 DNA have also been observed in spread preparations, but it is not clear whether these preparations already contained such molecules prior to spreading (19). The double-stranded RNA has a greater rigidity than the single-stranded RNA (12), and it may be that the former RNA is subject to shearing forces during spreading on monolayers. Breakage of the double strands by shearing would explain the distribution into discrete whole, half, and quarter molecules (6). RI spread in the absence of urea had a higher proportion of whole molecules than RI spread in the presence of urea (Fig. 10, 11). Since the RI spread in the presence of urea would have a slightly greater rigidity than that spread in the absence of urea, it would have a slightly greater susceptibility to shear. Synthetic polymers (polyisobutylene) as small as 500,000 in molecular weight are susceptible to shearing (5), but it has been reported that double-stranded RNA from cells infected with fr bacteriophage is not degraded in a blender at 10,000 rev/min (17).

The problem of unequivocally identifying the single-stranded component of RI is very difficult to solve. Single-stranded R17 RNA has four characteristics which distinguish it from double-stranded RF: (i) aggregation in the absence of urea, (ii) loss during spreading, (iii) sensitivity to ribonuclease, and (iv) length distribution around a single mode rather than the tri-modal distribution of RF. None of these characteristics is useful for the identification of the single-stranded component of individual RI molecules. In the present study, only RI approximately 1 μ in length was used for structure analysis. The 1-μ component was considered to be the double-stranded main chain, and the components of shorter length were considered to be the single-stranded branches.

**Fig. 16.** Histogram of λ for RI spread in the presence of urea; a total of 210 whole molecules of RI were analyzed.
FIG. 17. Replicative intermediate denatured with (a) dimethyl sulfoxide or (b) by heating at 37°C for 3 min; both samples were spread in the presence of urea. × 46,800.
This approach is in keeping with theoretical and experimental analysis of RI (11-13). It enabled us to determine the length distribution of branches and correlate branch length with position on the main chain. There is admittedly an unavoidable ambiguity in identifying a "starting end" of the main chain. However, this can be eliminated by taking a starting end such that the branch point would be of approximately the same length as the main chain. If one measured from the other end of the main chain, then \( y \) would equal \( L - \lambda \) where \( L \) is the maximum length of the main chain.

**Fig. 18.** Histogram of RI denatured with DMSO; a total of 860 molecules were analyzed.

**Fig. 19.** Histogram of RI denatured by heating (3 min, 97 C); a total of 389 molecules were analyzed.
But rearranging the analysis so that \( y = \lambda \) for all determinations does not detract from the established geometrical relationship between the branch and the branch point.

The RI used in this analysis had approximately one single strand per double strand (11, 12). Assuming a Poisson distribution of single strands, approximately 37% of the population should be unbranched whereas only 50% of the population had visible branches. This may be due to two factors: loss of branches by shearing during spreading and the presence of very short branches which cannot be distinguished from the background grain. The presence of a smaller proportion of longer branches in RI spread in the presence of urea may be indicative of losses by shear-

**Fig. 20.** Example of a mixture of RF and R17 RNA spread in the presence of urea; in this example the two types of molecules were simply mixed together and then spread. \( \times 46,800 \).
ing, since the single-stranded branches should be stiffer in the presence of urea; very long branches might be denatured from the double strand in urea, since only a few hydrogen bonds may bind it to the main chain.

Denatured RI behaves like single-stranded RNA in that it aggregates if spread in the absence of urea. Similar distributions were obtained after denaturation at low temperature in the presence of DMSO or at high temperature in the absence of DMSO. The highest peak corresponded to the complete RNA molecule. No molecules longer than R17 RNA were found, but molecules of all possible shorter lengths were found. Neither distribution could be fitted to the expected distribution of equal numbers of molecules of all possible chain lengths from \( N = 0 \) to 3,341, plus twice this number of \( N = 3,342 \) (12, 13). Considering the difficulties of accurate measurements, however, the distribution of heat-denatured RI (Fig. 19) approaches the theoretical distribution. Except for the peaks at 0.30 to 0.35 \( \mu \) and 0.40 to 0.45 \( \mu \), the frequency of 0 < \( N < 3,342 \) was approximately 0.5 and the frequency for \( N = 3,342 \) was approximately 1.0. Thus, this distribution would also favor an RI structure containing only one branch per double strand.

No branched molecules were found when RF and R17 RNA were mixed, even if the mixture was treated with phenol before examination by electron microscopy. This makes it unlikely that RI arises as an artifact by association of single-stranded viral RNA and double-stranded RNA during phenol extraction of infected cells.

For further investigation of structures such as RI, it is imperative that some ultrastructural technique be developed to distinguish single-stranded and double-stranded RNA. The analysis of the branches of RI could then be made without any ambiguity.

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