Replication of Bacteriophage Ribonucleic Acid: Some Properties of Native and Denatured Replicative Intermediate

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Purified replicative form (RF) and replicative intermediate (RI) prepared from *Escherichia coli* cells infected with the ribonucleic acid (RNA) bacteriophage R17 were denatured with dimethyl sulfoxide at 37 C or in aqueous solvents of low ionic strength at 97 C. Denaturation was demonstrated for RF and RI by an increase in specific infectivity and a striking change in the hyperchromicity curves after treatment. RI denaturation was also demonstrated by a shift in the buoyant density in Cs_2SO_4 from 1.619 to the buoyant density of single-stranded R17 RNA (1.627). Analysis of the denatured RI hyperchromicity curves and the equilibrium distributions of denatured RI in Cs_2SO_4 gradients revealed, however, a residual double-stranded component. Velocity sedimentation of denatured RI was performed, and the weight distribution of S values was calculated. From the known relation between molecular weight and S values, it was possible to transform the weight distribution into a number distribution of chain lengths. This distribution was compared with that predicted from the steady-state hypothesis for RI. Deviations from the predicted distribution may be due to the residual double-stranded component.

The replication of single-stranded viral ribonucleic acid (RNA) has been investigated by studying the structure of replicative intermediate (RI), which is double-stranded RNA template with bound nascent viral RNA (7, 8). Thus, some of the details of the kinetic process of RNA synthesis can be explored by structural analysis, since RI is believed to represent a steady state of molecules undergoing polymer synthesis (8).

In the first paper to enunciate this principle, the structure of RI was explored by measuring its hydrodynamic properties and comparing them with the properties of single-stranded viral RNA and replicative form (RF), which is pure double-stranded RNA (8). In the present paper, the emphasis will be on the population of molecules arising after denaturation of RI. According to the theoretical analysis of RI, complete denaturation should yield a population of single-stranded molecules with equal numbers of molecules from dinucleotides up to a chain length of $\lambda-1$, where $\lambda = 3342$ is the number of nucleotides in the viral RNA, plus a larger number of molecules of chain length λ , which is the contribution of the double-stranded template.

There is no possibility for *a priori* prediction of the average number of single strands per double-stranded template. When the theoretical analysis of RI was applied to the data on the relative amounts of single- and double-stranded component in the population of molecules of RI, it was estimated that the average number of single strands of any length was *one* per RI molecule (8). It must be emphasized that this number may vary from batch to batch of RI and may also depend on the method of preparation. Therefore, the number of molecules of nascent viral RNA could be more than one in native RI *in vivo*. After denaturation of preparations having one single strand per template, there should be $2X$ molecules of chain length λ and X molecules with chain lengths from $\lambda-1$ to 2 (8). The $2X$ molecules of length λ derive from the template with a molecular weight of 2.2×10^6 .

As a preliminary to an analysis of the population of molecules derived from RI by denaturation, it is necessary to investigate means by which RI can be denatured. Denaturation of RI was followed by increase in infectivity, changes in buoyant density, and changes in hyperchromicity.

MATERIALS AND METHODS

Preparation of RNA. Growth of *Escherichia coli* strain 3000 and bacteriophage R17 has been described (9). Preparation of RNA from purified bacteriophage R17 and preparation and purification of RF and RI have been described (7, 8, 20).

Denaturation of double-stranded RNA (RF or RI).

(i) For heat denaturation, approximately 20 to 40 μ g of RNA per ml was dialyzed overnight against PE [0.001 M potassium phosphate buffer, pH 6.6; 0.001 M sodium ethylenediaminetetraacetic acid (EDTA); ionic strength 0.012]. This RNA was then placed in a tightly stoppered tube, the total volume being 1 ml. The tube was plunged into a boiling-water bath, held there for 3 min, and then quick-frozen in a dry ice-alcohol bath. (ii) For denaturation with dimethyl sulfoxide (DMSO), double-stranded RNA was denatured with DMSO according to the method of Katz and Penman (15). Approximately 200 to 400 μ g of RNA was dialyzed against PE. One part of this RNA was mixed with six parts of DMSO (Spectro-quality Reagent; Matheson, Coleman and Bell, Cincinnati, Ohio), giving a final DMSO concentration of 85.7% (v/v). This was incubated at 37 C for 10 min and then quick-cooled in an ice bath. NaCl was added to a final concentration of 0.1 M (calculated only on the basis of the aqueous volume), and the RNA was precipitated by addition of 2 volumes of ethyl alcohol followed by incubation overnight at -20 C. The precipitated RNA was taken up in PE and dialyzed against PE before infectivity tests or analytical sedimentation studies.

Infectivity of RNA. These tests were carried out by infecting spheroplasts with RNA and plating the spheroplasts for infective centers, by use of standard assay techniques. The preparation and infection of spheroplasts were carried out by modifications of the procedure of Francke and Hofschneider (6). A culture of *E. coli* 3000 was grown overnight in TC Gl medium (7), diluted 1:2,000 in TC Gl, and allowed to grow to a titer of 2×10^8 cells per ml. The cells were harvested by centrifugation (5,000 rev/min) at room temperature for 10 min, and were then taken up in one-tenth the original volume in five parts tris(hydroxymethyl)aminomethane (Tris) buffer (0.1 M, pH 7.7) and three parts 40% sucrose. Then EDTA and lysozyme were added to digest the cell wall and form spheroplasts. An example of this procedure, for 100 ml of original culture, was as follows. (i) A 6.25-ml amount of 0.1 M Tris buffer (pH 7.7) was added; (ii) 3.75 ml of 40% sucrose was added; (iii) 0.26 ml of 0.1 M EDTA was added; (iv) 0.26 ml of lysozyme (Worthington Biochemical Corp., Freehold, N.J.) was added at 2 mg/ml. The cells were incubated for 10 min at room temperature, resulting in a complete conversion to spheroplasts as observed by dark-field microscopy. At this stage, further lysozyme action was inhibited by addition to the above sample mixture of 0.14 ml of 0.5 M $MgSO_4$ and 0.16 ml of 30% bovine serum albumin (Fraction V from bovine plasma, Armour Pharmaceutical Co., Kankakee, Ill.).

The spheroplasts were kept at room temperature for 30 min. Then 0.2-ml samples were exposed to 0.05

ml of appropriate dilutions of RNA (usually 10, 1, and 0.1 μ g/ml in 0.1 M Tris buffer, pH 7.7). After an additional 30 min at room temperature, 0.2 ml of an *E. coli* 3000 suspension (10^8 cells per ml) was added, followed immediately by the addition of 2 ml of spheroplast soft agar; this mixture was poured onto nutrient agar plates. The spheroplast soft agar had the following composition: Difco agar, 0.75%; sucrose, 3%; $CaCl_2$, 0.01 M; $MgSO_4$, 0.01 M; NaCl, 0.5%.

After autoclaving, the pH was adjusted between 7 and 8 with 1 N NaOH. Bottom agar was the modified MS agar previously used (9).

Hyperchromicity. Hyperchromicity curves were recorded on a Gilford model 2000 spectrophotometric recorder. Glycerin was circulated through Beckman thermospacers, and the temperature of the circulating bath was set at higher than 100 C. The temperature, measured directly in the cuvettes, increased from 25 to 100 C in less than 45 min. The data were corrected for the expansion of water and plotted as optical density (OD) at 260 $m\mu$ at any given temperature relative to that at 25 C.

Analytical centrifugation. Sedimentation velocity experiments and analysis of the results were described in a previous paper (8).

Distribution functions in the Cs_2SO_4 equilibrium gradient were determined by use of a 12-mm aluminum centerpiece. The solvent contained 0.001 M potassium phosphate buffer (pH 6.6) with 0.001 M sodium EDTA (PE). Solvent saturated at 25 C with Cs_2SO_4 (optical grade; Stanley H. Cohen Associates, Yonkers, N.Y.) was added to 1 to 2 μ g of RNA in PE, and the density of the solution was then determined by refractometry (high accuracy Abbe '60' refractometer, Bellingham and Stanley) by use of the equation $\rho^{25} = 13.6986 [\mu D]^{25} - 173233$ (13, 21). The final solvent density was adjusted to the appropriate value to band the particular RNA approximately in the center of the cell. Equilibrium centrifugation was carried out at 42,040 rev/min at 25 C for 24 to 48 hr. Under these conditions, no change in the concentration distribution of nucleic acid occurred after 24 hr.

Densitometer tracings of the photographic record of the equilibrium distribution were made by use of a Spinco model R Analytrol with a microdensitometer accessory. The distribution of Cs_2SO_4 in the analytical cell was calculated from the usual equation (21): $\rho_0 = \rho + (\frac{1}{2}\beta)\omega^2(r_0 + r'_e)(r_0 - r'_e)$, where ρ_0 = density at band center; ρ = original density of the solution; ω = angular velocity; r_0 = distance from rotor center to band center; $r'_e = [\frac{1}{2}(r_b^2 - r_a^2)]^{1/2}$; r_b = distance from rotor center to bottom of the cell; r_a = distance from rotor center to meniscus; $1/\beta$ = constant.

The constant $1/\beta$ was determined from the collection of data on the position of the band center for a given species of RNA centrifuged in Cs_2SO_4 solutions of variable original density. The average value of $1/\beta$ was 1.252×10^{-9} (cgs), which differs from that reported by Erikson and Szybalski (5). This may be due to several factors, such as the relative purity of the Cs_2SO_4 . The purity of the Cs_2SO_4 would affect the determination of ρ and thus alter the value of $1/\beta$.

RESULTS

Demonstration of denaturation of RF and RI: Infectivity assays. According to Amman, Delius, and Hofschneider (1), double-stranded RNA (RF) from cells infected with an RNA bacteriophage is not infectious by itself but can be converted to infectious material by heating. The conversion is due to denaturation of the double-stranded RNA, yielding at least one infectious single-stranded RNA, the "+" strand (1, 6, 12a). This conversion has been demonstrated for both RF and RI (6, 12a). There is also some suggestion that RI is infectious before heat denaturation and that the specific infectivity increases after heating (R. Erikson, E. Erikson, and J. Gordon, *J. Mol. Biol.* 22:257-268, 1966).

Data on the increase in infectivity after DMSO denaturation of preparations of RF or RI are reported in Table 1. The infectivity of a single batch of viral RNA varied by a factor of 10 in individual experiments, two of which are reported in Table 2. This variation was probably due to variation in competence of the spheroplasts.

TABLE 1. *Infectivity of viral-specific RNA species*

Prepn	PFU per μ g of RNA	Increase (fold) after DMSO treatment
R17 RNA (A) ^a	1.22×10^5	—
R17 RNA (B) ^a	1.35×10^4	—
Replicative form (RF).....	492	—
DMSO-denatured RF..	6,644	13.5
Replicative intermediate (RI).....	41	—
DMSO-denatured RI..	474	11.6

^a Standard RNA samples were assayed on two different batches of protoplasts. Batch A was used in the RF assay and batch B in the RI assay.

TABLE 2. *Buoyant density of native and denatured viral RNA species*

Expt no.	Sample	ρ_0
5	R17 RNA	1.627
7	R17 RNA	1.627
13	R17 RNA	1.628
1	RI	1.619
3	RI	1.619
8	RI	1.619
16	Denatured RI ^a	1.631
17	Denatured RI ^a	1.625
10	Denatured RI ^b	1.633
15	Denatured RI ^c	1.627

^a Treated for 3 min at 97 C.

^b Treated with 85.7% DMSO for 10 min at 37 C.

^c Treated with 85.7% DMSO for 10 min at 45 C.

Every test of infectivity of RF or RI was accompanied by a test of viral RNA. In a typical experiment, there was a 10- to 20-fold increase in infectivity after DMSO treatment of either RF or RI. Thus, the experiments of Hofschneider and co-workers on heat denaturation of RF and RI (12a, 15) and the present experiments with DMSO suggest that both methods may be useful in investigating the pattern of single-stranded molecules derived from RF and RI by denaturation. Most of the present data are concerned with RI, since conversion of RF to 27S single-stranded RNA has been well established (12a). Some comparative data on the denaturation of RF and RI are also presented.

Hyperchromicity. Figure 1 presents a comparison of hyperchromicity of R17 RNA, RF, and RI. As expected, the single-stranded RNA denatures over a very broad range of temperatures (2), the double-stranded RNA over a very short range (11, 16), and RI has a complex hyperchromicity owing to its partially single-stranded and partially double-stranded properties (7). Thus, denaturation of RF or RI should result in alterations in the hyperchromicity curve. This was best demonstrated with RF (Fig. 2), which appears to be completely converted to single-stranded RNA after DMSO denaturation (10 min at 37 C), at least according to the criterion employed in this experiment.

RI denatured by treatment for 10 min at 37 C with 85.7% DMSO still retained a small amount of double-stranded character as determined by the hyperchromicity test (Fig. 3A). This was retained even after more drastic treatment with DMSO (10 min at 45 C with 85.7% DMSO, Fig. 3B), or after heat denaturation (3 min at 97 C, Fig. 3C). The smallest amount of residual

FIG. 1. Temperature dependence of OD at 260 $m\mu$ of R17 RNA, RF, and RI, all in PE (ionic strength 0.012). The OD at 260 $m\mu$ is plotted as relative increase in OD at 260 $m\mu$ at any given temperature compared with that at 25 C.

FIG. 2. Relative increase in OD at 260 $m\mu$ as a function of temperature of native RF (○) and RF treated with DMSO (●). Both samples were measured in PE.

FIG. 3A-C. Relative increase in OD at 260 $m\mu$ as a function of temperature of native RI and RI treated as indicated. All samples were measured in PE. In Fig. 3C, heating at 97°C was followed by quick-freezing at -70°C prior to determining the hyperchromicity curve.

double-stranded component was found after heat denaturation.

Equilibrium distributions in Cs_2SO_4 . The buoyant density of a single-stranded viral RNA was invariably lower than that of double-stranded RF isolated from cells infected with that virus (cf. 4). Since RI seems to have an intermediate value of buoyant density according to equilibrium data in the preparative ultracentrifuge (3), buoyant density provides another criterion for denaturation. With the availability of highly

FIG. 4. Cumulative distribution of the equilibrium concentration of R17 RNA and RI preparations in a Cs_2SO_4 gradient. The cumulative distribution on a probability scale is plotted versus distance from the rotor center. (A) R17 RNA; (B) native RI; (C) mixture of RI and R17 RNA (ratio of RI to R17 RNA, 1.01).

purified RI, the buoyant density of RI was carefully compared with that of R17 RNA in Cs_2SO_4 in the analytical cell.

Some of the data on the buoyant densities are shown in Table 2. The average buoyant density of single-stranded R17 RNA was 1.627 and of RI, 1.619, values consistent with the reported values of 1.630 and 1.616, respectively (3). After denaturation, the values scattered somewhat but were all close to the value for single-stranded RNA. An analysis of the equilibrium distribution, however, indicated that double-stranded RNA was still present in the heated or DMSO-treated RI preparations. This analysis was carried out by

plotting on probability paper the cumulative distribution of the equilibrium concentration of nucleic acid versus distance from the rotor center. The height above base line of the densitometer tracing was taken as a measure of nucleic acid concentration. The distribution was started at the point nearest the rotor center. This plot has several advantages: (i) a Gaussian distribution is transformed into a straight-line plot; (ii) σ can be read directly as the abscissa height between ordinate values of 0.500 and 0.841; (iii) the mean, which should correspond to the peak for the Gaussian distribution, is read directly from the ordinate value of 0.50; (iv) as compared with the

FIG. 5. (A) Distribution of the equilibrium concentration of denatured RI. (B) Same as 5A, plotted as a cumulative distribution on probability paper.

FIG. 6. (A) Weight distribution of S value for RI denatured with 85.7% DMSO for 10 min at 37 C. This RI was centrifuged in a solvent of ionic strength 0.06 at 52,640 rev/min. (B) Number distribution of chain lengths for RI denatured with 85.7% DMSO for 10 min at 37 C. This is a transformation of the data presented in Fig. 6A. The arrow indicates the position of R17 RNA (molecular weight, 10^6 to 1.1×10^6 ; chain length, $\lambda = 3,342$).

plot of band height versus (band width)²/8 β (19), the probability plot is more accurate, since it avoids the large errors due to squaring the largest band widths, which are most difficult to measure because they are closest to the base line.

The Gaussian plot of the equilibrium distribution of bacteriophage R17 RNA is shown in Fig. 4A; that of RI, in Fig. 4B; and that of an artificial mixture of R17 RNA and RI, in a ratio of 1.01 RI to 1 R17 RNA, is shown in Fig. 4C. The R17 RNA distribution is Gaussian, as is the RI distribution. The Gaussian distribution of RI is unexpected and will be commented on in the Discussion. The two-component distribution of the mixture of RI and R17 RNA was clearly demonstrated by the probability plot (Fig. 4C). The peak of this distribution was located at a density of 1.614 g/cm³. The equilibrium concentration distributions for denatured RI are shown in Fig. 5A, and the probability plot of the cumulative distributions, in Fig. 5B. Under all conditions of denaturation, the distributions are skewed toward the buoyant density of single-stranded RNA, and the departure from the Gaussian distribution is clearly shown in Fig. 5B. The position of the peak in relation to distance from the rotor center depended on ρ and the position of the meniscus in the ultracentrifuge cell.

Thus, the experiments designed to demonstrate denaturation of RI and RF indicated that there was a residual double-stranded component present in RI, although the major component was denatured single-stranded RNA. The hyperchromicity curves indicated that heat-denatured RI had a smaller residual double-stranded component than DMSO-denatured RI.

Velocity sedimentation analysis of denatured RI. The above experiments, although interesting in themselves, were preliminary to the analysis of denatured RI. The following strategy was used. Since the theoretical analysis of RI viewed as a steady-state population predicted equal numbers of fragments with chain lengths of 2 to $\lambda-1$, then the most meaningful analysis would be a plot of the number distribution of chain lengths. This distribution can be derived from the weight distribution of S values (17) provided that the relationship between molecular weight and S value is known. Single-stranded R17 RNA in solutions of high ionic strength has, however, a sedimentation constant which is higher than that expected from the Spirin or Gierer relationship between S and M (7). Therefore, the ionic strength of the viral RNA was carefully adjusted according to the curve of dependence of the S value of this RNA on ionic strength (8) so that the S value of the complete molecule of molecular weight 10^6 to 1.1×10^6 would correspond to the

value predicted from the well-known relationship between S and M . In particular, an ionic strength of 0.06 M was used to obtain $S = 22.5$. To clarify and recapitulate, RI was denatured in solutions of low ionic strength as described in Materials and Methods. Just before performing a standard velocity sedimentation analysis, the ionic strength was adjusted to 0.06 M by adding the appropriate amount of NaCl. A weight distribution of S values [$q(S) = 1/C_0 \times \Delta C_0 / \Delta S_{20,w}^0 \times 100$] was calculated (17), plotted, and then transformed into a number distribution of chain lengths. The weight distributions [$q(S)$] are shown in Fig. 6A and Fig. 7A. The number distributions [$N(S)$] are shown in Fig. 6B and 7B.

The transformation from a weight distribution to a number distribution [$N(S)$] was done as follows. A molecular weight was assigned to each S value by use of the Spirin equation, $M = 1,550 \cdot$

FIG. 7. (A) Weight distribution of S value for RI denatured by heating in PE for 3 min at 97°C. Conditions for centrifugation, same as in Fig. 6A. (B) Transformation of the data presented in Fig. 7A to the number distribution of chain lengths. The arrow indicates the position of R17 RNA.

$S^{2.1}$ (cf. 7). The weight distribution $q(S)$ was then divided by the chain length to obtain $N(S)$. The chain length (number of nucleotides) was calculated for each S value by first calculating M and then multiplying this by $3,342/1.1 \times 10^6$, since the single-stranded RNA of molecular weight 1.1×10^6 has 3,342 nucleotides (18).

Figure 6A shows the weight distribution of S for RI denatured with DMSO, and Fig. 6B shows the transformation to the number distribution of chain lengths. Figures 7A and B show, respectively, the weight distribution of S and the transformation to the number distribution of chain lengths for heat-denatured RI. There was a pronounced bulge in the number distribution of DMSO-denatured RI (Fig. 6B) and a smaller bulge in the number distribution of heat-denatured RI (Fig. 7B). Since heat-denatured RI had less residual double-stranded component than DMSO-denatured RI, it is not unreasonable to speculate that this prominent deviation from the predicted distribution may be due to the double-stranded component. The height of the peak of complete viral RNA ($N = \lambda$) was about twice that of the plateau region for DMSO-denatured RI and 2.5 times the plateau region for heat-denatured RI.

DISCUSSION

As a preliminary to an analysis of denatured RI, several criteria for denaturation were studied. The infectivity of RI after treatment with DMSO under conditions which result in denaturation of the poliovirus replicative form (15) led to a 10- to 14-fold increase in specific infectivity for spheroplasts, measured as plaque-forming units (PFU) per microgram of RNA. This increase is comparable to the ninefold increase in RI infectivity after heat denaturation, reported by Erikson, Erikson, and Gordon (J. Mol. Biol. 22:257-268, 1966). A similar increase in specific infectivity was found for RF denatured with DMSO.

Changes in optical density during thermal denaturation of R17 RNA, RF, and RI provide a basis for a further analysis of the degree of denaturation. Single-stranded RNA denatures over a wide temperature range (2), whereas RF and other types of double-stranded RNA denature at higher temperatures and over a narrow temperature range (11, 14, 16). RI hyperchromicity curves have two distinct components owing to the single- and double-stranded properties of the molecules (7). All these features are illustrated in Fig. 1. Whereas the hyperchromicity of DMSO-denatured RF increases continuously with no detectable step, that of RI denatured under any condition has a step in the temperature range where double-stranded RNA denatures.

This suggests a residual double-stranded component, and the amount of this component can be estimated from the percentage increase in absorbance of the step region. The residual double-stranded component varied between 17 and 25% of the total with DMSO-treated RI and was 6.4% with heated RI.

Equilibrium distributions of both R17 RNA and RI in gradients of Cs_2SO_4 conformed to the Gaussian distribution. Although this was expected for the single-stranded RNA, it was not entirely expected for RI, which is composed of single-stranded RNA of $\rho_0 = 1.629$ and double-stranded RNA of $\rho_0 = 1.608$ to 1.606 (3, 4). The variable lengths of single-stranded RNA might be expected to confer a density heterogeneity on RI which has an average buoyant density of 1.619, intermediate between that of single-stranded R17 RNA and RF. However, it may be that the intermediate buoyant density of RI is not entirely due to the averaged contributions of RF and R17 RNA, but also due to some structural features peculiar to RI, such as the structure at the point where the single-stranded branch deviates from the double strand.

From the probability plot of the cumulative distribution, the σ^2 for R17 RNA was 2.02×10^{-3} , leading to a molecular weight estimate of 0.77×10^6 . The σ^2 for RI was 1.37×10^{-3} to 1.51×10^{-3} , leading to a molecular weight estimate of 1.01×10^6 to 1.14×10^6 . The molecular weight estimate for R17 RNA is too low by a factor 1.4 and that for RI by a factor 2.5, by use of 1.1×10^6 as the best estimate for R17 RNA (10, 18) and 2.9×10^6 as the theoretical weight average molecular weight of RI (7). The molecular weight estimates derived from the band width of equilibrium distributions of T2 and T4 deoxyribonucleic acid are known to be low by a factor of 2 (19), and the deviation found in the present determinations may be assumed to be due to the same type of error, whatever that may be. This fact, plus the pronounced deviation of the distributions of denatured RI from the Gaussian distribution, made it pointless to attempt an estimate of the molecular weight of the denatured RI from the band width of the equilibrium distribution in Cs_2SO_4 . However, the distributions clearly showed that, whereas the bulk of denatured RI was single-stranded ($\rho_0 = 1.627$ to 1.631), there was still a double-stranded component.

The number distribution of chain lengths was predicted to be composed of equal numbers of molecules with chain lengths of 2 to $\lambda-1$ plus a larger number of molecules with chain lengths of λ (cf. 7). The relative number of molecules of chain length λ depends on two factors. (i) The first factor is the average number of single

strands per double-stranded template, which was about 1 for the present preparations of RI (7). On this basis, therefore, there should be two molecules of chain length λ for every molecule of chain length less than λ . (ii) This assumes, however, that completion of chains, i.e., addition of the terminal nucleotide to a nascent chain of length $\lambda-1$, results in displacement from RI, so that the longest chain to be found associated with RI may be one of length $\lambda-1$.

From the number distribution, it is difficult to obtain more than a first-order approximation of the ratio of numbers of molecules of length λ to the number of molecules of length less than λ . This is because there is considerable inherent error in the estimate of the number distribution used here, especially at chain lengths less than about 200 (see next paragraph). Also, the variable amount of residual double-stranded RNA in the population of denatured RI will affect the result (see next paragraph). An estimate of the relative amounts of material of chain length λ and lengths $<\lambda$ was made by planimetry of a linear plot of N versus chain length. Note that for convenience only semilogarithmic plots are shown in Fig. 6B and 7B. This ratio is 0.4 rather than the predicted value of 2. This would indicate an upper value of five single strands (or five growing points) per replicative intermediate. Thus, the present experiments and earlier experiments (7, 8) indicate that there are probably between one and five growing points per RI molecule.

Between chain lengths of 200 and 1,100, there is a satisfactory fit of the number distribution to that predicted. Deviations occurring at very low chain lengths are due to two factors. Chains of very short length may be lost during dialysis of denatured RI. Also, the slope of the concentration distribution during velocity sedimentation is very small and, therefore, very difficult to measure at values corresponding to the smallest chain lengths. Thus, large errors arise in this portion of the weight distribution curve and subsequently in the number distribution transformation. The deviation from equal numbers of molecules between 1,200 and 3,000 was seen in all experiments. The amount of material in this region was somewhat higher in DMSO-denatured RI than in heat-denatured RI. This leads to the speculation that this represents the residual double-stranded material. When analysis of hyperchromicity curves for heat-denatured RI led to an estimate of 6.4% double strand, the area under the bulge in the number distribution curve was 6.2%. On the other hand, RI treated with DMSO for 10 min at 37 C had a 16% residual double-stranded component when analyzed by hyperchromicity, but the area under the bulge in the number

distribution curve was only 7%. In view of the difficulties inherent in this experiment and the problem of the residual double-stranded component, the fit of the number distribution of denatured RI to the predicted distribution is quite satisfactory. It is not clear as yet whether the double-stranded residue is a special class of double-stranded molecules or some partially denatured RI. No evidence of a special type of double-stranded RNA, such as circular RNA, was seen in electron micrographs of RF and RI (12) or denatured RI (Granboulan and Franklin, *in preparation*).

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