*DENATURATION AND RENATURATION OF VIRAL RNA, III. PURIFICATION OF THE COMPLEMENTARY STRAND OF R17 RNA**

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The general concepts of the replicative processes of most RNA viruses are now well established with a firm experimental basis in the descriptions of both *in vivo* and *in vitro* replication of the RNA bacteriophage group.[1-3] Two viral-specific double-stranded RNA structures play a key role in viral RNA replication. One of these structures is the replicative form (RF) which is a complete double-stranded structure consisting of viral RNA (+ strand) and RNA complementary to the + strand along its entire length (− strand).[4-7] The second structure is the replicative intermediate which is double-stranded RNA containing nascent single strands.[8-10]

Recent studies by Weissmann and his collaborators have challenged the presently accepted account of the molecular events involved in viral RNA replication by emphasizing the presence in the *in vitro* reaction system of excess − strands which are not hydrogen bonded.[11, 12] It is therefore of primary interest to study the properties of the RNA complementary to that found in viral particles.

Purification of complementary RNA from many RNA viruses presents a challenging chemical problem since there is little to distinguish the − strand from the + strand. For example, the base ratios of the RNA from bacteriophage R17[13] are A = 23.1, U = 25.7, G = 26.3, C = 24.9, and the ratio A + U/C + G is 0.953. Thus, the predicted base ratios of the complementary RNA would be A = 25.7, U = 23.1, G = 24.9, and C = 26.3, very similar to those of the + strand. The procedure for purification of R17 complementary RNA, described in this communication, is of *general applicability* and does not depend on any physical differences between + and − strands.

In principle, at least, the procedure is straightforward. Double-stranded RNA is denatured under mild conditions to minimize cleavage of phosphodiester bonds.[14] The denatured RNA is then annealed with *fragments* of + strands (viral RNA) and the resulting double-stranded RNA is purified. This double-stranded RNA, composed primarily of intact − strands annealed to small pieces of + strands, is then denatured and the − strands are readily isolated on the basis of their size. The procedure was made feasible by the availability of a convenient method to separate single-stranded and double-stranded RNA[9] and methods of carefully controlled denaturation and annealing of viral RNA.[14, 7]

Materials and Methods.—Production of R17 RNA fragments: Purified R17 RNA[10] was dialyzed against 0.01 *M* Tris, pH 7.2, containing 0.001 *M* EDTA (TE), and then sonicated at full power in the MSE ultrasonic disintegrator with 15-sec bursts followed by 1-min cooling periods (ice bath) for variable total periods of time. The size of the fragmented RNA was then examined by sedimentation in a 7–20% sucrose gradient and by chromatography on an 8% ago-gel column (Mann Research Labs., New York, N.Y.).

Denaturation of double-stranded RNA: Purified replicative form, replicative intermediate, or annealed RNA was denatured in dimethyl sulfoxide (DMSO) by the method of Iglewski and Franklin[14] except for the incubation of the DMSO-RNA solution at 42°C for 5 min.

Preparation of the negative strand: Completely denatured replicative intermediate (RI) was separated from RI with residual double-stranded properties by chromatography on cellulose.[9] One part of this denatured double-stranded RNA was mixed with 50 parts of fragmented R17 RNA, the RNA mixture was then precipitated at −20°C after the addition of 2 vol of ethyl alcohol and $^1/_{10}$ vol of potassium acetate, and then resuspended in 0.15 *M* NaCl, 0.015 *M* sodium citrate (SSC) containing 65% DMSO. The final concentration of the denatured double-stranded RNA was 2.66 μg/ml. This RNA solution was heated to 52°C for 1 min and then cooled to 49°C for 30 min, 48°C for 30 min, 46°C for 30 min, 44°C for 20 min, 42°C for 10 min, 40°C for 5 min, and then to 30°C at a rate of 0.3–0.5°C/min. The annealed double-stranded product was isolated from the single-stranded RNA by chromatography on cellulose.[9] The double-stranded RNA resulting from the annealing process was denatured in TE containing 85.7% DMSO, as described above. The denatured RNA was precipitated at −20°C after the addition of 2 vol of ethyl alcohol and $^1/_{10}$ vol of potassium acetate. The precipitate was resuspended in TE and incubated at 37°C for 15–20 min to dissociate RNA aggregates.[7] The RNA was then placed on a 7–20% sucrose gradient and centrifuged at 50,000 rpm for 3 hr at 5°C in the Spinco SW-65 rotor. The 27*S* fraction, which contained intact negative strands of RNA, was collected.

Infectious RNA assay: Escherichia coli strain 3000 was grown in 100 ml of MS broth containing 1 ml of 20% glycerol instead of glucose as the carbon source. The cells were harvested at 2–3 × 10^8 cells/ml and resuspended in 10 ml of 0.1 *M* Tris buffer, pH 7.7 (five parts) and 40% sucrose (three parts) (T-S-15). Then 0.26 ml of 0.1 *M* EDTA and 0.03 ml of lysozyme (1 μg/ml; Worthington Biochemical Corp., Freehold, N.J.) were added to form spheroplasts. After 20 min at room temperature, 0.14 ml of 0.5 *M* $MgSO_4$, 0.5 ml of 30% bovine serum albumin (fraction V from bovine plasma, Armour Pharmaceutical Co., Kankakee, Ill.), and 0.1 *M* Tris, pH 7.7, containing 60% sucrose (T-S-60) were added. The spheroplasts were incubated at room temperature for 30 min, sedimented, and resuspended in 5 ml of a solution composed of 2.5 ml of T-S-15, 2.5 ml of T-S-60, and 0.02 ml of 50% glucose solution and incubated at 5°C for 2 hr. The cells were again sedimented and resuspended in 5 ml of a solution containing 1.5 ml of T-S-15, 1.0 ml of T-S-15 containing 100 μg/ml of poly-L-ornithine,[15] 0.02 ml of 50% glucose, and 2.5 ml of T-S-60. A 0.2-ml sample of the RNA dilutions in 0.1 *M* Tris, pH 7.7, containing 100 μg/ml of *O*-(diethylaminoethyl) dextran (DEAE-dextran)[15] was exposed to 0.2 ml of the spheroplast suspension, incubated at 37°C for 1 min, and then for 20 min at room temperature. After the incubation period, 2 ml of spheroplast soft agar[16] and 0.2 ml of *E. coli* 3000, at 1 × 10^8 cells/ml were added and poured onto nutrient agar plates.

Results and Discussion.—Fragmentation of R17 RNA: Purified R17 RNA in TE was sonicated for various periods of time and then sedimented in a 7–20 per cent sucrose gradient. Prior to sonication the R17 RNA banded at 27*S* but already after five seconds of sonication more than 50 per cent of the 27*S* RNA was fragmented and formed a broad heterogeneous band (Fig. 1). After 20 seconds of

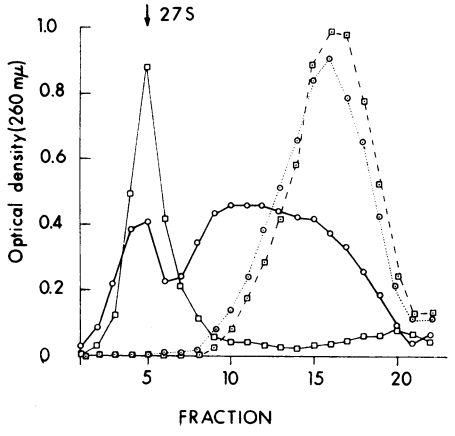


FIG. 1.—Fragmentation of R17 RNA. A preparation of purified R17 RNA (1000 μg/ml) was dialyzed for 24 hr against TE at 5°C. The RNA preparation was divided into 1-ml samples and sonicated for various periods of time before sedimentation analysis on a 4.4 ml 7–20% sucrose gradient (50,000 rpm, 3 hr, 5°C). — □ — Not sonicated; — O — sonicated for 5 sec; ···O··· sonicated for 30 sec; ——□—— sonicated for 120 sec.

sonication no RNA could be observed in the 27*S* region of the gradient, indicating complete fragmentation of the viral RNA. Sonication for 20 seconds produced a heterogeneous population of RNA having an average sedimentation coefficient of about 9*S* and this appeared resistant to further fragmentation. RNA sonicated for 90 seconds was completely devoid of intact R17 RNA as determined by sedimentation analysis and chromatography on ago-gel (Fig. 2).

Purification of the minus strand: The source of the complementary strand of R17 RNA was a mixture of RF and RI which was harvested at 45 minutes p.i. A mixture containing 400 μg of RF and RI plus 9 μg of RF and RI labeled with H^4-uridine as a marker was denatured in 85.7 per cent DMSO and chromatographed on cellulose (Fig. 3). The RNA that eluted as single-stranded RNA (90%) was used as a source of − strands, thereby eliminating partially denatured double-stranded RNA as an ultimate source of intact + strands. This step resulted in an improvement in the purity of the final preparation of − strands since the efficiency of annealing − strand preparations with R17 RNA increased by 10–15 per cent. The denatured H^3-labeled double-stranded RNA was annealed with an excess of unlabeled fragmented + strands and then chromatographed on cellulose (Fig. 3); 44 per cent of the RNA eluted as labeled double-stranded RNA in STE.

The annealed RNA was denatured in DMSO and the 27*S* RNA was isolated from a sucrose gradient (Fig. 4). The 27*S* band of labeled RNA was followed by some heterogeneous RNA which was probably degraded fragments of the 27*S* RNA.

To demonstrate that the labeled RNA sedimenting in the 27*S* region of the gradient was intact − strands, fractions from the gradient were pooled and annealed with unlabeled R17 RNA (Fig. 3). An average of 88 per cent of the labeled RNA following annealing eluted from a cellulose column as double-stranded RNA (Table 1). Therefore it appears that a minimum of 88 per cent of the RNA having a sedimentation coefficient of 27*S* is intact − strand. The 12 per cent which does not anneal may be an indication of the efficiency of annealing or of contamination with + strands. The same degree of annealing (88%) was noted for the heterogeneous band of RNA as for the 27*S* RNA (Table 1). Therefore the heterogeneous band of RNA may indeed be a degradation product of the 27*S* RNA.

The yield of purified intact negative strands was between 10 and 25 per cent of the estimated amount of − strands in the initial double-stranded RNA. The yield

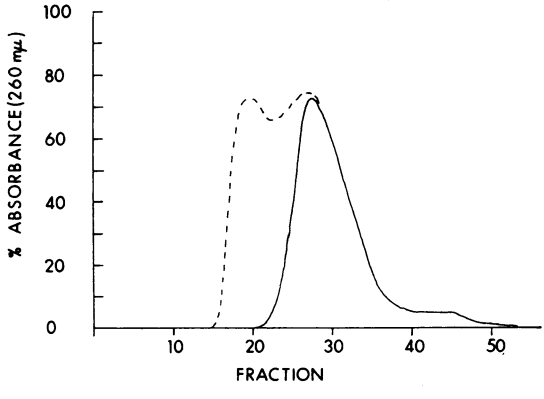
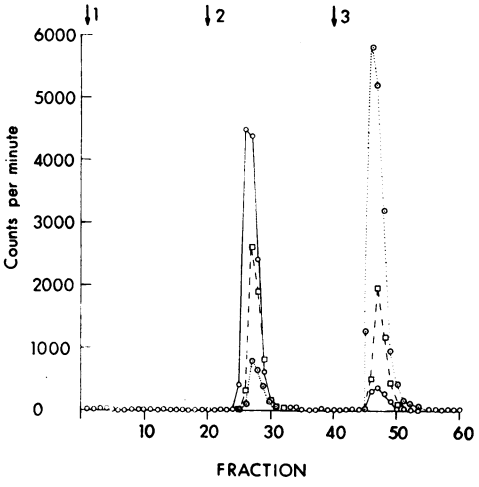
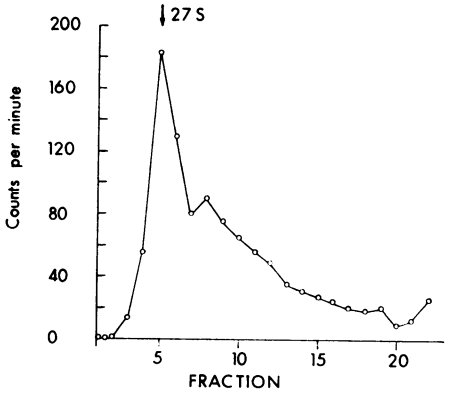


FIG. 2.—Chromatography of fragmented R17 RNA on ago-gel. Purified R17 RNA (1000 μg/ml) was dialyzed for 24 hr against TE at 5°C. The RNA was then sonicated with 15-sec bursts for a total of 90 sec. —— Fragmented R17 RNA; — fragmented RNA mixed with intact R17 RNA.





(*Left*) FIG. 3.—Cellulose chromatography of RNA. Preparations of denatured or annealed RNA were chromatographed on cellulose columns to quantitate the percent of single- and double-stranded RNA in a preparation. The RNA was eluted from the column in a stepwise manner with STE (0.1 *M* NaCl, 0.001 *M* EDTA, 0.05 Tris at pH 7.0) containing 35% ethyl alcohol (1); STE containing 15% ethyl alcohol (2); and STE (3). —— O —— Double-stranded RNA denatured in DMSO; —— □ —— denatured double-stranded RNA annealed with unlabeled fragmented R17 RNA; ····O···· negative strand preparation annealed with unlabeled R17 RNA.

(*Right*) FIG. 4.—Sedimentation analysis of the denatured RNA product which resulted from annealing denatured double-stranded RNA with fragmented R17 RNA. The denatured RNA was alcohol precipitated, resuspended in TE, layered on a 4.4-ml sucrose gradient (7–20%), and then sedimented at 50,000 rpm for 3 hr at 5°C in the Spinco SW-65 rotor.

of intact — strands was variable and apparently dependent on the amount of degradation of the RNA incurred during the purification procedure and also on the volume of the reaction mixture. The larger reaction volumes used to produce larger amounts of — strand resulted in lower yields of — strand.

Annealing fragmented R17 RNA with denatured RF uniformly labeled with H^3-uridine indicated that 43 per cent of the labeled RF annealled with the unlabeled R17 RNA. From the base ratios of $R17^{13}$ RNA, 47.1 per cent of the labeled uridine should be found in the — strand of RF. Since 43 per cent of the H^3-uridine from the denatured RF was incorporated into the annealed product, the efficiency of annealing was approximately 90 per cent.

Infectivity of the minus strand: For comparative assays of infectivity, R17 RNA and a denatured mixture of RF and RI were exposed to the same purification procedure as the — strand to eliminate the possibility that the elaborate procedures

TABLE 1

CELLULOSE CHROMATOGRAPHY OF THE PRODUCTS OF ANNEALING R17 MINUS-STRAND RNA WITH R17 PLUS-STRAND RNA

	Fractions* 3 and 4		Fractions 5 and 6		Fractions 7–12		Fractions 13–16	
Expt.	STE with 15% ETOH	STE	STE with 15% EtOH	STE	STE with 15% ETOH	STE	STE with 15% ETOH	STE
1	16	84	10	90	10	90	6	94
2	12	88	12	88	11	89	10	90
3	7	93	14	86	16	84	12	88
Average	12%	88%	12%	88%	12%	88%	10%	90%

Each pooled fraction was annealed with 400 μg of R17 RNA in a 3-ml vol of SSC containing 65% DMSO.
* Fractions correspond to the fractions collected from the sucrose gradient of Fig. 4.

TABLE 2

INFECTIVITY OF VIRAL RNA

Sample[a]	Specific infectivity (PFU/μg RNA)
R17 RNA	1.1×10^4
R17 RNA (treated)[b]	1.4×10^4
RF-RI (treated)[c]	1.5×10^3
negative strand (50:1)	50
RF-RI (treated)[d]	5.9×10^3
negative strand (25:1)	860

[a] All of the RNA samples used for infectious RNA assays were isolated from the 27*S* region of a sucrose gradient, with the exception of the untreated R17 RNA which was already homogeneous 27*S* RNA.

[b] Treated indicates the sample was subjected to the same experimental manipulations involved in the purification of the − strand except fragmented R17 RNA was not added.

[c, d] Represent two different batches of double-stranded RNA. The negative strand was obtained from batch *c* by annealing the denatured double-stranded RNA with a 50:1 excess of fragmented R17 RNA, while preparation *d* was annealed with a 25:1 excess of fragmented R17 RNA.

destroyed the infectivity of the RNA. The 27*S* fraction of R17 RNA had the same specific infectivity (PFU/μg RNA) after being exposed to the purification procedure as before treatment (Table 2). Also, the specific infectivity of the denatured RF-RI mixture which had been exposed to the purification procedure without addition of fragmented + strands was comparable to the infectivity reported for denatured RF or RI.[16] In contrast, the infectivity of the − strand preparations was less than 0.5 per cent that of R17 RNA. The infectivity associated with the − strand preparations increased when a lower concentration of fragmented + RNA was used to displace intact + strands in the purification procedure (Table 2). Therefore the residual infectivity associated with the − strand preparations could well be due to contamination of noninfectious − strands with a small amount of infectious + strands. This would be a reasonable conclusion since the annealing tests used to measure the purity of the − strand do suggest that a few per cent of the 27*S* RNA does not anneal with + strands and therefore could themselves be + strands.

The apparent lack of infectivity associated with the − strand is not surprising since the + strand contains all of the genetic information necessary to synthesize the three known structural and functional proteins of RNA bacteriophages, including the viral-specific RNA-dependent RNA polymerase.[17, 18] Recently Rüst and Sinsheimer[19] have isolated DNA complementary to bacteriophage φX-174 DNA using a heavy labeling technique, and this complementary DNA was infectious. The infectivity might be attributed to a cellular DNA polymerase which synthesized + strands using the infecting − strand as template.[19] The + strands could then direct the normal course of infection. The lack of infectivity of the R17 complementary RNA further emphasizes the absence of an RNA-dependent RNA polymerase in uninfected *E. coli*. At present the only role we may assign to complementary RNA is that of template for viral RNA synthesis, but further studies on this purified complementary RNA will be necessary to elucidate the biological role of this type of RNA.

Summary.—Denatured double-stranded RNA was annealed with fragmented R17 RNA which thereby excluded the intact + strand from the annealed double-stranded product. The intact − strands which were separated from the fragmented + strands were complementary to R17 RNA and were not infectious.

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