JOURNAL OF VIROLOGY, Aug. 1967, p. 793-803

Vol. 1, No. 4
Printed in U.S.A.

Denaturation and Renaturation of Viral Ribonucleic Acid

I. Annealing R17 Ribonucleic Acid with Denatured Replicative Form or with Denatured Replicative Intermediate

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Received for publication 25 April 1967

Purified replicative form (RF) and replicative intermediate (RI) prepared from *Escherichia coli* infected with R17 were denatured in 0.15 M NaCl, 0.015 M sodium citrate containing 65% dimethylsulfoxide. Denaturation of RF or RI was demonstrated spectrophotometrically, chromatographically, and by sedimentation analysis. Denatured RF or RI was annealed by carefully decreasing the temperature from 62 to 20 C. Annealing was accompanied by a decreased absorbance at 260 mμ. The decrease in absorbance during annealing appeared to be dependent upon the rate of cooling and the concentration of ribonucleic acid (RNA). Denatured RF or RI was annealed with R17 RNA which was labeled with ^{3}H-uridine. The annealed product was 73 to 82% resistant to 0.1 μg/ml of ribonuclease. Annealing R17 RNA with either denatured RF or RI resulted in the formation of a ribonuclease-resistant product with a sedimentation profile resembling that of native RI. Melting the annealed products in 85.7% dimethyl sulfoxide produced 27*S* single-stranded R17 RNA and a heterogeneous population of more slowly sedimenting RNA.

Three types of virus-specific ribonucleic acid (RNA) have been found to be associated with the replication of the bacteriophage R17. A single-stranded RNA molecule of molecular weight 1.1×10^6 is found within the protein capsid of the mature virus (19). Shortly after infection of the host cell by the virus, two other types of virus-specific RNA, replicative form (RF) and replicative intermediate (RI), can be detected (1, 6, 7, 24). These double-stranded RNA molecules are involved in the synthesis of the single-stranded viral RNA of the virion. Whereas RF is completely double-stranded RNA, RI is double-stranded RNA with bound nascent single-stranded viral RNA (8, 9).

Recently, techniques have been developed for the purification of all of these types of virus-specific RNA. Single-stranded RNA can best be isolated and purified by previous purification of mature virus (22). After purification of the virus, the RNA is separated from the protein coat by a technique such as phenol extraction. Techniques for the purification of the double-stranded RF (1, 8) and RI (5, 8) have also been developed. The ability to purify these types of virus-specific RNA has greatly stimulated studies of their chemical, physical, and biological properties (5, 9, 10).

The ability of denatured double-stranded RNA to anneal with single-stranded viral RNA has been demonstrated by Weissman et al. (2, 23). Extremely high temperatures (up to 120 C) were employed both for denaturation of the double-stranded RNA and also for the annealing process. These temperatures surely enhance phosphodiester bond hydrolysis. Furthermore, the double-stranded RNA used for annealing was already degraded by extensive treatment with pancreatic ribonuclease (2). In the present paper, conditions were examined which would allow the annealing of intact strands of denatured RF or RI with single-stranded R17 RNA with little hydrolysis of the phosphodiester-linked backbone of the molecules.

Dimethylsulfoxide (DMSO) has been used for the denaturation of deoxyribonucleic acid (DNA) and RNA (14, 15, 17). This organic solvent is a strong hydrogen-bond acceptor (16), and therefore enhances polynucleotide denaturation at much lower temperatures than do aqueous solvents. In the present study, DMSO was used to lower the denaturation temperature of RF and RI, and the possibility of annealing in the presence of DMSO was also examined. Since DMSO lowers the T_m of the thermal transition, it should

provide mild thermal conditions for the annealing process, provided it does not react with the bases of the denatured nucleic acid as do other reagents such as formaldehyde (20, 21). This paper describes some properties of the products resulting from the annealing of denatured RF and RI with bacteriophage R17 RNA.

MATERIALS AND METHODS

Preparation of RNA. Growth of *Escherichia coli* strain 3000 and bacteriophage R17 have been described (11). The following procedure was used to prepare R17 RNA labeled with ^{3}H-uridine. *E. coli* strain 3000 was grown in 330 ml of TC Gl medium (8) to a final concentration of 1.5×10^8 cells/ml. The cells were sedimented by centrifugation at 8,000 rev/min for 10 min in the GSA rotor of a Sorvall centrifuge. The cell pellet was washed with 80 ml of sterile 0.1 M NaCl and resedimented. The washed cells were suspended in 1.5 ml of TC Gl and infected with the bacteriophage R17 at a multiplicity of 10 plaque-forming units per cell. The virus was adsorbed to the cells for 3 min at 25 C before resuspending the cells in TC Gl medium to a cell concentration of 3.0×10^8 cells/ml. The TC Gl medium had been prewarmed to 37 C and contained 10 μc/ml of ^{3}H-uridine. The infected cells were incubated in a shaking water bath for 4 hr at 37 C. The virus was harvested by cooling the infected culture in an ice bath, adding 5% (v/v) chloroform, and shaking the infected culture for 10 min in the cold. The virus suspension was then decanted from the chloroform. The methods used for purification of bacteriophage R17 and of its RNA have been described (9). The preparation and purification of RF and RI also have been described in detail (8, 9).

Denaturation and annealing of RNA. For convenience, the single-stranded R17 RNA was mixed with either RF or RI and precipitated at −20 C after the addition of 2 volumes of absolute ethyl alcohol and one-tenth volume of 2 M potassium acetate. The precipitated mixture of nucleic acids was resuspended in a solution containing 0.15 M NaCl and 0.015 M sodium citrate (SSC; 4). DMSO (methyl sulfoxide; spectroquality reagent; Matheson, Coleman, and Bell, East Rutherford, N.J.) was added to the nucleic acid solution to a final concentration of 65% (v/v). The DMSO was stored over activated molecular sieve (Linde, type 5A, 60–80 mesh, Matheson, Coleman, and Bell) to remove water from it (16). The complete mixture was placed in a standard quartz cuvette of 10-mm path length with ground-quartz stopper. The total volume was 3 ml.

The denaturation and annealing of the material was followed by continuously recording the hyperchromicity of the sample at 260 mμ with a modified Beckman-Gilford spectrophotometer. Since DMSO has a high adsorbance at 260 mμ, the samples were read against a blank cuvette filled with SSC containing 65% DMSO. A deuterium light source was used, and absorbance was read with a Gilford model 220 photometer-indicator. The system was heated by circulating glycerol from a Haake water bath through the inner set of Beckman Dual Thermospacers. The system could also be cooled by circulating tap water through the outer set of thermospacers. This was usually done to prevent excessive heating of the monochromator or the photocell unit. The temperature of the sample in the cuvettes was measured directly by means of a thermocouple placed in one of the cuvettes (Digitec model 501 digital thermometer with 402 vinyl-sheathed thermistor).

The RNA sample was melted by increasing the temperature from 20 to 62 C. The sample was then annealed by carefully regulating the decrease in temperature. The sample was rapidly cooled at a rate of 1 to 2 degrees C per minute between 62 C and 48 to 49 C, or until a decrease appeared in the optical density (OD) of the RNA at 260 mμ. Then the sample was cooled extremely slowly between 48 and 40 C. In this temperature range, the temperature was decreased by one or two degrees at a time and kept at that temperature until there was no further decrease in the OD of the RNA at 260 mμ. Below 40 C, the sample was cooled at a rate of about 1 degree C per minute.

The data are presented without correction for thermal expansion of the solvent, since this factor was not available for mixtures of DMSO and water.

Column chromatography. Single-stranded RNA was separated from the annealed double-stranded RNA on a cellulose column. The preparation of the cellulose column and its operation have been described (8).

Sucrose gradient sedimentation. A 0.20-ml sample of RNA was placed on a 4.4-ml sucrose gradient (7 to 20% sucrose) containing a buffer composed of 0.1 M NaCl, 0.001 M ethylenediaminetetraacetate (EDTA), and 0.05 M tris(hydroxymethyl)aminomethane (Tris), *p*H 7.2 (STE). The gradients were centrifuged at 50,000 rev/min for 2.75 hr at 5 C in a Spinco SW 65 rotor. After centrifugation, 0.2-ml fractions were collected in 1.8 ml of STE. The samples were divided in half, and one half was treated with 0.1 μg of pancreatic ribonuclease per ml (Worthington Biochemical Corp., Freehold, N.J.) for 10 min at 37 C. The RNA was then precipitated by adding 0.1 mg of carrier yeast RNA (Pabst Laboratories, Milwaukee, Wis.) and 0.2 ml of 60% trichloroacetic acid with 0.9% sodium pyrophosphate. The precipitate was collected on a membrane filter (Millipore Corp., Bedford, Mass.), washed once with 10% trichloroacetic acid containing sodium pyrophosphate, and then with absolute alcohol. The radioactivity adhering to the dried filter was counted on a Nuclear-Chicago scintillation counter; as scintillator, 4 g of 2,5-diphenyloxazole (POP) per liter and 0.50 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (POPOP) per liter, dissolved in toluene were used.

Remelting of annealed products. The annealed RNA was dialyzed against 0.001 M EDTA and 0.01 M Tris, *p*H 7.2 (TE) for 24 hr at 5 C. One part of this RNA was mixed with six parts of DMSO to yield a final DMSO concentration of 85.7% (17). The DMSO-RNA solution was incubated at 37 C for 20 min, and was then quickly cooled in an ice bath. NaCl was added to a final concentration of 0.1 M, calculated on the basis of the aqueous volume. The denatured RNA was precipitated at −20 C after adding 2 volumes of ethyl alcohol and 0.1 volume of 2 M potassium acetate.

The precipitated RNA was resuspended in TE and incubated at 37 C for 15 min before sedimentation analysis in a 7 to 20% sucrose gradient. Without the preincubation in TE, various amounts of the labeled RNA sedimented to the bottom of the gradient, indicating an apparent aggregation of the alcohol-precipitated RNA. Preincubation in TE favored dissociation of any aggregated RNA, resulting in reproducible sedimentation patterns. The gradient was fractionated, and the ribonuclease resistance of the denatured RNA was determined.

RESULTS

Spectrophotometric measurements of denaturation and renaturation. Both the double-stranded RF and RI of the bacteriophage R17 had a characteristically sharp thermal transition (9) when denatured in the presence of SSC containing 65% DMSO (Fig. 1a, 1b). The T_m of the transition was 50 C. When RI was heated from 20 to 30 C, an initial increase in the adsorbance at 260 mμ was observed, which does not occur during denaturation of RF (cf. Fig. 1a, 1b). This initial increase in adsorbance is due to the dissociation of helical regions within single-stranded RNA molecules (3). The helix-coil transition of the single-stranded component of RI was complete at 30 C.

The initial stages of annealing of RF or RI were accompanied by an increase in the adsorbance which occurred between 62 and 50 C (Fig. 4b, 6b). The same increase in adsorbance was observed when the samples were cooled at rates between 0.1 and 2 degrees C per minute. Between 50 and 49 degrees C, the adsorbance of the denatured RNA started to decrease. The decrease in adsorbance at 260 mμ was rapid between 49 and 46 C. Between 49 and 40 C, the temperature was decreased by 1 degree at a time and was kept at that level until there was no further decrease in the OD of the RNA at 260 mμ. The time the sample was held at each temperature was approximately 25 to 30 min. With each decrease in temperature by 1 degree, there was an initial rapid decrease in OD at 260 mμ which diminished with an increase in time at that temperature. When there was no further decrease in the OD of the RNA at 260 mμ, the temperature was again decreased by 1 C. Similar kinetics were observed at lower annealing temperatures, except that the rate of decrease in OD diminished as the temperature of annealing approached 30 C. Below 30 C, there was again a rapid decrease in the absorbance of the RNA at 260 mμ. The procedure described in Materials and Methods resulted in a 90 to 100% decrease in the absorbance of RNA after denaturation. The total time required to anneal the sample was 2.5 to 3 hr. The decrease in the adsorbance appeared to be dependent on the concentration of RNA. Figure 1a illustrates a typical absorbance pattern when low concentrations of RF or RI were employed for denaturation and annealing. A concentration of about 0.4 OD_{260} units/ml or greater was required to obtain a 90 to 100% decrease in absorption upon slow cooling of the sample.

To eliminate the possibility that complete reversibility of the absorbance during denaturation and annealing might be caused by incomplete denaturation, the samples were heated to 62 C and then rapidly cooled in an ice bath. Under conditions of rapid cooling, little or no annealing of completely separated RNA strands should occur. The effect of rapid cooling on the absorb-

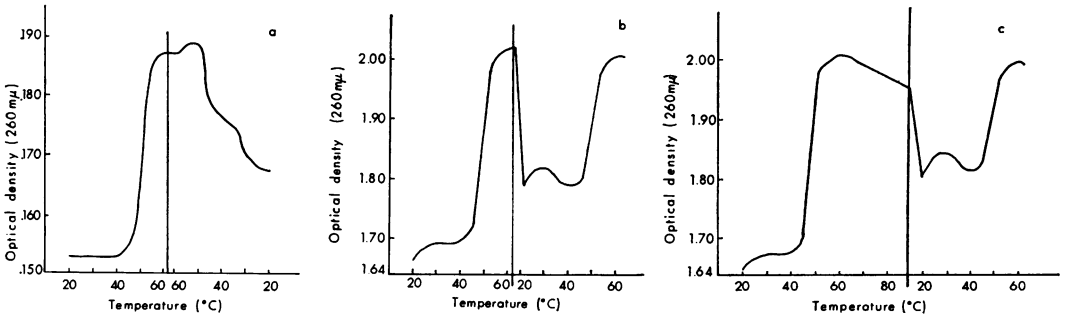

FIG. 1a. *Effect of temperature on the optical density of replicative form (RF) during denaturation and annealing at low concentrations. An 18-μg amount of RF was dissolved in 3 ml of SSC containing 65% DMSO; this was heated from 20 to 60 C, and then slowly cooled from 62 to 20 C.*

FIG. 1b. *Effect of temperature on the optical density of replicative intermediate (RI) during denaturation followed by rapid cooling. A 170-μg amount of RI was dissolved in 3 ml of SSC containing 65% DMSO. The RNA was heated to 62 C and then rapidly cooled in an ice bath. The chilled RNA was then reheated to 62 C. The absorbance at 260 mμ was recorded between 20 and 62 C.*

FIG. 1c. *Same as 1b, except the RI was heated to 90 C before chilling in an ice bath.*

ance at 260 mμ, the chromatographic behavior, and the sedimentation rate of the denatured RNA were examined. Rapid cooling only resulted in a 60% decrease in the absorbance of the denatured RNA (Fig. 1b). The decrease in absorbance was independent of the final temperature of denaturation (Fig. 1b, 1c). Upon reheating the denatured material, there was an initial increase in the absorbance between 25 and 30 C. The initial increase was followed by a decrease in absorbance, which was then followed by a sharp rise in absorbance coincident with the temperature of denaturation for native RI or RF. The sharp rise in absorbance began at 42 C and was complete at 58 C. This phenomenon could be repeated by again rapidly cooling and reheating the same sample. This secondary melting occurred even though the sample was heated 40 C beyond the T_m of the double-stranded RNA. The denatured RNA samples were again rapidly cooled in an ice bath and chromatographed on a cellulose column. The denatured RNA was eluted from the cellulose

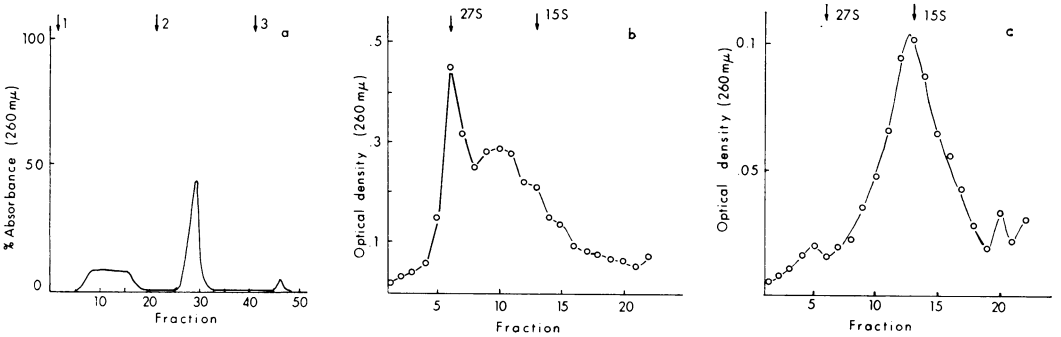

FIG. 2a. *Cellulose chromatography of denatured RI. RI was denatured at 62 C in SSC containing 65% DMSO and then rapidly cooled in an ice bath. The denatured RNA was placed on a cellulose column and eluted in a stepwise manner with STE containing 35% ethyl alcohol (1); STE containing 15% ethyl alcohol (2); and STE (3).*

FIG. 2b. *Sedimentation analysis of the RNA eluting in SSC containing 15% ethyl alcohol. The denatured single-stranded RNA eluting from cellulose in STE containing 15% ethyl alcohol was precipitated with 2 volumes of ethyl alcohol. The RNA was resuspended in TE, layered on a 7 to 20% sucrose gradient, and centrifuged at 50,000 rev/min for 2.75 hr at 5 C in a Spinco SW 65 rotor.*

FIG. 2c. *Sedimentation analysis of the RNA eluting in STE. The RNA eluting from cellulose in STE was precipitated with 2 volumes of ethyl alcohol, resuspended in TE, and sedimented in a sucrose gradient as described in 2b.*

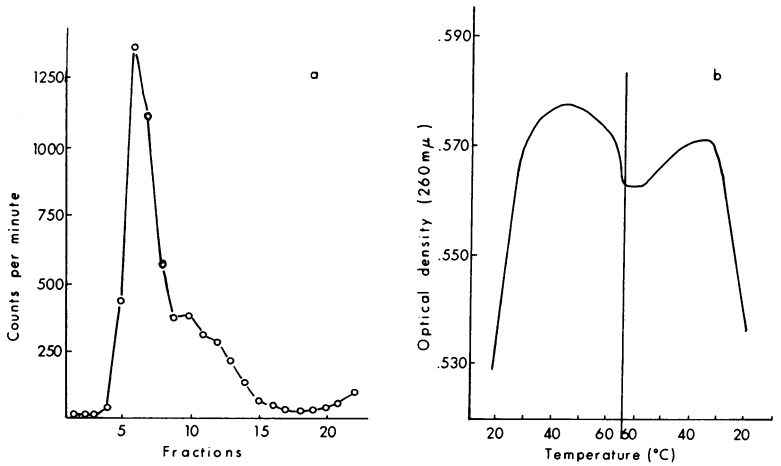

FIG. 3a. *Sedimentation analysis of R17 RNA. R17 RNA was phenol-extracted from purified bacteriophage. A portion of the RNA was layered on a 4.4-ml sucrose gradient (7 to 20%) and centrifuged at 50,000 rev/min for 2.75 hr at 5 C in a Spinco SW 65 rotor.*

FIG. 3b. *Temperature dependence of the optical density of R17 RNA at 260 mμ. R17 RNA suspended in SSC containing 65% DMSO was slowly heated to 65 C and then slowly cooled to 20 C while the absorbance at 260 mμ was recorded continuously.*

column. The denatured RNA was eluted from the cellulose by stepwise elution with STE containing 35% ethyl alcohol (35% ETOH-STE), 15% ETOH-STE, and STE. Single-stranded RNA elutes in 15% ETOH-STE and double-stranded RNA elutes in STE (8). Figure 2a shows an elution pattern of denatured RI. The pattern was identical for samples denatured at 62 or 90 C and then rapidly cooled in an ice bath. The material eluting in 35% ETOH-STE was not alcohol-precipitable and did not sediment in a 7 to 20% sucrose gradient centrifuged at 50,000 rev/min for 2.75 hr. This material was probably DMSO which has a high adsorbance at 260 mμ. Approximately 94% of the RNA eluted in 15% ETOH-STE, indicating that the material was single-stranded RNA (8). The remaining 6% of the material eluted in STE. It will be shown that the material eluting in STE is double-stranded RNA contaminated with a small amount of single-stranded RNA which did not elute in the 15% ETOH-STE.

Sucrose gradient analysis was employed to determine the sedimentation characteristics of the material eluting from the cellulose column in 15% ETOH-STE (Fig. 2b). When RI was denatured at 62 C, a sharp 27*S* band was observed, which was followed by a broad band of heterogeneous sedimenting material. The 27*S* band appears to be complete single strands of R17 RNA. The 27*S* band of RNA is followed by a broad band of heterogeneous sedimenting RNA which may be due to the nascent single-stranded fragments associated with RI (10).

Figure 2c shows the sedimentation analysis of six pooled samples of material denatured at 62 to 90 C and eluting from cellulose columns in STE. A small band of material occurred near the bottom of the sucrose gradient and had a sedimentation coefficient of 27*S*. This material was probably single-stranded RNA which had not been completely eluted from the cellulose column by 15% ETOH-STE (8; Franklin, Salditt, and Hinckley, *in preparation*). The major band of material in the gradient had a sedimentation coefficient of 15*S*, and was probably double-stranded RNA because of its elution from cellulose in STE.

Annealing denatured RF with R17 RNA. The R17 RNA used for annealing was labeled with ^{3}H-uridine. Its sedimentation profile in a 7 to 20% sucrose gradient is shown in Fig. 3a. This single-stranded RNA produced a typical adsorbance pattern when heated from 20 to 65 C in SSC containing 65% DMSO and then cooled to 20 C (Fig. 3b). With an increase in temperature, there was the usual helix-coil transition within the single-stranded RNA molecules, and this was complete at 30 C. The hyperchromicity was very small, since the single-stranded RNA was already almost completely denatured at ambient temperature. This is in contrast to the rather large hyperchromic effect obtained in aqueous solutions of moderate ionic strength (10, 13). The absorbance of the sample remained constant from 30 to 65 C except for the slight decrease in absorbance of the RNA between 45 and 65 C, which was due to the thermal expansion of the solution. Upon cooling the sample, there was an initial increase in the absorbance of the RNA, which was probably due to the decrease in volume of the RNA solution.

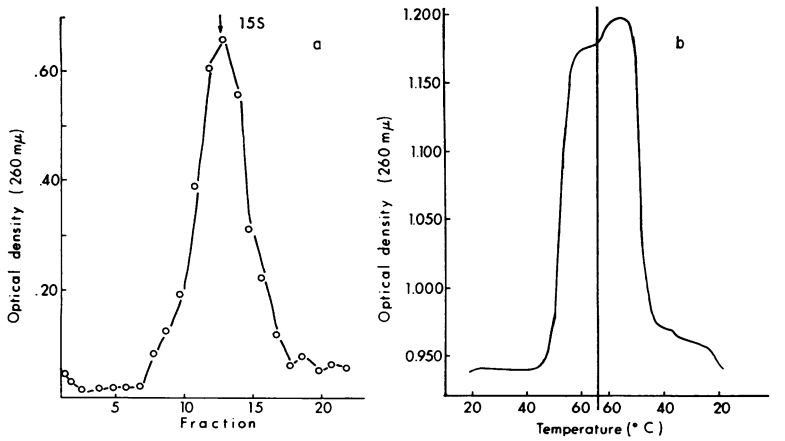

FIG. 4a. *Sedimentation analysis of unlabeled RF. RF was layered on a 4.4-ml sucrose gradient (7 to 20%) and centrifuged at 50,000 rev/min for 2.75 hr at 5 C in a Spinco SW 65 rotor.*

FIG. 4b. *Effect of temperature on the optical density of RF during denaturation and annealing. A 113-μg amount of RF was dissolved in 3 ml of SSC containing 65% DMSO. The RNA was heated to 62 C and then slowly cooled from 62 to 20 C.*

Cooling the RNA below 30 C resulted in a decrease in absorbance, which was associated with reformation of the single-stranded RNA secondary structure.

The RF used for the denaturation and annealing studies had a typical sedimentation profile (Fig. 4a) and absorbance pattern at 260 mμ when heated to 62 C and then slowly cooled to 20 C (Fig. 4b).

A mixture of single-stranded R17 RNA and RF produced the absorbance pattern shown in Fig. 5a. This figure has a combination of the absorption characteristics of R17 RNA (Fig. 3b) and of RF (Fig. 4b). It is also identical to the absorbance pattern of RI (Fig. 6b). These comparisons illustrate the double-stranded nature of RF as compared to the combined double- and single-stranded nature of RI.

Unlabeled RF was denatured in the presence of R17 RNA labeled with ^{3}H-uridine by heating the mixture to 62 C in SSC containing 65% DMSO as usual. The denatured RF was then annealed with the labeled R17 RNA by carefully cooling the mixture to 20 C (Fig. 5a). The optimal temperature of annealing in SSC containing 65% DMSO was 2 to 3 C below the T_m as compared

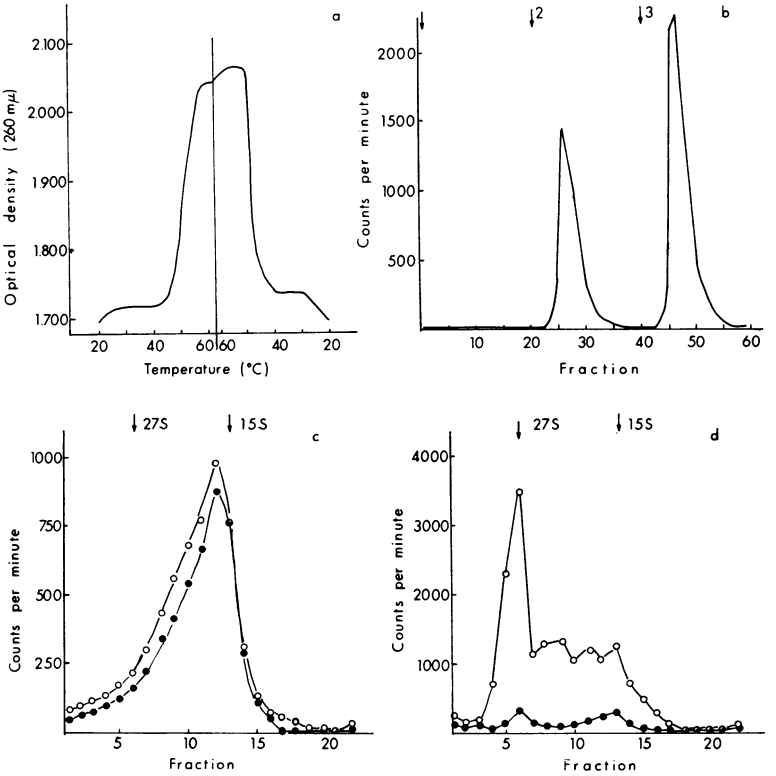

FIG. 5a. *Effect of temperature on the optical density during denaturation of RF and its annealing with R17 RNA. A 144-μg amount of RF was mixed with 64 μg of R17 RNA and suspended in 3 ml of SSC containing 65% DMSO. The RNA was heated from 20 to 62 C and then slowly cooled from 62 to 20 C.*

FIG. 5b. *Elution pattern of denatured RF annealed with R17 RNA. After annealing denatured RF with R17 RNA, the product was chromatographed on a cellulose column. The RNA was eluted from the cellulose in a stepwise manner with STE containing 35% ethyl alcohol (1); STE containing 15% ethyl alcohol (2); and STE (3).*

FIG. 5c. *Sedimentation analysis of the RNA eluting from cellulose in STE. The annealed RNA eluted from cellulose in STE was precipitated with 2 volumes of ethyl alcohol. The RNA was resuspended in TE, layered on a sucrose gradient (7 to 20%), and centrifuged at 50,000 rev/min for 2.75 hr at 5 C in a Spinco SW 65 rotor. One half of each fraction was untreated (○) and the other half was treated with ribonuclease, 0.1 μg/ml, 10 min, 37 C (●).*

FIG. 5d. *Sedimentation analysis of the denatured product resulting from annealing R17 RNA with denatured RF. The annealed RNA eluting from cellulose in STE was dialyzed against TE and then denatured in TE containing 85.7% DMSO at 37 C. The denatured RNA was precipitated with 2 volumes of ethyl alcohol, resuspended in TE, and sedimented in a sucrose gradient as described in 5c. One half of each fraction was untreated (○) and the other half was treated with ribonuclease, 0.1 μg/ml, 10 min, 37 C (●).*

TABLE 1. *Ribonuclease resistance of R17 RNA annealed with replicative form*

Expt	Ribonuclease resistance			Material eluting in STE
	Total material after annealing	Material eluting in STE	STE fraction after denaturation[a]	
	%	%	%	%
1	57	82	17	70
2	78	81	19	71
3	61	82	18	66
Avg	65	82	18	69

[a] The RNA was dialyzed against TE and then denatured in 85.7% DMSO at 37 C for 20 min. The denatured RNA was precipitated with ethyl alcohol and resuspended in STE before treatment with ribonuclease.

with the optimal annealing temperature for DNA which was approximately 25 C below the T_m (18). After annealing, the labeled R17 RNA became 65% resistant to digestion with 0.1 μg of ribonuclease per ml (10 min at 37 C; Table 1). The annealed material was chromatographed on a cellulose column. The labeled RNA eluted from the column in two fractions (Fig. 5b). The first fraction was eluted in 15% ETOH-STE and was not resistant to ribonuclease treatment. The second fraction which eluted in STE comprised 69% of the radioactivity and was 82% resistant to 0.1 μg of ribonuclease per ml (Table 1).

A portion of the resistant material was sedimented in a 7 to 20% sucrose gradient (Fig. 5c). The sedimentation profile of the annealed RNA was similar to that of native RI (8). The frontal portion of the band extends toward the bottom of the gradient and is more sensitive to digestion by ribonuclease, but not as sensitive as native RI (8). The peak of the material has a sedimentation coefficient of 15*S*.

The remainder of the annealed ribonuclease-resistant material was dialyzed against TE and melted in 85.7% DMSO at 37 C. The denatured RNA was 18% resistant to 0.1 μg of ribonuclease per ml (Table 1). The denatured RNA was concentrated by alcohol precipitation, resuspended in TE, heated for 10 min at 37 C, and sedimented in a 7 to 20% sucrose gradient (Fig. 5d). A sharp band was observed with a sedimentation coefficient of 27*S*. This band appears to be intact single-stranded R17 RNA molecules. A heterogeneous population of more slowly sedimenting RNA was also observed. There was an increase in the ribonuclease resistance in the 15*S* region of the gradient where double-stranded RNA (RF) sediments. The heterogeneous population of RNA molecules having sedimentation coefficients of less than 27*S* may be due to incomplete melting of the annealed RNA as well as to partial degradation of the 27*S* RNA.

Annealing denatured RI with R17 RNA. Unlabeled native RI, which produces a typical broad sedimentation profile (Fig. 6a) and exhibits a melting and annealing pattern (Fig. 6b), was mixed with R17 RNA labeled with ^{3}H-uridine and alcohol-precipitated. The precipitated RNA was resuspended in SSC containing 65% DMSO, denatured by increasing the temperature to 62 C, and annealed by carefully decreasing the temperature to 20 C (Fig. 6c). The annealed R17

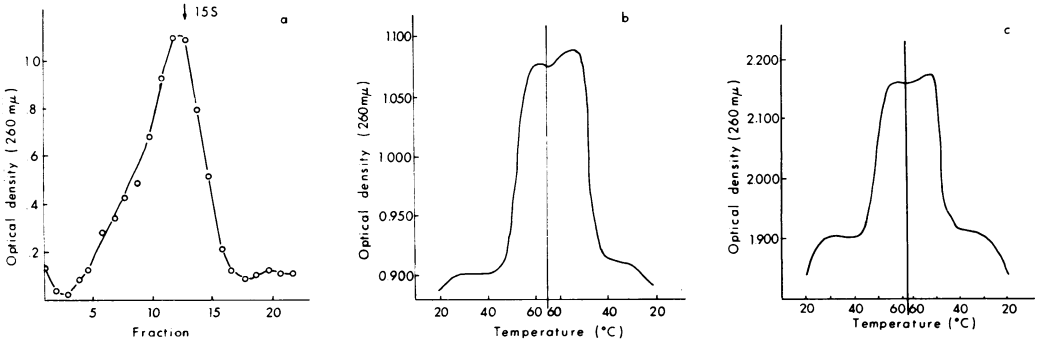

FIG. 6a. *Sedimentation analysis of unlabeled RI. RI was layered on a 4.4-ml sucrose gradient (7 to 20%) and centrifuged at 50,000 rev/min for 2.75 hr at 5 C in a Spinco SW 65 rotor.*

FIG. 6b. *Effect of temperature on the optical density of RI during denaturation and annealing. A 100-μg amount of RI was dissolved in 3 ml of SSC containing 65% DMSO. The RNA was heated from 20 to 62 C and then was slowly cooled from 65 to 20 C while continuously recording the adsorbance at 260 mμ.*

FIG. 6c. *Effect of temperature on the optical density during the denaturation of RI and its annealing with R17 RNA. A 156-μg amount of RI was mixed with 64 μg of R17 RNA and suspended in 3 ml of SSC containing 65% DMSO. The RNA was heated from 20 to 62 C and then slowly cooled from 62 to 20 C.*

RNA became 41% resistant to 0.1 μg of ribonuclease per ml. After determining the ribonuclease resistance, the RNA was chromatographed on a cellulose column. A distinct fraction containing 47% of the total radioactivity eluted in STE as double-stranded RNA and was now 73% resistant to ribonuclease (Table 2). A portion of the ribonuclease-resistant material was placed on a 7 to 20% sucrose gradient and centrifuged at 50,000 rev/min for 2.75 hr (Fig. 7a). The sedimentation profile of the denatured RI annealed with R17 RNA was similar to the sedimentation profile of denatured RF annealed with R17 RNA (Fig. 5c). Thus, the annealing of R17 RNA with denatured RF or RI yielded a product whose sedimentation profile resembled that of native RI.

TABLE 2. *Ribonuclease resistance of R17 RNA annealed with replicative intermediate*

Expt	Ribonuclease resistance			Material eluting in STE
	Total material after annealing	Material eluting in STE	STE fraction after denaturation[a]	
	%	%	%	%
1	43	78	16	43
2	43	71	19	46
3	38	70	—	53
Avg	41	73	17	47

[a] The RNA was dialyzed against TE and then denatured in 85.7% DMSO at 37 C for 20 min. The denatured RNA was precipitated with ethyl alcohol and resuspended in STE before treatment with ribonuclease.

The remainder of the annealed material was dialyzed against TE and melted in 85.7% DMSO. This melted material was 17% resistant to ribonuclease (Table 2). After melting, the RNA was precipitated, and its sedimentation pattern was examined in a 7 to 20% sucrose gradient (Fig. 7b).

The melting of the annealed R17-RI RNA produced a pattern similar to the one observed after the melting of the annealed R17-RF RNA. A sharp 27*S* band was observed which corresponded to the sedimentation rate of intact R17 RNA. This band was followed by a broad band of heterogeneous material which was partially resistant to 0.1 μg of ribonuclease per ml. The fractions containing the 27*S* RNA were collected and resedimented in a 7 to 20% sucrose gradient. A single band having a sedimentation coefficient of 27*S* was observed.

DISCUSSION

The initial objective of this study was to investigate the denaturation of double-stranded RNA (RF or RI) by using DMSO to decrease the T_m. The temperature of denaturation of

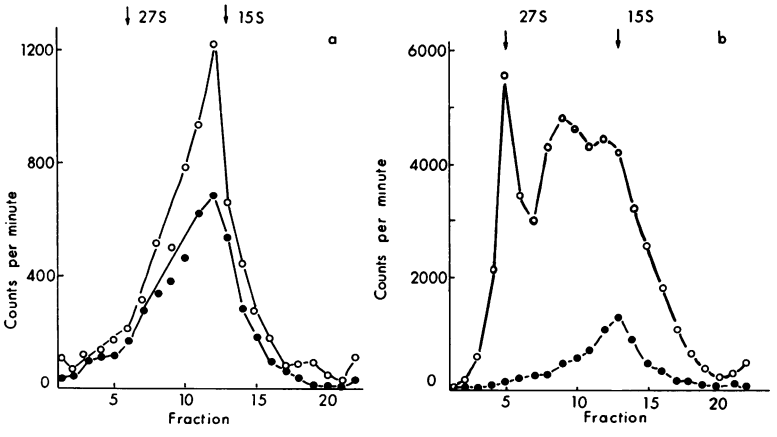

FIG. 7a. *Sedimentation analysis of the product resulting from annealing denatured RI with R17 RNA. The annealed RNA which elutes from cellulose in STE was precipitated with 2 volumes of ethyl alcohol. The RNA was resuspended in TE, layered on a 7 to 20% sucrose gradient, and centrifuged at 50,000 rev/min for 2.75 hr at 5 C in a Spinco SW 65 rotor. One half of each fraction was untreated* (○) *and the other half was treated with ribonuclease, 0.1 μg/ml, 10 min, 37 C* (●).

FIG. 7b. *Sedimentation analysis of the denatured product resulting from annealing R17 RNA with denatured RI. The annealed RNA was dialyzed against TE and then denatured in TE containing 85.7% DMSO at 37 C. The denatured RNA was precipitated with 2 volumes of ethyl alcohol, resuspended in TE and sedimented in a sucrose gradient as described in 7a. One half of each fraction was untreated* (○) *and the other half was treated with ribonuclease, 0.1 μg/ml, 10 min, 37 C* (●).

double-stranded RNA is dependent upon the ionic strength and the DMSO concentration of the RNA solution. As the ionic strength of the solution decreases, the T_m of the thermal transition is lowered. Also, an increase in the concentration of DMSO causes a decrease in the temperature of denaturation (*unpublished data*). Various ionic strengths and DMSO concentrations were examined to obtain the optimal DMSO and salt concentrations which would favor mild temperatures for both the denaturation and annealing of double-stranded RNA. SSC containing 65% DMSO was chosen for the denaturation and annealing of RF and RI, because it allows the denaturation and annealing processes to occur between 40 and 62 C, and because the RNA and salt remain soluble in this medium.

Although spectrophotometric measurements during the denaturation of RNA indicate maximal hyperchromicity at 58 C in SSC containing 65% DMSO, a measure of hyperchromicity during denaturation is not necessarily a true measure of strand separation. DNA strand separation is not directly related to the measured hyperchromicity. In the case of DNA, no strands have separated until 75% of full hyperchromicity is obtained, and even at full hyperchromicity a large fraction of the strands may be held together (12). To obtain RNA strand separation when maximal hyperchromicity is reached, the samples were subjected to rapid cooling. Rapid cooling of denatured double-stranded RNA should result in a single-stranded product. The effect of rapid cooling on the hyperchromicity, chromatographic behavior, and sedimentation profile of the denatured RNA was examined.

Rapid cooling caused a 60% reduction in the absorbance of the denatured RNA. The decrease in absorbance was independent of heating the sample to the point of maximal hyperchromicity or even to 30 C beyond this point. Upon reheating the rapidly cooled RNA samples, a sharp thermal transition occured coincident with the thermal transition of native double-stranded RNA. Similar observations have been reported for *Diplococcus pneumoniae* DNA by Doty et al. (4). Rapid cooling of denatured RNA may result in the formation of short complementary helical regions. As the sample is slowly heated from 20 to 30 C, the imperfect helical regions melt, releasing RNA chains that are at a favorable temperature (35 to 42 C) to anneal and form long complementary helices. Then these newly renatured helices enter the temperature range in which they remelt (45 to 58 C), thus producing a characteristic sharp thermal transition curve.

The bulk of the rapidly cooled denatured RNA elutes from a cellulose column as single-stranded RNA. A sedimentation profile of the eluted RNA indicates that the material is composed of an RNA species having a sedimentation coefficient of 27*S* plus a heterogeneous population of RNA with lower sedimentation coefficients. Rapid cooling of RNA denatured at temperatures ranging from 62 to 90 C also results in the formation of a small quantity of double-stranded RNA, as indicated by chromatographic and sedimentation analysis. This double-stranded RNA may be the result of a slight amount of renaturation upon rapid cooling or possibly of incomplete denaturation. Since the proportion of residual double-stranded material appears independent of the temperature of denaturation, it probably represents a slight renaturation during rapid cooling.

Renaturation, being an intermolecular process, is dependent upon the concentration of RNA (Fig. 1; *unpublished data*). The decrease in absorbance accompanying the renaturation of RF is dependent upon the concentration of RNA (*unpublished data*). An RNA concentration of about 0.4 OD_{260} units is required for 90 to 100% reduction in the absorbance of the RNA from the maximal absorbance of the denatured RNA to the original absorbance of the unheated RNA sample.

Another indication of the intermolecular processes involved in annealing is the demonstration of the annealing of labeled R17 RNA plus strands with minus strands from unlabeled RF or RI, producing a ribonuclease-resistant labeled RNA. These data could not be obtained with labeled RF or RI, for then the production of ribonuclease-resistant labeled material could also be interpreted as indicating incomplete denaturation.

The changes in absorbance which accompany the decrease in temperature during annealing can be divided into two classes, those associated with annealing and those associated with helix-coil transition within single-stranded RNA. The absorbance of single-stranded RNA in SSC containing 65% DMSO remains constant when cooled from 62 to 30 C, except for changes in absorbance due to the thermal expansion of the solution. Decreasing the temperature below 30 C results in a decrease in absorbance which is associated with helical regions within the single-stranded RNA. Therefore, it may be assumed that, upon annealing of double-stranded RNA under the conditions described here, the decrease in absorbance between 62 and 30 C is associated with the annealing process.

The initial decrease in temperature following denaturation of double-stranded RNA is accompanied by a further increase in the absorb-

ance of the RNA. The increased absorbance at 260 mμ is due to a decrease in the volume of the RNA solution as it is cooled.

R17 RNA appears to anneal more efficiently with denatured RF than with denatured RI, since a higher percentage of the labeled R17 RNA becomes resistant to ribonuclease when annealed with denatured RF. The decreased efficiency of annealing of denatured RI with R17 RNA may be due to an interference by the nascent single-stranded fragments associated with RI.

The products resulting from annealing denatured RF or RI with R17 RNA are similar. The sedimentation profile and ribonuclease resistance of both annealed products resemble the characteristics of native RI. The higher sensitivity of the frontal portion of the sedimentation pattern would indicate that they have both a single- and a double-stranded component. The skewness of the distribution towards higher *S* values suggests that the single-stranded portion of the molecules may be heterogeneous with respect to the number of single strands and their chain length. The properties of the annealed material will be described in the following report (14*a*).

Denaturation of the products resulting from annealing R17 with denatured RF or RI produces similar results. Denaturation results in the production of a band of ribonuclease-sensitive material having a sedimentation coefficient of 27*S*. This material appears to be the original intact single-stranded R17 RNA. Following the 27*S* RNA is a broad band of heterogeneous sedimenting material. A portion of the RNA which sediments in the 15*S* region of the gradient appears to be partially resistant to ribonuclease. The annealed RI-like material would be expected to sediment in this portion of the gradient. The RI-like material may have a similar resistance to denaturation in 85.7% DMSO, as has been reported for native RI (10). The broad band of heterogeneous RNA may be composed of partially denatured double-stranded RNA and degraded single-stranded 27*S* RNA.

ACKNOWLEDGMENTS

We are grateful for the technical assistance provided by Yvonne Edwards, who prepared the nucleic acids used in this study.

This investigation was supported by Public Health Service grant AI-07645 from the National Institute of Allergy and Infectious Diseases and by Grant GB 5365 from the National Science Foundation.

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