

# Structural Units of Reovirus Ribonucleic Acid and Their Possible Functional Significance

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Reovirus contains ribonucleic acid (RNA) equivalent in amount to a molecular weight of approximately  $10^7$  daltons. On isolation, this RNA is invariably broken into fragments of three different sizes. The three pieces have been separated from each other by chromatography on methylated albumin-kieselguhr columns. Denaturation of the three fragments of RNA in dimethyl sulfoxide led to separation of the strands, as suggested by sucrose gradient sedimentation patterns and by the second-order kinetics of reannealing. Molecular weights of  $0.8 \times 10^6$ ,  $1.4 \times 10^6$ , and  $2.4 \times 10^6$  were determined for the double-stranded fragments from the sedimentation rates of the single-stranded RNA obtained by denaturation. There was little or no homology among the three classes of denatured RNA when taken in pairs in hybridization tests. The three pieces of double-stranded RNA, therefore, did not result from random breaks in the original viral RNA molecule. Virus-specific single-stranded RNA formed in infected cells, and previously found to be largely if not entirely messenger RNA transcribed from the viral genome, was also separated into three size classes by sedimentation through sucrose gradients. Each class of single-stranded RNA corresponded in size to one of the three fragments of double-stranded RNA. The largest piece of single-stranded RNA hybridized uniquely with the largest fragment of denatured viral RNA. By whatever means this fragment of double-stranded RNA may be joined into the viral RNA molecule, it seems to act as a specific unit for transcription of an uninterrupted messenger RNA of equivalent length.

The reovirus virion contains double-stranded (dsRNA) with a molecular weight equivalent to about  $10^7$  daltons (5, 6, 12). If this RNA exists in the particle as a single unbroken filament, it should have a contour length of 5 to 6  $\mu$  (4, 7, 12), and occasional filaments of this length, and longer, have been seen in electron micrographs. Nevertheless, the bulk of the RNA, extracted from virions by a variety of methods, consists of much shorter lengths. Contour lengths of these fragments have been carefully measured, and they fall into three main classes at 1.1, 0.6, and 0.35  $\mu$  (3a, 4, 9). Present evidence, based on electron microscopy, suggests that the fragments arise from breakage of the long filaments of RNA during isolation from the virion. The question then arises whether or not the breaks are introduced in a random manner. If the breaks are found not to be random, it can be inferred that there are specific weak points in the viral RNA molecule. Indeed, it has recently been suggested by Dunnebacke and Kleinschmidt (3a) that neither the lengths of the three major RNA fragments seen

in the microscope nor the frequency of their appearance is consistent with the hypothesis of random breakage.

In the present paper, we provide further evidence that the fragments of viral RNA are not caused by random breaks in the molecule. The three size-classes of dsRNA seen by electron microscopy have been separated by column chromatography and denatured. It has been shown that there is little or no homology among the three classes of denatured RNA when taken in pairs in hybridization tests. Further, the single-stranded RNA (ssRNA) formed in infected cells (10, 17) and shown to be largely messenger RNA (mRNA) transcribed from the viral genome (14, 17) has been separated into three fractions on the basis of sedimentation rate. Each fraction of this ssRNA corresponds in size to the denatured RNA from one of the classes of dsRNA.

The largest piece of ssRNA hybridizes uniquely and completely with denatured RNA from the longest segment of denatured dsRNA,

suggesting that, in the infected cell, this part of the viral RNA molecule may function as a "transcription unit."

#### MATERIALS AND METHODS

**Cells and virus.** Suspension cultures of L cells, media, and reovirus type 3 were used as described previously (10, 19). Cultures were infected by allowing virus, at an added multiplicity of 10 plaque-forming units (PFU) per cell, to adsorb for 2 hr at room temperature to L cells in suspension at a concentration of  $10^8$  cells/ml. The cells were then centrifuged, resuspended to a concentration of  $5 \times 10^5$  cells/ml in prewarmed medium, and placed at 37 C. This time is regarded as time-zero.

**Solutions.** LTM buffer: 0.14 M LiCl, 0.01 M tris-(hydroxymethyl)aminomethane (Tris) chloride (pH 7.4), 0.001 M  $MgCl_2$ . LAM buffer: 0.14 M LiCl, 0.01 M acetate (pH 5.2), 0.001 M  $MgCl_2$ . STE buffer, 1.0 M: 1.0 M NaCl, 0.01 M Tris chloride (pH 7.4), 0.001 M ethylenediaminetetraacetate (EDTA) (0.7 M and 0.3 M STE buffers contain 0.7 M NaCl and 0.3 M NaCl, respectively). TE buffer: 0.01 M Tris chloride (pH 7.4), 0.001 M EDTA. SDS buffer: 0.1 M NaCl, 0.01 M Tris chloride (pH 7.3), 0.5% sodium dodecyl sulfate.

**Labeled compounds.** Uridine-5- $^3H$  (25 c per mmole) and uridine-2- $^{14}C$  (25 mc per mmole) were obtained from New England Nuclear Corp., Boston, Mass.

**Purification of  $^{14}C$ -labeled reovirus and extraction of its RNA.** A culture was infected as described above and, at time-zero, 0.5  $\mu$ g of actinomycin D per ml was added (10, 15). After 2 hr, 0.02  $\mu$ g of  $^{14}C$ -uridine per ml was added. Virus obtained from this culture was concentrated with ammonium sulfate, treated with deoxyribonuclease, crystalline chymotrypsin, and then with genetrin-113, as described by Gomatos and Tamm (6) and Kudo and Graham (10). The resulting solution was layered over 0.5 ml of 60% sucrose solution and centrifuged in the SW25 rotor of a Spinco model L centrifuge for 2 hr at  $65,000 \times g$  at 4 C. Approximately 1 ml of fluid containing the virus was withdrawn from the bottom of the tube, dialyzed against phosphate-buffered saline, layered over 30 ml of 10 to 50% sucrose gradient, and centrifuged for 90 min at  $65,000 \times g$ . A single peak of  $^{14}C$  was found two-thirds of the way down the tube, and coincided with the hemagglutination (HA) activity. The central fractions of this peak were combined and dialyzed against LTM buffer.

RNA was extracted from this purified virus by shaking with water-saturated phenol. After three such extractions 200  $\mu$ g of yeast RNA (Calbiochem, Los Angeles, Calif.) per ml was added as carrier, followed by three volumes of ethyl alcohol. After several hours at -20 C, the precipitated RNA was washed with ethyl alcohol to remove residual phenol and was dissolved in 0.3 M STE buffer.

**Extraction of labeled dsRNA from infected cells.** A 1-liter culture was infected, and 0.5  $\mu$ g of actinomycin D per ml was added at time-zero. After 2 hr, 20  $\mu$ g of  $^{14}C$ -uridine (or 100  $\mu$ g of  $^3H$ -uridine) was added. At 16 to 20 hr after infection, the cells were centrifuged, washed twice with ice-cold LTM buffer, frozen, and

stored at -70 C. In this system, synthesis of dsRNA and viral maturation were almost complete by 20 hr postinfection: at that time, there was little cell lysis and the virus was very firmly cell-associated. The frozen cells were suspended in 50 ml of LAM buffer and 5 ml of 5% SDS was added. The mixture was stirred for 2 min at room temperature and then deproteinized by shaking it three times with phenol at room temperature. After this extraction, the RNA was precipitated by the addition of 3 volumes of 95% ethyl alcohol at -20 C. The precipitate was dissolved in 2 ml of LTM buffer containing 10  $\mu$ g per ml of electrophoretically pure, ribonuclease-free deoxyribonuclease (General Biochemicals Inc., Chagrin Falls, Ohio), and was stirred for 10 min at room temperature. Sodium chloride was added to 1 M concentration, and after 18 hr at 4 C the resulting precipitate of ssRNA was removed by centrifugation, leaving the dsRNA in solution. It was assumed that 1 OD<sub>260</sub> unit was equivalent to 45  $\mu$ g of RNA per ml.

**Extraction of labeled single-stranded virus-specific RNA from infected cells.** This procedure was similar to that employed for the preparation of dsRNA from infected cells, except that LTM buffer was used instead of LAM, and immediately after the phenol extraction 0.02 ml of a 10% Macaloid suspension (W. M. Stanley, Jr., Ph.D. Thesis, Univ. of Wisconsin, Madison, 1964) was added to the aqueous layer. This mixture was stirred for 10 min and the Macaloid was removed by centrifugation. Three volumes of ethyl alcohol was added to precipitate the RNA, which was then dissolved in a small volume of LTM buffer and treated with ribonuclease-free deoxyribonuclease for 10 min. The RNA was again precipitated with ethyl alcohol and dissolved in 0.5 ml of SDS buffer (8).

**Methyl albumin-kieselguhr (MAK) column analysis of dsRNA.** dsRNA prepared from infected cells was adjusted to contain 0.6 M NaCl and passed into a MAK column (13) where the dsRNA was retained. A linear gradient of NaCl was applied to the column with the use of 150 ml each of 0.7 M and 1.0 M STE buffer. A flow-rate of 15 to 20 ml per hr was maintained, and 3.8-ml fractions were automatically collected. Optical density at 260  $m\mu$  of the eluate was recorded continuously with a Gilson Medical Electronics recorder and fraction collector, and  $^{14}C$  was recorded continuously by scintillation counting with a Packard Tri-Carb model 320E flow counter.

**Sucrose gradient sedimentation analysis of RNA.** Generally, such analyses were carried out with 5 to 20% linear sucrose gradients by use of an SW50 rotor in a Spinco model L or L2 centrifuge at 20 C. The exact conditions are specified for each experiment. As a sedimentation marker, L-cell RNA, labeled either with  $^3H$ -uridine or  $^{14}C$ -uridine, whichever was appropriate, was added to the sample just before sedimentation analysis.

**Denaturation of double-stranded RNA with dimethyl-sulfoxide (DMSO; 8).** Ten volumes of DMSO were added to one volume of a solution of  $^{14}C$ - or  $^3H$ -labeled dsRNA which had been dialyzed against either 0.3 or 0.01 M STE, and the mixture was incubated at 37 C. To test the sensitivity of the denatured RNA to ribonuclease action, 0.1 ml of the incubation

mixture was withdrawn at intervals into 2.0 ml of 0.3 M STE buffer containing 4  $\mu$ g of ribonuclease. Digestion with ribonuclease was carried out for 30 min at 37 C and was stopped by the addition of 0.3 ml of 50% trichloroacetic acid and 1 drop of 0.5% bovine serum albumin. The precipitate was filtered on a membrane filter (type HA; Millipore Corp., Bedford, Mass.), washed with 5% trichloroacetic acid, dried, and counted in a liquid scintillation counter.

For analysis of the denatured RNA by sucrose gradient sedimentation, five volumes of 95% ethyl alcohol was added to the DMSO-RNA mixture, and yeast RNA was added as a carrier until the solution became slightly turbid. When 0.01 M STE solution was used in the denaturation experiment, an appropriate amount of NaCl was added to bring the concentration to 0.3 M before addition of the ethyl alcohol. The precipitate, which was allowed to form during 18 hr at 4 C, was collected by centrifugation, washed with 95% ethyl alcohol, and dissolved in TE buffer. This solution was then heated for 3 min at 60 C and cooled rapidly in an ice bath, to dissociate aggregates of denatured RNA that formed during the ethyl alcohol precipitation.

**Annealing of denatured RNA.** Appropriate amounts of denatured RNA were pipetted into small tubes, and the salt concentration was adjusted to give 0.3 M STE buffer in a final volume of 1 ml. The tubes were tightly stoppered and placed at the desired temperature for a predetermined interval. In fact, all such annealing tests were carried out at 72.5 C except for the single experiment, to be described later, in which the optimal temperature for annealing was determined. At the end of the incubation period, the tubes were transferred rapidly into an ice bath, and 1 ml of 0.3 M STE buffer containing 4  $\mu$ g of ribonuclease was added. The tubes were then incubated at 37 C for 30 min. One drop of 0.5% bovine serum albumin and 0.3 ml of 50% trichloroacetic acid were added; the resulting precipitate was then filtered, washed, dried, and assayed for radioactivity in a liquid scintillation counter. As a control, the ribonuclease sensitivity of denatured RNA was determined before and after being kept at 0 C for the same interval as the samples at 72.5 C.

**Radioactivity assays.** All determinations of radioactivity were made by scintillation counting in a Packard Tri-Carb spectrometer (19).

## RESULTS

**Isolation of three main fragments of viral RNA.** Attempts were made initially to separate the three fragments of viral RNA by sedimentation through sucrose gradients. Figure 1 shows the results of such an analysis of RNA which was isolated from purified,  $^{14}$ C-labeled reovirus. Three rather well-separated peaks of dsRNA are evident, sedimenting at rates of approximately 15S, 13S, and 10.5S. Application of larger amounts of RNA to the gradient, however, resulted in poorer separation of the peaks and

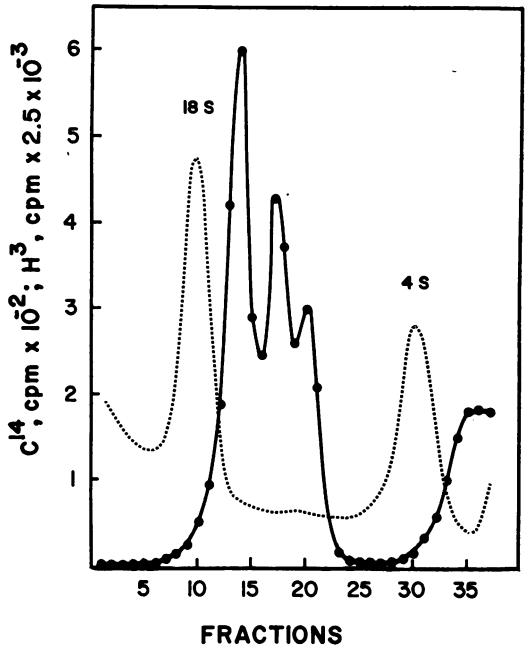


FIG. 1. Sucrose gradient sedimentation analysis of RNA extracted from purified,  $^{14}$ C-labeled reovirus. Symbols: ●, viral RNA  $^{14}$ C; dotted line, L-cell RNA labeled with  $^3$ H-uridine. Centrifuged at 49,500 rev/min for 230 min at 20 C.

necessitated the use of a different method for separating the three fragments on a larger scale.

It has been shown (10, 17) that double-stranded viral RNA may be eluted as an isolated fraction from an MAK column just ahead of L-cell ribosomal RNA. In the earlier experiments, rather steep gradients of sodium chloride were used and the RNA was eluted as a narrow peak at 0.7 to 0.8 M NaCl. When the MAK column was prepared as described by Mandel and Hershey (13) and a flat gradient of NaCl was applied at a slow rate of flow, the viral RNA could be separated into three fractions that were eluted between 0.75 and 0.85 M NaCl, as shown in Fig. 2. In this experiment, the dsRNA was labeled with  $^{14}$ C and isolated directly by extraction from infected cells, as described in Materials and Methods, rather than from purified virus; by this method, large amounts of dsRNA could be obtained with relative ease without first going through the procedure of virus purification with its attendant loss of material. Double-stranded RNA thus obtained was indistinguishable from that isolated from purified virus, whether analyzed by MAK column chromatography, sedimentation in sucrose or cesium sulfate gradients,

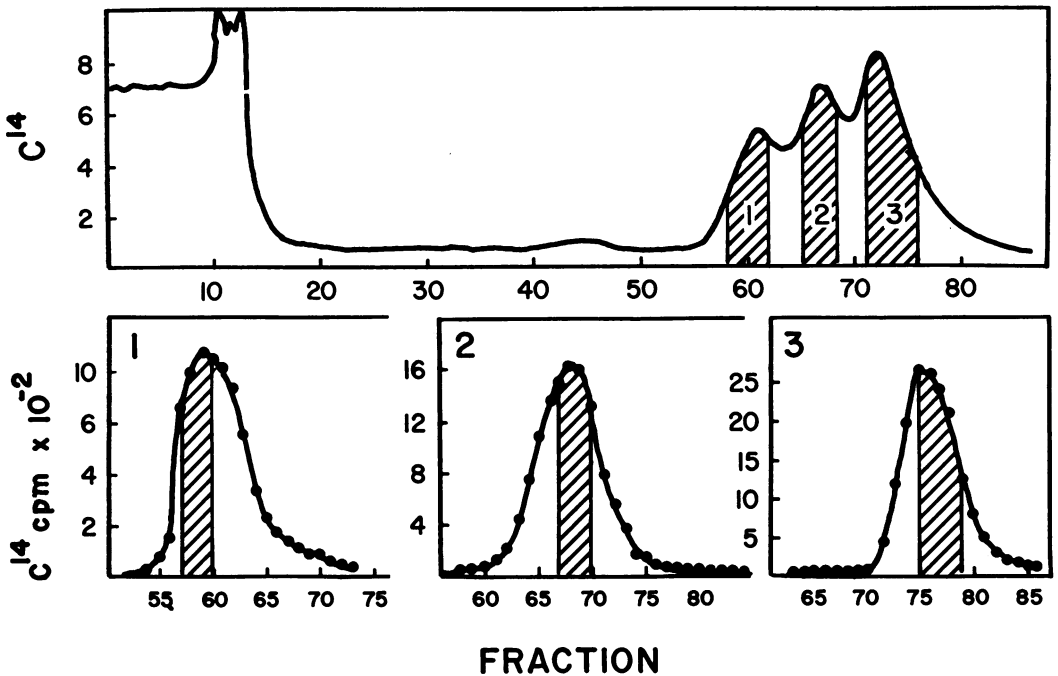


FIG. 2. Separation of three  $^{14}\text{C}$ -labeled viral RNA fragments on a MAK column. Upper panel shows the  $^{14}\text{C}$  trace obtained during elution: full scale deflection of the recording pen represented 1,000 counts/min. Double-stranded RNA was eluted between fractions 55 and 80. Lower panel shows the  $^{14}\text{C}$  profiles obtained when material from the shaded areas, 1, 2, and 3 in upper panel, was rerun on separate MAK columns.

or for base ratio (17), and was used in all succeeding experiments.

Fractions of peaks 1, 2, and 3 (Fig. 2) were combined, as represented by the shaded areas, and each of the combined quantities was rerun on a separate MAK column, with the same conditions of elution as before. The elution patterns obtained in these runs are shown in the lower three panels of Fig. 2, which are numbered to correspond to the three main peaks in the original column analysis. Eluted RNA was again combined, as illustrated by the shaded areas in the lower panels of Fig. 2, and the three dsRNA fractions thus obtained will be designated dsRNA-1, dsRNA-2, and dsRNA-3. Double-stranded RNA separated in this way was used in the denaturation and homology tests described later.

The sedimentation analysis of each of these fractions is shown in Fig. 3. Reference to the L-cell RNA sedimentation markers indicates a sedimentation rate of approximately 15S for dsRNA-1, 12.5S for dsRNA-2, and 10.5S for dsRNA-3. Applying the formula of Doty et al. (3),  $S = 0.063 1M^{0.37}$ , where  $S$  is the sedimentation constant and  $M$  is the molecular weight; the molecular weight of dsRNA-1 is  $2.7 \times 10^6$ ,

of dsRNA-2 is  $2 \times 10^6$ , and of dsRNA-3 is  $1 \times 10^6$  daltons. It should be emphasized that these estimates of molecular weight are only approximate, since the formula of Doty et al. was derived empirically for estimating the molecular weights of DNA and may not be strictly applicable in the present instance.

*Denaturation of the separated dsRNA fragments.* Reovirus RNA is highly resistant to the action of low concentrations (5  $\mu\text{g}/\text{ml}$  or less) of ribonuclease at salt concentrations of 0.1 M or greater. When heated to 100 C in 0.01 M NaCl for 3 min and quick-chilled, the RNA becomes almost completely sensitive to ribonuclease, indicating breakage of a majority of the bonds holding together the two strands, if not complete strand separation (6, 14, 15, 17). Nevertheless, because of the possibility that single-strand breaks might be introduced at the high temperature, an additional and more gentle method of denaturation was sought for comparison. This was provided by the observation of Katz and Penman (8) that high concentrations of DMSO denatured the double-stranded form of poliovirus RNA at 37 C.

The results in Fig. 4 show that, at a concentration of 90% (v/v) DMSO, 90% of reovirus

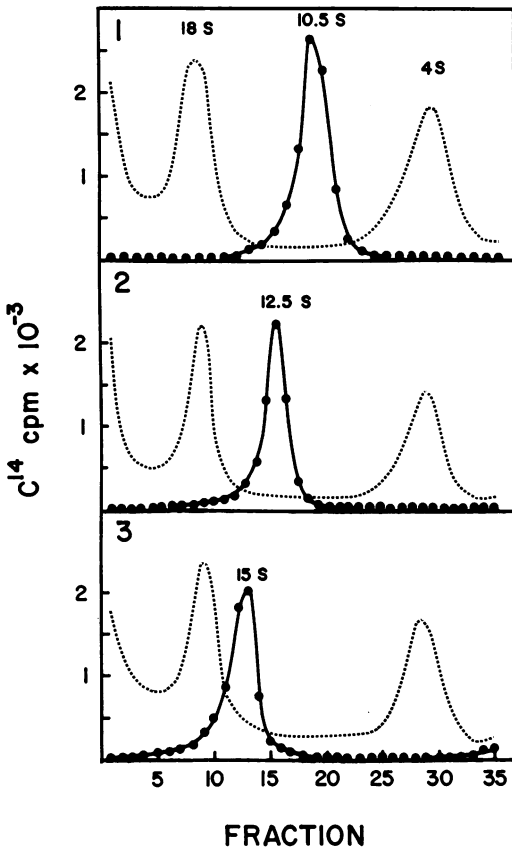


FIG. 3. Sedimentation analysis of the three viral RNA fragments after MAK column separation. (1) dsRNA-1, (2) dsRNA-2, (3) dsRNA-3; the dsRNA fraction numbers correspond to those in Fig. 2. Symbols: ●, dsRNA  $^{14}\text{C}$ ; dotted line, L-cell RNA labeled with  $^3\text{H}$ -uridine. Centrifuged at 49,500 rev/min for 230 min at 20 C.

RNA (dsRNA-3) became sensitive to ribonuclease action within 10 min at 37 C. Sedimentation analysis of dsRNA-3 melted for 30 min in this way and then precipitated with ethyl alcohol showed one main peak of ribonuclease-sensitive material, but a pellet of ribonuclease-sensitive RNA was also found to collect at the bottom of the centrifuge tube. On the supposition that such pellets represented denatured RNA that had become aggregated during the ethyl alcohol precipitation, RNA, melted by DMSO treatment and then precipitated with ethyl alcohol, was redissolved in TE buffer and heated at 60 C for 3 min prior to sedimentation analysis. This gentle heating caused all of the more rapidly sedimenting material to travel with the one major peak.

Sedimentation analyses of dsRNA-1, dsRNA-

2, and dsRNA-3, separated as described in the previous section and denatured according to the procedure just outlined, are shown in Fig. 5. Each denatured fraction gave rise to one major peak of ribonuclease-sensitive RNA. Some ribonuclease-sensitive material trailed the main peak found for both denatured dsRNA-2 and dsRNA-3. This trailing material is thought to be RNA that has been only partly melted; i.e., the two strands are incompletely separated. Similar sedimentation patterns were observed when the DMSO treatment was carried out for 180 min or at temperatures up to 80 C, or when the dsRNA was heated in TE buffer for 5 min at 100 C.

Thus, the three main peaks shown in Fig. 5 are presumed to represent the separated single-stranded components of the three fractions of double-stranded viral RNA, and some evidence will be provided in the next section that this presumption is correct. Reference to the L-cell RNA components used as sedimentation markers indicates that melted RNA from dsRNA-1 sediments at 14S, that from dsRNA-2 at 18.5S, and that from fraction 3 at 24.5S. The respective molecular weights of single-stranded RNA, calculated from the formula of Spirin (18),  $M = 1,550S^{2.1}$  where  $M$  is the molecular weight

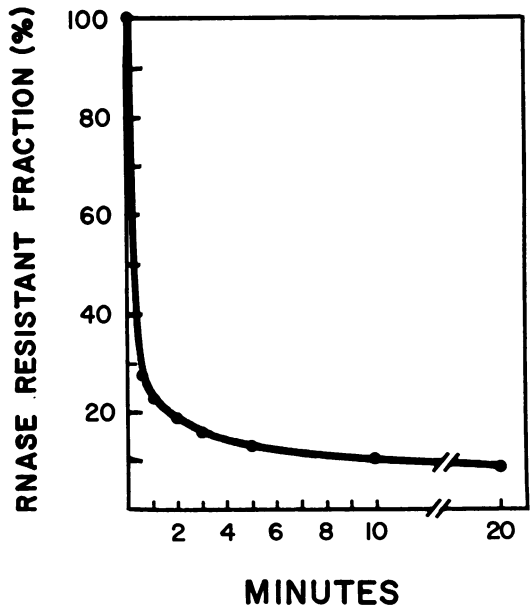


FIG. 4. Denaturation of dsRNA-3 with DMSO determined by decrease in ribonuclease resistance. Fraction dsRNA-3 (Fig. 2), 7.5  $\mu\text{g}$ , 18,000 counts/min of  $^{14}\text{C}$ , in 0.1 ml of 0.3 M STE buffer, was mixed with 1 ml of DMSO.

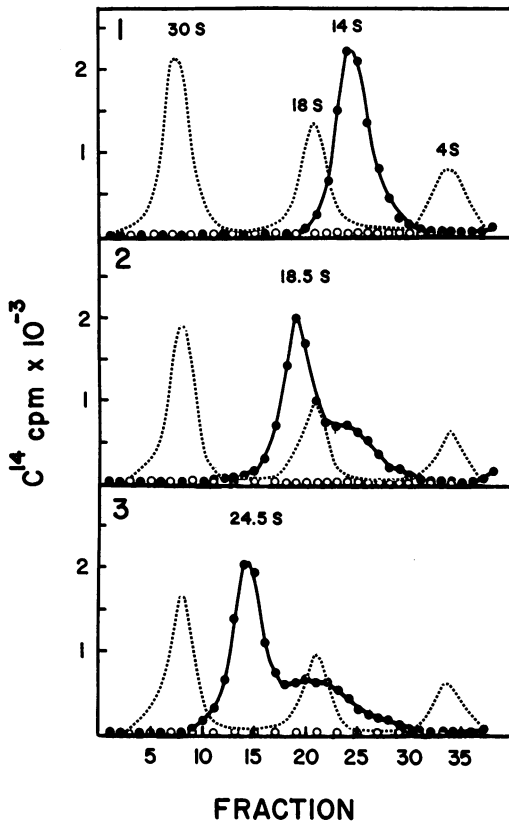


FIG. 5. Sedimentation of dsRNA-1, dsRNA-2, and dsRNA-3 after DMSO denaturation. After denaturation for 30 min with DMSO, the RNA, 3  $\mu$ g, 19,500 counts/min of  $^{14}\text{C}$ , was dissolved in 0.1 ml of TE buffer, heated to 60 C for 3 min, and layered over a sucrose gradient. Sedimentation was for 135 min at 49,500 rev/min at 20 C. Panel 1, dsRNA-1, panel 2, dsRNA-2, panel 3, dsRNA-3. Symbols:  $\bullet$ ,  $^{14}\text{C}$ -labeled, denatured dsRNA;  $\circ$ ,  $^{14}\text{C}$  insensitive to ribonuclease; dotted line,  $^3\text{H}$ -labeled, L-cell RNA components.

and  $S$  is the sedimentation rate, are  $0.4 \times 10^6$ ,  $0.7 \times 10^6$ , and  $1.2 \times 10^6$ .

**Annealing of denatured dsRNA.** A study was then undertaken of the reannealing of the denatured fractions of dsRNA, with the ultimate purpose of determining whether any homology existed among the three fractions.

To determine the proper conditions for annealing,  $^{14}\text{C}$ -labeled dsRNA (not separated into the three fractions) was denatured by heating for 3 min to 100 C in 0.01 M STE and was quickly chilled. Figure 6 shows the extent of reannealing of this melted RNA when kept in 0.3 M NaCl for 30 min at different temperatures, as measured by the conversion of RNA from a ribonuclease-sensitive to a ribonuclease-insensitive form. The

optimal temperature for annealing was 72.5 C, and all experiments on annealing of RNA were henceforth carried out at this temperature.

It was mentioned toward the end of the previous section that the two strands of viral RNA were presumed to be cleanly separated by DMSO treatment or by heating to 100 C in dilute salt solution. If this is the case, the rate of reannealing of denatured RNA should be concentration-dependent and obey second-order kinetics, assuming that zipping-up of the two strands is relatively rapid once effective collision occurs. Figure 7 shows the results of an experiment on the rates of reannealing of the three fractions of dsRNA that had been denatured by heating to 100 C. In Fig. 7,  $(C_0 - C)/C$  has been plotted against  $C_0 t$ , where  $(C_0 - C)/C = k C_0 t$  and  $C_0$  = the initial concentration of denatured viral RNA, in moles per liter;  $C$  = the concentration of unpaired viral RNA at any given time (i.e., the ribonuclease-sensitive fraction, in moles per liter);  $t$  = the time of annealing, in seconds; and  $k$  = the second-order rate constant.

This method of plotting the results of annealing tests according to the second-order rate equation has been fully discussed by Britten and Kohne (2). The linear portion of the curve (Fig. 7) accounting for upwards of 70% of the RNA, and more in other experiments, indicates a dependence of reannealing on second-order kinetics and shows that collision frequency of the two strands is the rate-limiting factor. Similar results were found for RNA denatured by DMSO. It is inferred from these results that denaturation had completely separated the two strands of each of the RNA fragments. At low values of  $C_0 t$ , there is some departure from linearity in the kinetics of reannealing (Fig. 7). There are

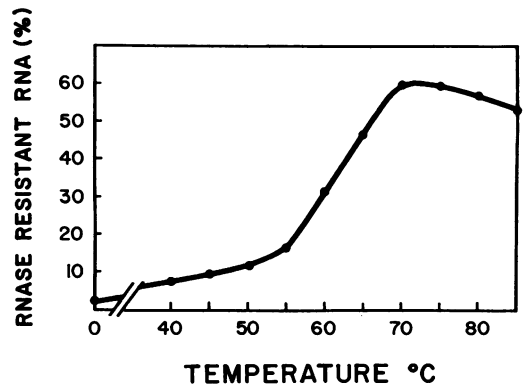


FIG. 6. Effect of temperature on reannealing of denatured dsRNA. Extent of annealing was determined by resistance to digestion by ribonuclease.

several possible explanations of this interesting phenomenon, but further work will be required to decide among them.

*Cross-annealing among the three denatured fragments of viral RNA.* In this section, we consider the question of possible homology among denatured RNA obtained from each of the three isolated dsRNA fragments. The experimental plan consisted of mixing a relatively small and constant amount of  $^3\text{H}$ -labeled dsRNA of one fragment with increasing amounts of  $^{14}\text{C}$ -labeled dsRNA of each of the three fragments in 0.01 M STE. All of these mixtures were heated at 100 C for 5 min to denature the RNA, and then were quickly chilled. The salt concentration was then

increased to 0.3 M STE, and the samples were placed at 72.5 C for 30 min. Each sample was then assayed for its content of ribonuclease-resistant  $^3\text{H}$  and  $^{14}\text{C}$  to get a measure of the extent of reannealing of the melted RNA. The results of these tests are shown in Fig. 8.

Considering, first, panel A-1, the extent of reannealing of  $^{14}\text{C}$ -labeled RNA-1 increased rapidly with increasing concentration, up to 1  $\mu\text{g}/\text{ml}$ . The reannealing of  $^3\text{H}$ -labeled RNA-1 followed the same course, as it should. Panels B-2 and C-3 show the self-annealing of denatured dsRNA-2 and dsRNA-3, respectively; the results in general are similar to those in A-1. Panel A-2 shows that there was little homology between denatured dsRNA-1 and dsRNA-2. It is true that with increasing concentrations of  $^{14}\text{C}$ -labeled RNA-2 the amount of hybridization with  $^3\text{H}$ -labeled RNA-1 increased (A-2), and a similar trend is shown in the results in panel A-3. Nevertheless, in the reciprocal tests, B-1 and C-1, respectively, there was virtually no increase in the amount of  $^3\text{H}$ -containing hybrid as the concentration of  $^{14}\text{C}$ -labeled RNA was increased. The results of these reciprocal tests suggest that the increasing hybridization of  $^3\text{H}$ -labeled dsRNA shown in panels A-2 and A-3 resulted from some cross-contamination of the  $^3\text{H}$ -labeled dsRNA-1 with  $^3\text{H}$ -labeled dsRNA-2 and dsRNA-3.

We conclude from this set of results that there is little or no homology among the three fragments of viral RNA and, in consequence, that these fragments did not arise from random breaks in the original molecule. It will be shown below that these fragments may have some functional significance, since at least one of them will hybridize along its full length with a fraction of ssRNA of similar length derived from infected cells.

*Fractionation of the ssRNA formed in infected cells.* In addition to the double-stranded viral RNA formed in reovirus-infected cells, at least an equal amount of single-stranded virus-specific RNA accumulates (10, 17). Most of this ssRNA is associated with polyribosomes engaged in virus-specific protein synthesis; it is mRNA involved in virus-specific protein synthesis and is transcribed from a single strand of the viral RNA (14, 17). Although the ssRNA was broadly distributed from approximately 35S to 10S in sedimentation analyses, it was previously pointed out (10) that it seemed to fall into at least two fairly distinct fractions. Better centrifuge technique has improved the resolution in this type of analysis and, as shown in Fig. 9A, the ssRNA can, in fact, be separated into three well-defined peaks.

In the experiment of Fig. 9A, cycloheximide

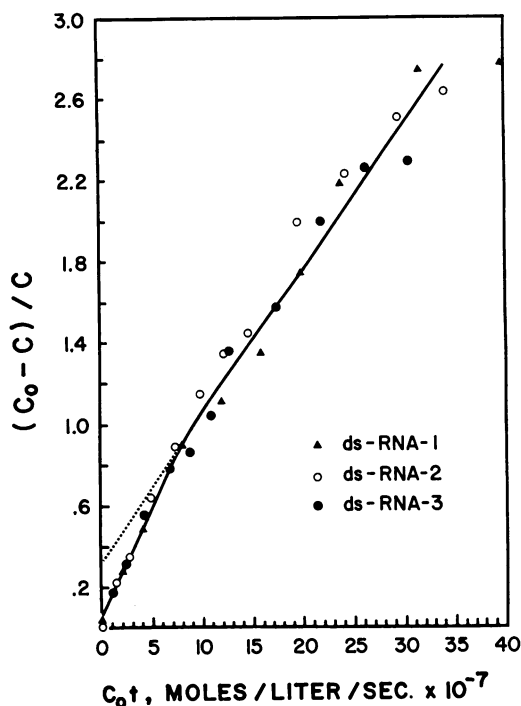


FIG. 7. Plot of the kinetics of reannealing of denatured dsRNA fragments according to the second-order equation. For symbols, see text. The  $^{14}\text{C}$ -labeled RNA had been denatured by heating the three separated fractions of dsRNA to 100 C for 5 min in 0.01 M STE and quick chilling. The salt concentration was adjusted to 0.3 M STE buffer. Final concentrations were dsRNA-1, 0.28  $\mu\text{g}/\text{ml}$ ; dsRNA-2, 0.34  $\mu\text{g}/\text{ml}$ ; dsRNA-3, 0.44  $\mu\text{g}/\text{ml}$ . A 1-ml amount of each solution was transferred to each of a series of tubes which were tightly closed, heated at 72.5 C for intervals varying from 0 to 140 min, and then quickly chilled. The amount of ribonuclease-resistant  $^{14}\text{C}$  was then determined in each sample. Concentrations, in moles per liter, were calculated from the molecular weights of the denatured RNA fragments given in the text.

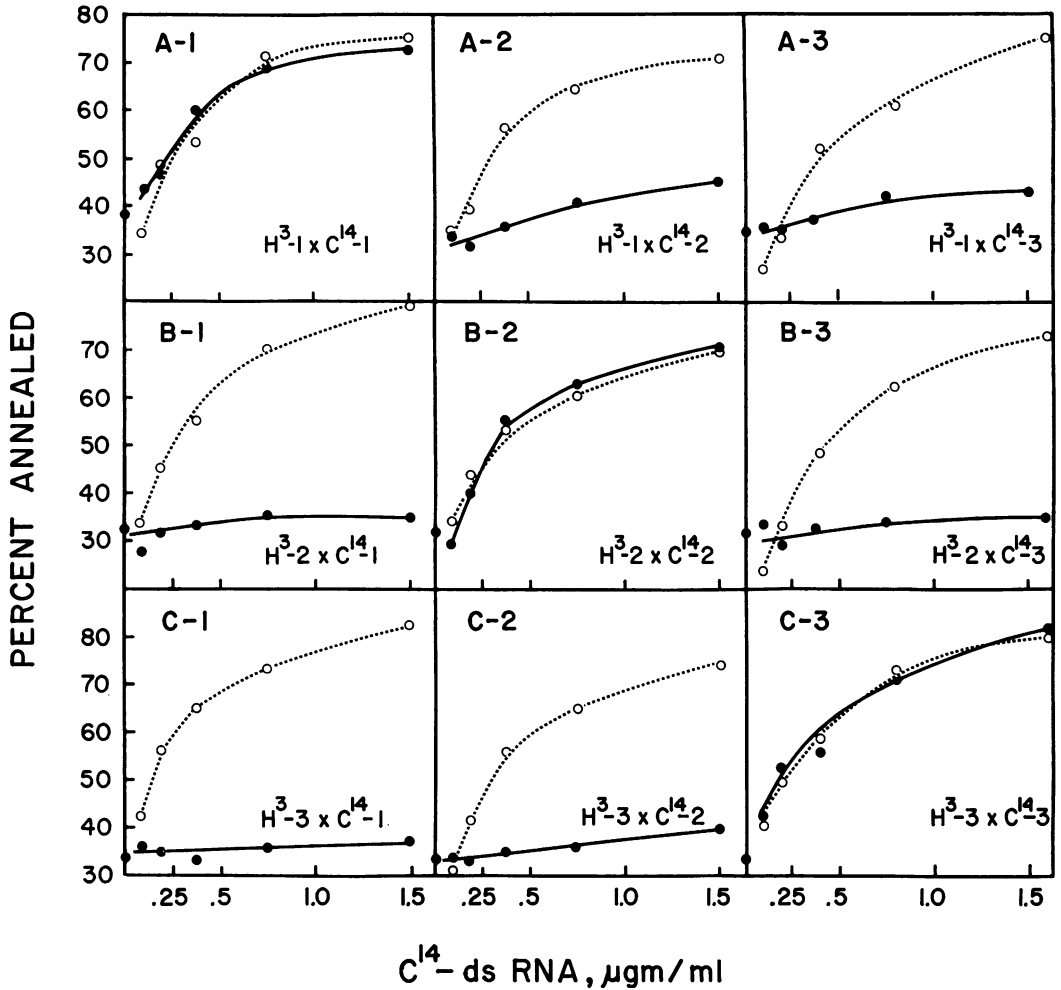


FIG. 8. Analysis of cross-annealing between the separated, denatured dsRNA fragments 1, 2, and 3. Series A, 0.07  $\mu\text{g}$ , 800 counts/min of  $^3\text{H}$ , of dsRNA-1 was mixed with increasing amounts of  $^{14}\text{C}$ -labeled dsRNA-1 (A-1), dsRNA-2 (A-2), or dsRNA-3 (A-3) prior to denaturation and reannealing. Series B, same as series A, but using 0.07  $\mu\text{g}$  of  $^3\text{H}$ -labeled dsRNA-2 per test. Series C, same as series A, but using 0.07  $\mu\text{g}$  of dsRNA-3 per test. Symbols:  $\circ$ ,  $^{14}\text{C}$ -labeled RNA insensitive to ribonuclease;  $\bullet$ ,  $^3\text{H}$ -labeled RNA insensitive to ribonuclease.

was added at 8 hr after infection with actinomycin D, and the cells were labeled with uridine- $^3\text{H}$  between 9 and 11 hr postinfection. It has been shown previously that cycloheximide added at 8 hr rapidly blocks further synthesis of viral RNA, whereas virus-specific ssRNA accumulates to an increased extent (19). Figure 9B shows the results of the control experiment from which cycloheximide was omitted. Three peaks of ssRNA can again be seen, but the two lighter peaks are obscured by the presence of dsRNA. Using the L-cell markers as references, the three peaks of Fig. 9A had sedimentation rates similar to those found for the ssRNA derived by DMSO

denaturation of the three fragments of viral RNA (Fig. 5). It seemed from this result that the three classes of ssRNA might have been transcribed from the three fragments of dsRNA we had isolated (Fig. 3). The remainder of this paper describes the separation of the three classes of ssRNA and demonstrates, by homology tests between them and the three fractions of melted dsRNA, that this is a plausible hypothesis.

In an experiment similar to that represented by Fig. 9A, but on a larger scale, cuts were taken from the gradient as indicated by the sections 1, 2, 3, and 4 on the figure. Each of the resulting four samples was heated at 60 C for 3 min in



TE buffer to break up possible aggregates and then was centrifuged separately through sucrose gradients. Figure 10 depicts the results thus obtained; the panels are numbered to correspond to the cuts taken in Fig. 9A. The slowest moving peak of material, panel 1, seemed reasonably homogenous. Peak 2 contained some peak 1 material. Peaks 3 and 4 contained all three classes of ssRNA, demonstrating, particularly for panel 4, that considerable aggregation of the various classes of ssRNA had occurred and had been largely discharged by the gentle heat treatment before sedimentation analysis. Samples were taken out of the gradient as indicated by the shaded areas of panels 1, 2, and 3 (Fig. 10), and these will be designated as fractions ssRNA-1, ssRNA-2, and ssRNA-3, in order of increasing sedimentation rate, as the dsRNA fragments were numbered.

$^3\text{H}$ -labeled ssRNA from each fraction was then mixed with DMSO-denatured RNA from the corresponding fractions of  $^{14}\text{C}$ -labeled dsRNA, and the three mixtures were sedimented

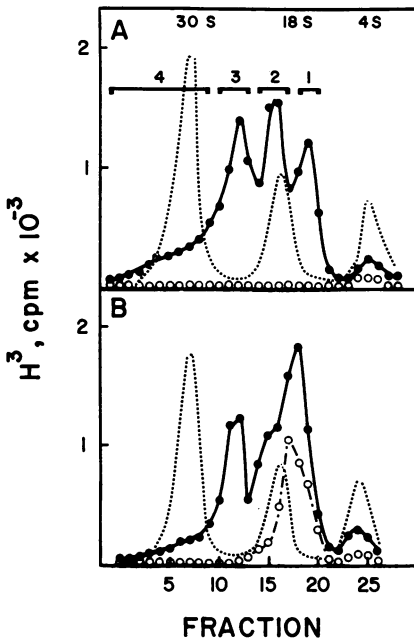


FIG. 9. Sedimentation of RNA extracted from virus-infected cells labeled in presence and absence of cycloheximide. (A) Cycloheximide, 20  $\mu\text{g}/\text{ml}$ , and actinomycin D, 2  $\mu\text{g}/\text{ml}$ , added at 8 hr postinfection and labeled with uridine- $^3\text{H}$  from 9 to 11 hr postinfection. RNA was extracted at 11 hr postinfection. (B) No cycloheximide, other conditions as in A. Symbols: ●, total  $^3\text{H}$ -labeled RNA; ○, ribonuclease-resistant  $^3\text{H}$ -labeled RNA; dotted line,  $^{14}\text{C}$ -labeled L-cell RNA sedimentation markers.

through sucrose gradients. The results (Fig. 11) show that for each of the three fractions there is close correspondence between the sedimentation rates of ssRNA and the denatured dsRNA. The trailing  $^{14}\text{C}$ -labeled material in these gradients, also shown in Fig. 5, has been ascribed tentatively to incompletely melted dsRNA. Thus, each fraction of ssRNA corresponds very closely in molecular length to one fraction of dsRNA. The next question at issue was whether a given fraction of ssRNA would hybridize uniquely with its corresponding fraction of melted dsRNA.

*Cross-hybridization between the fractions of ssRNA and denatured dsRNA.* Figure 12 represents the results of an experiment in which denatured dsRNA-3 was annealed with ssRNA-1, ssRNA-2, and ssRNA-3. Hybridization occurred only between ssRNA-3 and denatured dsRNA-3 (panel 3), and the homology was almost complete. There was no homology between dsRNA-3 and the other two classes of ssRNA.

It should be noted that these ssRNA fractions, especially ssRNA-1 and -2, contained a small amount of unlabeled dsRNA which would have accumulated in the infected cells before the addition of cycloheximide (19). A control test was therefore carried out to find whether this dsRNA would have influenced the annealing tests. Double-stranded RNA (unfractionated) was added to each of ssRNA-1, -2, and -3, and the mixtures were heated to 72.5 C in 0.3 M STE buffer. Both before and after the heating, approximately 3% of the ssRNA was resistant to ribonuclease. There was thus no conversion of ssRNA to a ribonuclease-resistant form, indicating that the presence of a small amount of undenatured dsRNA had little effect on the results of Fig. 12.

To find whether ssRNA-3 might be uniquely transcribed from dsRNA-3, ssRNA-3 was hybridized with each of the three fractions of denatured dsRNA, with the results shown in Fig. 13. Curves for self-annealing of the three double-stranded fragments have been omitted from the figure, but all were similar to those shown for  $^{14}\text{C}$ -labeled dsRNA-3 in Fig. 12. Again, homology between ssRNA-3 and dsRNA-3 was almost complete, but some homology was also observed between ssRNA-3 and both dsRNA-1 and dsRNA-2. This latter result is most simply interpreted on the assumption that a small amount of dsRNA-3 is present as a contaminant in both dsRNA-1 and 2, since such contamination was revealed in the experiments of Fig. 8. It should be emphasized that the present experiments would in principle be much more sensitive in picking up such cross-contamina-

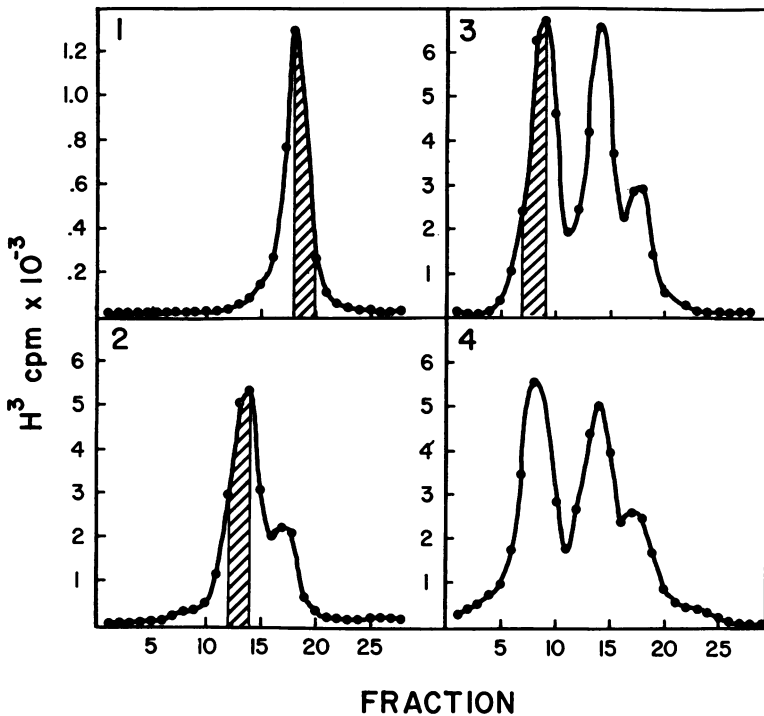


FIG. 10. Sedimentation of three classes of ssRNA after separation in a sucrose gradient and heating at 60 C for 3 min. Sedimentation in SDS buffer for 150 min at 49,500 rev/min at 20 C in the SW50 rotor.

tion than were those of Fig. 8. Any contamination of another fraction by dsRNA-3 would result in significant hybridization of ssRNA-3 because of the low concentration of ssRNA-3 employed. To get some estimate of the amount of presumed contamination that could be present, apparent second-order rate constants were calculated from equation 1 and the data of Fig. 13 at the point where annealing was 50% complete, i.e., where  $(C_0 - C)/C$  is unity. Relative values for these constants were unity for the reaction dsRNA-3 versus ssRNA-3, 0.14 for dsRNA-2 versus ssRNA-3, and 0.03 for dsRNA-1 versus ssRNA-3. On the assumption that the amount of ssRNA-3 added in these annealing tests was, in all cases, negligible compared with the amount of dsRNA-3 present, these values are an approximate measure of the amount of dsRNA-3 that may be present in the fractions dsRNA-2 and dsRNA-1, namely, 14 and 3% of the total RNA in the respective fractions. Further purification of the dsRNA fractions by sedimenting them through sucrose gradients prior to repeating the tests of Fig. 13 made little change in the picture. This result shows that any contamination of the other fractions by dsRNA-3 must have resulted from some breakdown of dsRNA-3 at an early stage in the preparation.

As an additional argument, if the values just calculated were assumed to represent the lengths of regions in dsRNA-2 and dsRNA-1 homologous to ssRNA-3, one would expect the two lower curves in Fig. 13 to have reached a maximum at low concentrations of dsRNA; they show no tendency to do this. It is concluded that ssRNA-3 is uniquely transcribed from dsRNA-3 in the infected cells.

In the remaining tests of the pattern, ssRNA-2 showed homology with denatured dsRNA-1, and ssRNA-1 with dsRNA-2, as well as with dsRNA of their equivalent length. This cross-hybridization was so extensive that no clear interpretation of the results was possible. It might be pointed out, however, that dsRNA-1 and dsRNA-2 are the two fractions most likely to be cross-contaminated and the most difficult to purify, and further experiments along these lines will have to await improved methods of preparing fractions dsRNA-1 and 2.

#### DISCUSSION

Viral RNA as isolated routinely in the present work was broken into lengths that fell into three size classes. We have shown in a previous section that the sedimentation rates of dsRNA-1, dsRNA-2, and dsRNA-3 (Fig. 5) after dena-

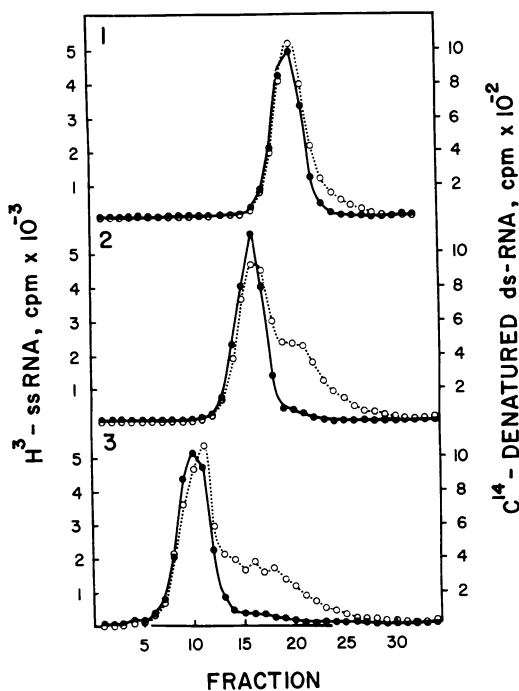


FIG. 11. Sedimentation of the three classes of separated ssRNA with the three denatured dsRNA fragments. The three classes of  $^3\text{H}$ -labeled ssRNA were separated as shown in Fig. 10 and  $^{14}\text{C}$ -labeled dsRNA fragments were separated as shown in Fig. 2. After denaturation of the dsRNA with DMSO, ssRNA-1 was mixed with dsRNA-1, ssRNA-2 with dsRNA-2, and ssRNA-3 with dsRNA-3. Each mixture was heated at 60 C for 3 min and sedimented through a sucrose gradient prepared in SDS buffer for 140 min at 49,500 rev/min at 20 C. Panel 1, RNA-1; panel 2, RNA-2; panel 3, RNA-3. Symbols: ●,  $^3\text{H}$ -labeled ssRNA; ○, denatured  $^{14}\text{C}$ -labeled dsRNA.

turation were 14S, 18.5S, and 24.5S, respectively. Molecular weights of  $0.4 \times 10^6$ ,  $0.7 \times 10^6$ , and  $1.2 \times 10^6$  daltons, respectively, were calculated for these fragments from the Spirin relationship between sedimentation constant and molecular weight. Prior to denaturation, the double-stranded pieces must then have had molecular weights of approximately  $0.8 \times 10^6$  daltons for dsRNA-1,  $1.4 \times 10^6$  for dsRNA-2, and  $2.4 \times 10^6$  for dsRNA-3. These latter values are in agreement with molecular weights calculated for the three main fragments seen in electron micrographs, namely  $0.8 \times 10^6$ ,  $1.4 \times 10^6$ , and  $2.5 \times 10^6$  daltons (3a, 4), on the assumption that  $1 \mu$  is equivalent to  $2.3 \times 10^6$  daltons. Clearly, the three fractions of dsRNA we have been studying can be identified with the three major lengths seen by electron microscopy.

Estimates of the relative amounts of dsRNA-1, dsRNA-2, and dsRNA-3 can be arrived at by summing the areas under elution profiles resulting from MAK column chromatography (Fig. 2) or sedimentation analyses (Fig. 1). Results of several such analyses are given in Table 1. On the assumption that the viral RNA molecule contains two lengths of dsRNA-3, the three fragments of dsRNA fall into the molar ratios of 3 dsRNA-1, 3 dsRNA-2, and 2 dsRNA-3. This assigned distribution can be little better than an approximation when one considers the errors involved in assessing areas under poorly separated peaks (Fig. 2) and the variation from one analysis to another (Table 1). Even so, these estimates are within the range of the much more accurate results provided by Dunnebacke and Kleinschmidt (Table 1) from measurement of the frequency distribution of contour lengths in an electron microscope. Whether the several pieces within each size range are identical to one another or not is still unknown. Moreover, the order in which they occur in the viral RNA molecule has still to be worked out. From our results, the molecule of viral RNA would have a weight of  $11.4 \times 10^6$  daltons before fragmentation; a weight of approximately  $12.4 \times 10^6$  daltons would be derived

TABLE 1. Relative amounts of the three dsRNA fragments

Expt	Relative amt <sup>a</sup>		
	dsRNA-1	dsRNA-2	dsRNA-3
1	1	1.4	1.8
2	1	1.4	1.8
3	1	1.5	2.0
4	1	2.2	2.9
5	1	1.5	2.5
Average	1	1.5	2
Relative molar ratio <sup>b</sup>	3	3	2
No. of fragments per molecule <sup>c</sup>	4-6	3	2

<sup>a</sup> Determined from areas under the three peaks of MAK column analyses (Fig. 2) of RNA extracted from infected cells for experiments 1 to 4, and of sucrose gradient analysis (Fig. 1) of RNA from purified virus for experiment 5. Amount of dsRNA-1 in each analysis taken as unity.

<sup>b</sup> Average relative amount divided by molecular weight of the fraction. The viral RNA molecule is assumed to contain two pieces of dsRNA-3.

<sup>c</sup> Results of Dunnebacke and Kleinschmidt (3a) for number of fragments per viral RNA molecule.

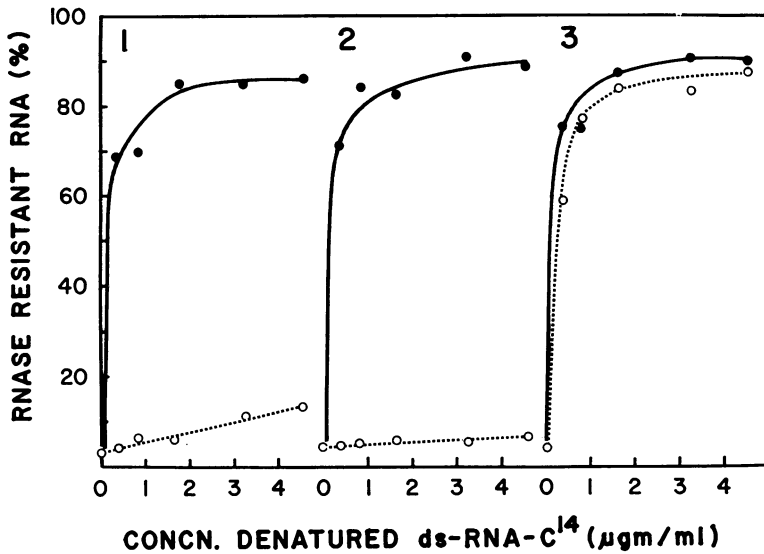


FIG. 12. Hybridization tests of the three classes of  $^3\text{H}$ -labeled ssRNA with denatured dsRNA-3 labeled with  $^{14}\text{C}$ . Each point represents a mixture annealed at  $72.5^\circ\text{C}$  for 3 hr in  $0.3\text{ M}$  STE buffer, quick-chilled, and tested for ribonuclease sensitivity. The amount of ssRNA per test was approximately  $0.01\ \mu\text{g}$ . Panel 1, ssRNA-1 and dsRNA-3; panel 2, ssRNA-2 and dsRNA-3; panel 3, ssRNA-3 and dsRNA-3. Symbols:  $\circ$ ,  $^3\text{H}$ -labeled RNA insensitive to ribonuclease;  $\bullet$ ,  $^{14}\text{C}$ -labeled RNA insensitive to ribonuclease.

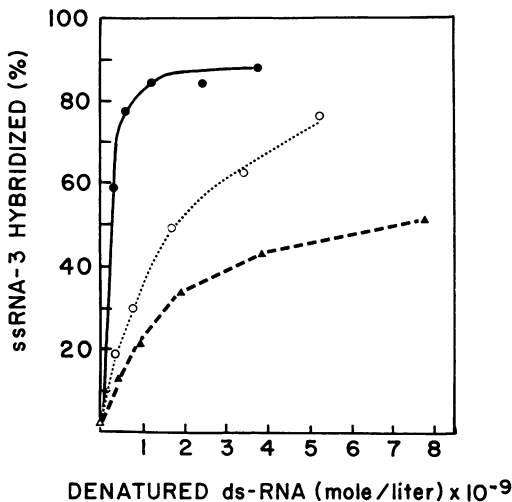


FIG. 13. Hybridization tests of ssRNA-3 with the three classes of denatured dsRNA. Experimental conditions as in legend to Fig. 12. Symbols:  $\bullet$ , ssRNA-3 versus dsRNA-3;  $\circ$ , ssRNA-3 versus dsRNA-2;  $\blacktriangle$ , ssRNA-3 versus dsRNA-1.

from the data of Dunnebacke and Kleinschmidt (3a).

The breaks occurring in the viral RNA molecule during isolation are not introduced at random. Such a mechanism is excluded by our ob-

ervation that little or no hybridization occurs between the various denatured double-stranded fragments. Further, as pointed out by Dunnebacke and Kleinschmidt (3a), the contour lengths and their frequency distribution are inconsistent with a hypothesis of random breakage. Neglecting the unlikely possibility that these segments never are joined together in the virion, the only reasonable alternative is to postulate that there are preferred regions in the viral RNA molecule where breakage can readily occur. Whether the preferred regions result from single-strand nicks as in T5 phage deoxyribonucleic acid (1), or from double-stranded segments being held together by protein linkers or by short stretches of ssRNA, is a subject for further investigation.

Iglewski and Franklin (7a) have recently observed that RNA isolated from reovirus by sodium perchlorate treatment gave rise on heat denaturation to three single-stranded fragments that seem to correspond to the denatured dsRNA-1, -2, and -3 described in the present paper. DMSO denaturation, on the other hand, produced one fraction equivalent in sedimentation rate to denatured dsRNA-3 and another sedimenting at  $14\text{S}$ . The authors suggested that the latter was incompletely melted dsRNA. One could explain these results on the grounds that bonds holding dsRNA-1 and dsRNA-2 together, end to end, are stronger than those that hold

dsRNA-3 into the viral RNA molecule; sodium perchlorate might then break the viral RNA molecule into two species of roughly equivalent length, one consisting of dsRNA-3 and the other of 1 unit of dsRNA-2 with 2 units of dsRNA-1. It would need further to be postulated that heat denaturation of this mixture breaks all remaining bonds holding together the structural units, as well as separating the strands, whereas DMSO leads only to strand separation and introduces no further breaks. This hypothesis can be readily tested and is of interest, since it suggests that bonds between the various double-stranded structural units can be broken selectively.

By whatever means the various lengths of dsRNA may be held together in the viral RNA molecule, at least one of them seems to act as a "unit of transcription." For the longest segment of dsRNA, an equivalent length of ssRNA (mRNA) is synthesized. This ssRNA is homologous throughout its length with one strand of dsRNA-3. As pointed out above, there are probably two such lengths of dsRNA-3 in the viral RNA molecule, which may or may not be identical. At any rate, each, with a molecular weight of some  $2.4 \times 10^6$  daltons, contains information to code for 5 to 7 proteins, and our results indicate that this information is transcribed in one unbroken piece of mRNA. The smallest pieces of dsRNA probably contain information for only one or two proteins. Pieces of ssRNA were made equivalent in length to the segments of dsRNA-1, but the homology tests were ambiguous in determining whether they were uniquely specified by dsRNA-1. A similar ambiguity existed in the homology experiments with dsRNA-2 and the two smaller classes of ssRNA. This is an important problem to clarify. Should it turn out that each ssRNA is uniquely transcribed from one of the three corresponding lengths of dsRNA, it ought to be feasible to relate some of the virus-specific functions in the infected cell with definite physical segments of the viral genome.

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