Purification and Characterization of Poliovirus-induced Infectious Double-stranded Ribonucleic Acid

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SUMMARY

Ribonuclease-resistant infectious double-stranded RNA has been isolated in milligram quantities from poliovirus-infected HeLa cells. Purification was accomplished by eliminating high molecular weight single-stranded RNA by differential NaCl precipitation and chromatographing the remaining RNA on columns of methylated albumin-Kieselguhr. The product has the following physicochemical and biological properties: (a) Homogeneity with respect to size, as determined by sedimentation analysis; (b) an S0, w of 17.2, which is compatible with the molecular weight of 4 x 10^6 anticipated for poliovirus double-stranded RNA; (c) buoyant density in Cs2SO4 of 1.60 g cm^-3; (d) abrupt hyperchromic shift of 25% on heating, the value of Tm (temperature at midpoint) being dependent upon ionic environment; (e) nucleotide composition consistent with a base-paired, double-stranded secondary structure; (f) infectivity which is partially resistant to RNase and resistant to formaldehyde; (g) specific infectivity 30-fold greater than that of poliovirus single-stranded RNA; (h) optimal conditions for assay of infectivity which differ from those of single-stranded RNA.

It is concluded that the RNase-resistant infectivity derives from information contained in a double-stranded molecule and that the replicative cycle initiated by double-stranded RNA must differ from the cycle initiated by single-stranded RNA. The fact that double-stranded RNA is infectious has definite implications regarding the possible functional role of this material in the virus growth cycle.

Infection with a wide variety of small, ribonucleic acid-containing bacterial, plant, and animal viruses induces the formation of RNase-resistant, double-stranded RNA (1, 2). This material has been termed the "replicative form" of viral RNA (3), although its exact role in viral replication has yet to be determined. Infected bacteria represent the most convenient source of replicative form RNA, and preparations on a large scale have been reported (4, 5). However, animal virus replicative form is of unique interest because, in contradistinction to that of bacterial and plant viruses, it is intrinsically infectious (3, 6). This fact implies that the double-stranded molecule embodies the entire information content of the viral genome. It therefore seemed advantageous to develop a purification procedure which would provide infectious double-stranded RNA in quantities sufficient to allow correlated physicochemical and biological studies.

We have prepared RNase-resistant infectious RNA from poliovirus-infected HeLa cells, utilizing the techniques of differential precipitation with NaCl and chromatography on columns of methylated albumin-Kieselguhr in a manner similar to that described by Amman, Delius, and Hofschneider (4) for M12 phage-specific RNA. The product has been shown to be identical with the poliovirus double-stranded RNA previously obtained by zonal centrifugation (7, 8). In addition, experiments have been performed to show that infectivity is a property of the double-stranded molecule and cannot be attributed to contamination by single-stranded RNA or whole virions.

METHODS

Propagation of Cells and Virus—Strain S1 HeLa cells were grown in suspension at cell densities between 2 and 5 x 10^6 cells per ml in Eagle's medium supplemented with 5% horse serum. Infection with Mahoney strain Type 1 poliovirus and purification of the virus were carried out according to previously described methods (9). Cells for preparation of large quantities of RNA were grown in 20-liter carboys and collected in a Servall Szent-Györgi-Blum continuous flow centrifuge.

Labeling of Infected Cells with ³²P—Cells concentrated in suspension (4 x 10^6 cells per ml) were incubated with actinomycin D (gift of Merek Sharp and Dohme), 5 µg per ml, for 1 hour at 37°, then infected in phosphate-free medium in the presence of carrier-free ³²P-orthophosphate (International Chemical and Nuclear Corporation City of Industry, California) at 25 µC per ml. Under these conditions, transfer RNA is the only form of host cell RNA that is labeled (10).

Extraction of RNA from Infected Cells—Infection was allowed to proceed for 5 hours, after which the cells were sedimented by centrifugation (1500 rpm, 5 min, International PR-2 centrifuge) and resuspended at a density not exceeding 3 x 10^6 cells per ml in one of two buffers: (a) 0.02 M sodium phosphate, pH 7.2, containing 0.15 M NaCl, or (b) 0.01 M sodium acetate, pH 5.1, containing 0.1 M NaCl and 0.35% sodium dodecyl sulfate. The cell suspension was stirred with an equal volume of phenol (Mallinekrodt, reagent grade) at 60° for 10 min and cooled in an
ice bath. After separation of phases by centrifugation (2000 rpm, 15 min), the aqueous phase and interphase were extracted twice more, with the use of fresh phenol. The aqueous phase was then withdrawn and treated once with phenol. RNA was precipitated with ethanol (final concentration, 70%) at -18°C, and the precipitate was washed once with a 2:1 mixture of ethanol-0.15 M NaCl at 0°C. We have found no difference in the yield either of total RNA or of specific forms of RNA when extracted in the above two buffer systems.

**Differential Salt Precipitation of RNA**—RNA was dissolved in 0.02 M sodium phosphate, pH 7.2, at a concentration of 1 to 2 mg per ml, and NaCl was added to a final concentration of 1 M. Initial experiments were performed with 10% (w/v) (1.7 M) NaCl, but this higher concentration was found to have no advantage over 1 M. The solution was frozen and stored at -18°C, then thawed at approximately 4°C, and the flocculent precipitate was collected after centrifugation (2000 rpm, 20 min, 0°C). After the precipitate was washed with 1 M NaCl solution, the wash and original supernatant were pooled and the precipitate was dissolved in 0.02 M sodium phosphate, pH 7.2, containing 0.15 M NaCl. The supernatant fraction contained 20% of the original absorbance at 2600 A. Precipitation will occur at 4°C without any evidence of degradation of RNA, but freeing the salt-RNA solution yields a more rapid and efficient precipitation of high molecular weight single-stranded RNA.

**Chromatography of RNA on Methylated Albumin-Kieselguhr**—Methyl-esterified bovine serum albumin (Armour, Fraction V) was prepared according to the procedure of Mandell and Hershey (11), except that esterification was carried out at 37°C for 5 days, as recommended by Hayashi, Hayashi, and Spiegelman (12). Kieselguhr (Johns-Manville Hyflow Super-Cel) was heated at 600°C for 30 min to destroy organic residue, suspended in 0.4 M NaCl-0.05 M sodium phosphate buffer, pH 6.8 (1 g of Kieselguhr per 10 to 20 ml of buffer), and coated with methylated albumin by the addition of a 1% solution of albumin in water (1 ml of albumin solution per 3 g of Kieselguhr). Columns composed of small upper and lower neutral layers (Kieselguhr only; approximately 5% of the total column bed) and a single intervening albumin-containing layer served for both analytical and preparative purposes. The capacity was usually 1 mg of RNA per g of Kieselguhr bed, but varied depending on the individual batch of methylated albumin. Chromatography was performed at 35°C to provide optimal recovery of RNA and facilitate adequate resolution of virus-specific RNA from host cell ribosomal RNA (13). The columns were loaded with RNA at concentrations never exceeding 0.5 mg per ml in 0.6 M NaCl-0.05 M sodium phosphate buffer, pH 6.8. At this salt concentration, acid-soluble nucleotides, inorganic 32P, and over 95% of cellular soluble RNA pass directly through the column.

Elution of high molecular weight RNA was accomplished with linear NaCl gradients produced by adding 1.6 M NaCl dropwise to 0.6 M NaCl. A flow rate of 0.5 ml per min was maintained with a Sigmamotor peristaltic pump. Absorbance of column eluates at 2600 A was recorded in a Cary spectrophotometer fitted with a flow cell with a 1-mm light path. Radioactivity was monitored continuously by passing the eluate through a coil (volume, 0.5 ml) of narrow gauge polyethylene tubing over which was mounted an end window Geiger-Müller tube connected to a Nuclear-Chicago rate meter. However, the count profile was always verified by plating a 20-μl sample from each fraction for counting in a low background counter. The refractive index of individual fractions was measured on an Abbé refractometer and converted to NaCl concentration with the use of an empirically derived standard curve.

Columns have been stored at room temperature and reused over the course of 1 week, but this is advisable only for preparative purposes. Recovery of RNA, initially around 80%, improves as a column is reused, but the salt concentration at which a given type of RNA elutes decreases.

**Titration of Infectious RNA**—Titers of infectious RNA were determined with a plaque assay (14) in which HeLa cells suspended in agar in the presence of one or more polybasic compounds are used.

**Absorbance Calculations**—The ε 260, mφ of poliovirus double-stranded RNA has not yet been determined. Approximate concentrations of purified double-stranded RNA were calculated with the value ε 260, mφ for single-stranded poliovirus RNA (in 0.02 M sodium phosphate, pH 7.2) of 233 dig-1 cm-1. For purposes of comparison, we note that the ε 260, mφ of MS2 bacteriophage double-stranded RNA is 210 dig-1 cm-1, and that of single-stranded MS2 RNA is 251 dig-1 cm-1 (5).

**Zonal Centrifugation in Sucrose Density Gradients**—Portions of RNA preparations, 2 to 4 ml, were layered over preformed gradients of 5 to 20% sucrose (in 0.15 M NaCl, 5 mM Tris-HCl, pH 7.5, and centrifuged at 33,000 rpm for 96 hours was carried out in a Spinco model L centrifuge with an SW25 rotor at approximately 4°C. After centrifugation, the tubes were punctured at the bottom, the effluent was analyzed for 2600-A absorbance, and 1.0-ml fractions were collected for determination of radioactivity. Sedimentation coefficients were estimated by the method of Martin and Ames (15), with the peaks of HeLa cell ribosomal RNA as points of reference.

**Equilibrium Density Gradient Centrifugation in Cs2SO4**—Solubility of Cs2SO4 (obtained in crystalline form from Gallard-Schlesinger Chemical Manufacturing Corporation, New York) in 5 mM Tris-HCl, pH 7.5, p = 1.60, were prepared to contain no more than 50 μg of RNA in a volume of 5 ml. Centrifugation at 33,000 rpm for 96 hours was carried out in a Spinco model L centrifuge with an SW25 rotor at approximately 4°C. Incomplete resolution of the species of RNA was obtained with shorter periods of centrifugation, and larger amounts of RNA led to zones of precipitate which entrapped material of different density (this work was completed before Lozeron and Szybalski (16) reported that formaldehyde suppresses the precipitation of RNA in Cs2SO4). The bottom of the centrifuge tube was punctured, and alternate fractions of 0.03 and 0.06 ml were collected. The smaller fractions were utilized for determination of radioactivity, the larger fractions for chemical analysis and measurements of refractive index. Densities were determined from the refractive index according to the method of Hearst and Vinograd (17). If infective titrations were to be performed, successive 0.15-ml fractions were collected to provide sufficient material for dilution.

**Measurement of Acid-insoluble Radioactivity**—Samples were precipitated with 5% trichloroacetic acid after the addition of 1 mg of yeast RNA. Precipitates were washed once with 5% trichloroacetic acid, dissolved in 1% NH3OH, and plated for counting in a low background counter.

**Determination of RNase-resistant Radioactivity**—RNase resistance was determined with the RNA in 2X1 solution of 1 The prefixes, 2X and 0.01X, indicate concentration multiples or dilutions of the stated reagent.
0.15 M NaCl-0.015 M sodium citrate, pH 7.0, unless otherwise indicated. Pancreatic RNase (Worthington) was added to the desired concentration, and incubation was carried out at 37°, usually for 30 min. At the conclusion of the incubation, the samples were chilled in an ice bath, carrier RNA and trichloroacetic acid were added in quick succession, and the precipitates were processed as above.

Nucleotide Composition—32P-labeled RNA and 500 μg of unlabeled HeLa cell ribosomal RNA were precipitated with 2.5% perchloric acid and hydrolyzed with 0.3 M KOH at 37° for 16 hours. The resulting nucleotides were separated electrophoretically, and the relative amount of isotope in each nucleotide was determined according to the procedure of Sebring and Salzman (18). Results are expressed as mole percentages.

Thermal Hyperchromicity—RNA preparations, at a 2600-A absorbance of about 0.5, were dialyzed to the desired electrolyte conditions, sealed in microcuvettes (300 to 400 μl), and heated in a Gilford spectrophotometer equipped to record both the optical density at 2600 A and the temperature of the samples. Results are presented as optical densities at ambient temperatures, without correction for solvent expansion.

Analytical Ultracentrifugation—All experiments were performed in a Spinco model E ultracentrifuge with bright Durall rotors and Kel-F centerpieces. Conventional boundary sedimentation analysis was performed at 52,640 rpm with the RNA in 0.05 M sodium phosphate buffer and various concentrations of NaCl. Sedimentation coefficients were corrected to standard conditions, s20, w. Zone sedimentation was carried out according to the method of Vinograd et al. (19), with a gap-transfer centerpiece (20). Centrifugation was at 44,770 rpm and 7.9°. The lamellar solution consisted of RNA at a 2600-A absorbance of 1.8 in 20 μl of 0.05 M sodium phosphate buffer, pH 6.8. The bulk solution was 1.0 M NaCl-0.2 M sodium phosphate buffer, pH 7.8, in D2O. Its viscosity has not been determined, so correction of sedimentation coefficients calculated from the zone sedimentation data is not presently feasible.

Reaction with Formaldehyde—Preparations of RNA were treated with 1% formaldehyde (Baker, reagent grade) in 0.02 M sodium phosphate-0.004 M sodium citrate buffer, pH 7.1, containing 0.05 M NaCl (16) at 22°. Aliquots were withdrawn at various times and were diluted no less than 100-fold into 0.02 M sodium phosphate, pH 7.2, containing 0.15 M NaCl and 1 mM MgCl2, for assay of infectivity.

RESULTS

Chromatographic Analysis of 32P-labeled RNA from Infected Cells—RNA derived from mammalian cells infected with poliovirus has been analyzed in several laboratories by chromatography on columns of methylated albumin-Kieselguhr. Infectious 35-S single-stranded viral RNA was found to elute later (i.e. at a higher salt concentration) than the majority of host cell ribosomal RNA (21). A second species of infectious, virus-specific RNA was found to elute in advance of ribosomal RNA (6, 22). This material was partially RNase-resistant, and had a buoyant density lower than that of single-stranded RNA. It was therefore considered identical with the double-stranded replicative form RNA isolated by zonal centrifugation following digestion of contaminating single-stranded RNA by RNase (8).

The fact that chromatography on columns of methylated albumin-Kieselguhr can effectively resolve double-stranded RNA from host cell and viral species of single-stranded RNA makes it possible to purify poliovirus replicative form without exposure to RNase. However, the limited capacity of these columns represents a major restriction on their use when considered in terms of the large quantities of RNA that have to be processed in order to obtain useful amounts of double-stranded RNA. This problem was solved by eliminating high molecular weight single-stranded RNA by differential salt precipitation prior to chromatography.

Fig. 1a illustrates the chromatography of unfractionated RNA extracted from 32P-labeled infected cells. As described above, two major types of labeled viral RNA are differentiated. One of these is eluted as a homogeneous fraction at 0.8 M NaCl, the other in a broader distribution at 1.0 M NaCl. Host cell ribosomal RNA accounts for the large optical density peaks. The poor separation of 16-S (eluting earlier) from 28-S RNA is characteristic of the behavior of mammalian ribosomal RNA when chromatographed on methylated albumin-Kieselguhr.

If the RNA is fractionated by treatment with 1 M NaCl prior to chromatography, the late eluting species of viral RNA, which corresponds to the single-stranded RNA found in mature virions (21), and the bulk of the ribosomal RNA are precipitated (Fig. 1c). The other major fraction of viral RNA is soluble in 1 M NaCl (Fig. 1b). On the basis of previous reports (6, 22), this early eluting, soluble peak of viral RNA was presumed to be double-stranded replicative form. To verify this, poliovirus double-stranded RNA, purified by sucrose gradient centrifugation after digestion with RNase (8), was chromatographed on methylated albumin-Kieselguhr and was shown to be eluted at the same salt concentration and the same position with reference to RNase.
to ribosomal RNA as the salt-soluble material. This early eluting RNA was then prepared in quantity and subjected to further analysis as described below.

A third, small peak of labeled RNA, which overlaps the initial portion of 16-S ribosomal RNA (Fig. 1, a and c), appears to be virus specific and salt precipitable. Preliminary experiments have shown that there is RNase-resistant infectivity eluting in this same region, but there is presently no good evidence as to the nature of either the labeled RNA or the infectivity.

**Large Scale Preparation of Double-stranded RNA**—To obtain double stranded poliovirus RNA in quantities sufficient to allow physicochemical studies, the procedures of NaCl precipitation and chromatography described above were applied to large amounts of RNA extracted from cells infected in the absence of radioisotopes. Fig. 2 depicts a representative chromatographic run, in which a small sample of purified, $^{32}$P-labeled double-stranded RNA was added to serve as a marker. A large peak of infectious RNA and the labeled marker RNA elute simultaneously. The amount of marker RNA added represented less than 1% of the final infectivity. A minor portion of cellular soluble RNA is adsorbed to the column under the chromatographic conditions used and is eluted as a heterogeneous peak in advance of the double-stranded RNA. Any residual DNA contaminant would be eluted from the column at around 0.6 M NaCl, and thus would be eliminated during loading.

**Ultracentrifugation**—The RNA eluted from methylated albumin-Kieselguhr at 0.8 M NaCl was analyzed by ultracentrifugation for relative homogeneity with respect to size. Zonal centrifugation of $^{32}$P-labeled material (Fig. 3) through a 5 to 20% sucrose gradient gave a single peak. The estimated sedimentation coefficient was 14-S. This corresponds to the S value for RNase-resistant RNA previously identified by zonal centrifugation of unfractionated RNA extracted from infected cells (7, 8). Assays for infectious RNA have demonstrated coincidence between the peaks of radioactivity and plaque-forming units.

Analysis of a large scale preparation by the zone sedimentation technique of Vinograd et al. (19) is illustrated in Fig. 4. There is a slowly sedimenting component (no more than 10% of the ultraviolet-absorbing material) which probably represents soluble RNA, present because of the trailing which occurs during elution of RNA from the methylated albumin-Kieselguhr column. The progressive broadening of the leading edge of the major zone with time of centrifugation is characteristic of concentration-dependent sedimentation (23), and is reminiscent of the behavior of native double-stranded DNA under similar conditions (10).
The nucleotide composition of RNA from mature poliovirus is taken from a previous study (8). All analyses were performed on RNA which first had been separated in CsSO₄ equilibrium density gradients. Results are expressed as mean value ± standard error of the mean, and are derived from no less than six separate determinations.

<table>
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<tr>
<th>Mature poliovirus observed</th>
<th>Double (+,-) calculated</th>
<th>Double observed</th>
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<tr>
<td>Uridine, 23.8 ± 0.2</td>
<td>25.6</td>
<td>26.2 ± 0.1</td>
</tr>
<tr>
<td>Guanosine, 23.2 ± 0.3</td>
<td>23.5</td>
<td>23.1 ± 0.0</td>
</tr>
<tr>
<td>Adenosine, 29.3 ± 0.1</td>
<td>26.5</td>
<td>26.7 ± 0.1</td>
</tr>
<tr>
<td>Cytidine, 23.8 ± 0.2</td>
<td>23.5</td>
<td>24.0 ± 0.1</td>
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The $s_{20,W}$ of the major component, determined from four separate boundary sedimentation experiments, is 17.2 ± 0.2. On the basis of a molecular weight of $2 \times 10^4$ for poliovirus single-stranded RNA (24), the double-stranded replicative form is assumed to have a molecular weight of $4 \times 10^4$. This corresponds to an $s_{20,W}$ of 16.9, calculated from the relationship, $s_{20,W} = 0.0882 M^{0.346}$ suggested by Studier (25) for native DNA. Considering the magnitude of experimental error involved in such determinations, the correlation between observed and calculated $s_{20,W}$ values for poliovirus double-stranded RNA is quite close. However, these results must be considered tentative, because the calculations are based on the assumption that the partial specific volume and the relationship between $s_{20,W}$ and molecular weight are similar for double-stranded RNA and DNA. Gomatos and Steckenuis (26) have reported that calculation of the molecular weight of reovirus double-stranded RNA from sedimentation data yields values almost double those calculated from molecular lengths measured in electron micrographs.

Equilibrium Density Gradient Centrifugation—Labeled double-stranded RNA, obtained by chromatography, had a buoyant density in CsSO₄ of 1.60 g cm⁻³, in agreement with the results for RNase-resistant RNA purified by zonal centrifugation (8), and contrasting with single-stranded viral and ribosomal RNA, which have a buoyant density of 1.65 g cm⁻³ (8). Infectious RNA from the large scale preparation of purified double-stranded RNA also was found at a density of 1.60 g cm⁻³.

Nucleotide Composition (Base Ratios)—The nucleotide composition of $^{32}P$-labeled, chromatographically purified RNA is identical with that previously reported for double-stranded RNA isolated by sequential zonal and equilibrium density gradient centrifugation (8). Results are presented in Table I. The analytical values for single-stranded RNA derived from mature virus were used to calculate the nucleotide composition anticipated for a base-paired molecule consisting of a strand of viral (“plus”) RNA and a complementary (“minus”) strand. The base ratios of the purified, RNase-resistant RNA are in close agreement with the calculated values. In the previous study (8), it was shown that about 50% of the label in the double-stranded RNA will specifically anneal with unlabeled single-stranded viral (plus) RNA, and that this fraction of the labeled RNA has a nucleotide composition approximating that of the theoretical minus strand.

Thermal Denaturation—Nucleic acids with hydrogen-bonded, double-stranded secondary structure undergo transition from ordered helical form to a random coil structure when heated above a critical temperature (27). This transition is characterized by the temperature at its midpoint, $T_m$. Initially, when only trace quantities of double-stranded replicative form RNA were available, the $T_m$ was determined by utilizing the fact that disruption of ordered secondary structure is accompanied by loss of RNase resistance (28). This is illustrated in Fig. 5. Aliquots of $^{32}P$-labeled RNase-resistant RNA, in 0.01X a solution of 0.15 M NaCl-0.015 M sodium citrate, pH 7.0, were maintained at the indicated temperatures for 15 min, then quenched in an ice bath and assayed for acid precipitable radioactivity after digestion with RNase (50 μg per ml) in 2X a solution of 0.15 M NaCl-0.015 M sodium citrate, pH 7.0. The $T_m$ obtained in this manner is approximately 87°. Loss of RNase resistance definitely represents a helix to coil transition, because the density of the labeled material shifts from that of double-stranded to that of single-stranded RNA after the above heating procedure (8).

Fig. 5 also illustrates determinations of $T_m$ as calculated from the hyperchromic shift that accompanies denaturation of double-stranded nucleic acids (29). In comparison with the result obtained from transition of RNase resistance, the $T_m$ determined in 0.01X a solution of 0.15 M NaCl-0.015 M sodium citrate, pH 7.0, by the optical procedures is 82°. The optical measurements of $T_m$ have a precision of ±0.5°, and we consider them more definitive than those derived from the transition to nuclease sensitivity. Regardless of the salt conditions, denaturation of double-stranded poliovirus RNA occurs over a range of 10–12°, with a hyperchromic shift of 25 to 27%. MS2 double-stranded RNA melts over a range of about 20° and has a hyperchromicity of 26% (5).
TABLE II
Specific infectivity of single- and double-stranded RNA

Purified RNA preparations were titered with a procedure (14) in which the assay cells are incubated with polybasic compounds prior to exposure to RNA. Where indicated in the table, one or both of the polycations used were included in the incubation mixture.

<table>
<thead>
<tr>
<th>Polycation used in assay</th>
<th>Titer</th>
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<tbody>
<tr>
<td></td>
<td>Single-stranded RNA</td>
</tr>
<tr>
<td>plaque-forming units/µg RNA</td>
<td>plaque-forming units/µg RNA</td>
</tr>
<tr>
<td>None</td>
<td>$1.6 \times 10^4$</td>
</tr>
<tr>
<td>Polyornithine</td>
<td>$6.7 \times 10^4$</td>
</tr>
<tr>
<td>DEAE-dextran</td>
<td>$1.6 \times 10^6$</td>
</tr>
<tr>
<td>Polyornithine and DEAE-dextran</td>
<td>$6.7 \times 10^4$</td>
</tr>
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Reduction of salt concentration leads to a proportionate lowering of the $T_m$ of nucleic acids (30). However, at extremely low salt concentrations, the $T_m$ begins to rise, and this has generally been attributed to trace amounts of divalent cations (5, 30). Thus, the $T_m$ of double-stranded poliovirus RNA is raised to 92° when determined at the NaCl concentration (0.0015 M) of 0.01X a solution of 0.15 M NaCl-0.015 M sodium citrate, pH 7.0, but in the absence of citrate ion. The addition of 50% methanol reduces the $T_m$ in 0.01X a solution of 0.15 M NaCl-0.015 M sodium citrate, pH 7.0, to 72°.

Infectivity of Double-stranded RNA—Poliovirus-induced double-stranded RNA is capable of initiating the infective cycle in susceptible cells and giving rise to complete, infectious progeny virus. In Table II, the specific infectivity of double-stranded RNA purified by chromatography on columns of methylated albumin-Kieselguhr is compared with that of single-stranded RNA derived from purified virus by phenol extraction. The conditions necessary in each case for maximal titers are different, a fact which suggests that one or more early events in the initiation of the replicative cycles of these two RNA forms are different. This impression is further substantiated by a striking difference in the rate of uptake of single- and double-stranded RNA by the host cell. Penetration of double-stranded RNA is complete within 5 min, but single-stranded RNA is taken up over a period of 1 hour. The best titers obtained with double-stranded RNA are 30-fold higher than those observed with single-stranded RNA, but this difference may simply reflect the relative RNase resistance of the double-stranded molecule.

Previous studies (8, 31), in which the degradation of labeled RNA to an acid-soluble state was used as a measure of RNase susceptibility, showed that the presence of NaCl or divalent cation was essential to the RNase resistance of double-stranded RNA. The data in Table III reiterate this point for the case of acid-precipitable radioactivity and show that the RNase resistance of infectivity is similarly dependent upon salt concentration. However, the resistance is not absolute, for large reductions in infectivity can be effected by high enzyme concentrations. These results serve to emphasize the importance of eliminating RNase digestion from purification procedures if a biologically active product is desired. In contrast to the relative resistance of double-stranded RNA, the infectivity of single-stranded RNA

$^1$G. Koch, J. M. Bishop, and N. Quintrell, manuscript in preparation.

$^2$FIG. 6. The effect of formaldehyde on the infectivity of single- and double-stranded poliovirus RNA. •, single-stranded RNA; ○, double-stranded RNA. Conditions of inactivation and other experimental details are described under “Methods.”
would be completely abolished by an enzyme concentration as low as 0.001 µg per ml, regardless of the salt concentration.

**Effect of Formaldehyde on Infectious RNAs**—The reaction of formaldehyde with polynucleotides involves free amino groups of the constituent purines and pyrimidines (32–34). Neither double-stranded DNA (32, 33) nor double-stranded RNA (35), in which the amino groups are engaged in interstrand hydrogen bonding, will bind formaldehyde to any significant extent. On the other hand, formaldehyde reacts strongly with denatured DNA (30) and inactivates infectious single-stranded viral RNA (32). In their original description of RNase-resistant RNA, obtained from cells infected with mouse encephalomyocarditis virus, Montagnier and Sanders (3) reported that the infectivity of this RNA was not inactivated by formaldehyde. Fig. 6 illustrates resistance to formaldehyde in the case of poliovirus double-stranded RNA, and depicts the kinetics of inactivation of single-stranded RNA for purposes of comparison. The experiment was done in a low salt environment because higher salt concentrations impede the binding of formaldehyde to RNA, presumably by conferring some degree of ordered, hydrogen-bonded secondary structure (32).

**DISCUSSION**

The property of RNase resistance provides a convenient tool for both the characterization and the purification of double-stranded RNA, but resistance to enzymatic attack is far from absolute. As illustrated above (Table III), elimination of nucleic exposure from purification procedures is critical to the preservation of maximal infectivity. Moreover, preparation of large quantities of MS2 phage double-stranded RNA, with the use of exhaustive digestion with RNase, results in a product the sedimentation coefficient of which is reduced to almost half the value anticipated on the basis of the estimated molecular weight (5). The present chromatographic procedure was developed to obviate this problem and to facilitate the handling of RNA from large quantities of infected cells.

It seems clear that the infectivity of animal virus replicative form RNA derives from information contained in a double-stranded molecule. This view is supported by the following facts. (a) The infectivity is relatively resistant to RNase, and the extent of this resistance is determined by factors, such as salt and divalent cation concentration, which influence the integrity of secondary structure. (b) The conditions required for maximal infectivity of single- and double-stranded RNA are different, and the specific microenvironments of the two RNA forms, determined under optimal conditions, differ by more than 30-fold. (c) Plaque forming ability electrophromographs with the double-stranded RNA, and is found in the same zone in equilibrium density gradients of Cs₂SO₄; both techniques effect good separation of single- and double-stranded RNA. (d) The infectivity of double-stranded RNA is not affected by treatment with formaldehyde, whereas single-stranded RNA is rapidly inactivated. (e) As will be detailed elsewhere (37), double-stranded RNA is less sensitive to biological inactivation by ultraviolet light than is the homologous single-stranded RNA.

The specific infectivity of double-stranded RNA is 30-fold higher than that of single-stranded RNA (14). Even under optimal conditions, however, only 1 in 50,000 molecules of double-stranded RNA yields an infective center in our assay system. Attempts to separate infectious from noninfectious molecules by chromatography on columns of methylated albumin-Kieselguhr or hydroxylapatite, by sucrose gradient centrifugation, or in equilibrium density gradients of Cs₂SO₄ were unsuccessful. In the absence of demonstrable physical heterogeneity of the double-stranded RNA preparation, and in view of the fact that many preparations of intact poliovirus show a particle to plaque-forming unit ratio of 1000:1, it is reasonable to assume that essentially all molecules in a double-stranded RNA preparation are potentially infective.

Some special mechanism must be invoked to explain the initiation of infection by double-stranded RNA. In the usual infection, the incoming single-stranded RNA probably participates in the organization of a polyribosome and thus directs the immediate synthesis of virus-specific proteins (38). In the case of double-stranded RNA, three alternative initial events can be envisaged. (a) Direct translation into polypeptide of the incoming RNA. Efforts to demonstrate messenger activity in vitro for double-stranded polynucleotides, however, have been unsuccessful (39, 40). (b) Strand separation. No model of the enzymatic reaction required for this alternative is presently known. (c) Transcription of double-stranded RNA into single-stranded RNA with host cell RNA polymerase. To date, this possibility has not been tested with physically intact, biologically active double-stranded RNA and homologous host cell polymerase. However, double-stranded reovirus RNA is inactive as a primer for the RNA polymerase of Escherichia coli (41).

The functional role of replicative form double-stranded RNA in the virus growth cycle is not yet clear, and the evidence that it is essential to replication is mainly indirect. Recent work in several laboratories (42–44) has led to the suggestion that the replication of single-stranded RNA proceeds by way of a complex structure (generally referred to as “replicative intermediate”) with the following characteristics: partial resistance to RNase, heterogeneous sedimentation behavior, buoyant density intermediate between that of single- and that of double-stranded RNA, insolubility in 2 M LiCl and 1 M NaCl, and a biphasic hyperchromic shift induced by heating. If it is assumed that double-stranded RNA is an integral component of this intermediate (S, 42–44), then the facts that the double-stranded molecule is infectious and that it yields intact single strands on solvent denaturation (45) limit the permissible hypotheses concerning the nature of the active complex and the detailed mechanism of replication.

One widely discussed model (46–48) involves the sequential synthesis of plus strands against a stable complementary single strand, implying that several partially completed strands are attached to the template. A double-stranded RNA derived from such a replicative complex would presumably have gaps in the plus strand which would have to be repaired by covalent linkage before the molecule could conform to the limiting factors discussed above. Alternatively, it is necessary either to view the bulk of the double-stranded RNA isolated from infected cells as a side product consisting of inactive template with a completed plus strand attached or to attribute infectivity to the complementary (template) strand. Another model, proposed previously on the basis of nucleotide composition data (8), envisages the synthesis of progeny plus strands against a stable template composed of intact plus and minus strands. This

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3 Also, M. Girard, D. Baltimore, and J. Darnell, personal communication (1966).
would give a replicative intermediate which could be reconciled with virtually all of the available data. Whatever the relationship of double-stranded RNA to replication may be, the fact that it is intrinsically infectious should be taken into account when considering replicative mechanisms, and lends considerable interest to the study of its properties.

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