*AN INTERMEDIATE IN THE SYNTHESIS OF POLIOVIRUS RNA**

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The pairwise complementarity of the nucleotide residues in nucleic acids[1] provides a simple mechanism for the specification of the sequence of residues in a nucleic acid. A molecule with a given sequence will specify a molecule with a *complementary* sequence and, in turn, the *complementary* molecule can specify the sequence of the original one. In the case of single-stranded RNA viruses, it is thus likely that a strand of *complementary* RNA will be the intracellular template for the synthesis of *viral* RNA (the molecule which exists in mature particles). This argument is strengthened by the finding of double-stranded RNA in cells infected with single-stranded RNA viruses.[2–4]

The hypothesis that the complementary molecule of RNA, in a double-stranded RNA, is the template for viral RNA synthesis requires that there be a region of hydrogen bonding between the growing molecule of viral RNA and the complementary RNA. As the new chain of viral RNA elongates, either it displaces the viral strand of the double-stranded RNA or it is not hydrogen-bonded except in proximity to the growing point. In either case, the growing strand of viral RNA plus its template will form a complex which will be partially single- and partially double-stranded. Since more than one molecule of nascent viral RNA could be attached to one double-stranded molecule, such complexes might be fairly large and easily distinguishable from true double-stranded RNA by their content of single-stranded RNA.

We shall present evidence in this paper for the existence of such a complex of single- and double-stranded RNA in cells infected with poliovirus. It is similar to a structure first identified in bacteria infected with an RNA bacteriophage[5] which was named the replicative intermediate (RI).[6] We shall retain this nomenclature.

The RI is most easily detected after exposure of cells to radioactive uridine for brief periods of time. A double-stranded portion can be isolated from the RI by treatment with RNase and it behaves like authentic double-stranded RNA.[2, 3] The existence of single-stranded RNA in the complex is indicated by the precipitability of the RI in solutions of high ionic strength and by its density in cesium sulfate. During the exponential period of RNA synthesis,[7] the RI contains the majority of the RNase-resistant RNA in the cell, but at the end of infection, totally double-stranded RNA is the predominant species.

A preliminary report of these results has appeared.[16]

Materials and Methods.—General procedures: The growth of suspension cultures of HeLa cells and their infection with poliovirus has been described.[7, 8] Phenol extraction of RNA was carried out at 60°C in the presence of 0.5% SDS.[9] RNA was concentrated by precipitation with 2 vol of ethanol after bringing the aqueous solution of RNA to 0.2 *M* NaCl. Sucrose gradients were made with 15–30% sucrose dissolved in SDS buffer.[7] Gradients were analyzed for absorbance at 260 mμ in a continuous flow cell by a Gilford recording spectrophotometer and the acid-precipitable radioactivity determined as previously described.[10]

Pulse-labeling of infected cells: At 3 hr after infection, when the rate of viral RNA synthesis was maximal,[7] a 10-ml culture of infected cells, treated with 5 μg/ml of actinomycin at the time of

infection, was exposed to H^3-uridine.[7] After a specified time, the culture was poured into a chilled tube containing 5 ml of acetate buffer, 1 ml of 10% SDS, and 15 ml of redistilled phenol previously equilibrated with acetate buffer. Phenol extraction was then carried out at 60°C.[9] This procedure ensures that all RNA synthesis is terminated at the time of sampling. Pouring cells onto iced saline does not completely stop synthesis—partially completed chains seem to be elongated and even finished while cells are kept at 0°C.[11]

Preparation of P^{32}-labeled double-stranded poliovirus RNA: The total RNA from a culture of 4×10^7 infected actinomycin-treated cells, which had been exposed throughout the viral growth cycle to $P^{32}O_4$, was prepared by phenol extraction and ethanol precipitation.[3] The RNA was dissolved in 1 ml of SDS buffer containing 0.1% SDS, and an equal volume of 4 *M* LiCl was added. The mixture was incubated at 4°C for 16–18 hr, and the flocculent precipitate was removed by centrifugation at 20,000 *g* for 20 min. The supernatant, which contained only double-stranded RNA and terminal labeled transfer RNA[3] was layered over a 28-ml 15–30% sucrose gradient made in SDS buffer and centrifuged for 16 hr at 25,000 rpm in the SW25.1 rotor of the Spinco ultracentrifuge. The acid-precipitable radioactivity of the fractions of the gradient was determined on small aliquots,[10] and the region containing the 18*S* peak of double stranded RNA was pooled.

Equilibrium sedimentation of pulse-labeled RNA: RNA was prepared from 4×10^7 infected cells which had been exposed to 0.5 mC of H^3-uridine for 2.5 min. The ethanol-precipitated RNA was precipitated with 2 *M* LiCl as described for the preparation of double-stranded RNA. The RNA which precipitated was collected by centrifugation and dissolved in 1 ml of SDS buffer. One-tenth ml of this preparation was mixed with about 2,000 cpm of P^{32}-labeled double-stranded RNA, 0.5 ml of 2 × SSC, 1.5 ml of a cesium sulfate solution of density 1.975 (as determined by refractive index), and H_2O to give a final volume of 2.4 ml. An RNase-treated sample was prepared by mixing the RNA's and 2 × SSC with 0.1 ml of 1 mg/ml pancreatic RNase and left at room temperature for 30 min. Cesium sulfate was then added and the volume adjusted to be equal to the other sample. The samples were overlaid with 2 ml of Bayol F mineral oil and were centrifuged at 18° for 65 hr at 37,000 rpm in the SW50 rotor of the Spinco model L-2 ultracentrifuge. Fractions of 2 drops were collected and diluted to 2 ml with 2 × SSC. The position of the band of single-stranded RNA was determined by measuring the optical density of each fraction at 260 mμ. This band, which represents mainly ribosomal RNA, is coincident with the band of single-stranded viral RNA.[2] One half of each fraction was treated with 100 μg of pancreatic RNase, and the treated and untreated portions of the fractions were precipitated with 1 ml of 25% trichloroacetic acid. Yeast RNA (200 μg, Sigma, commercial grade) was added to the fractions, and the precipitate was collected on Millipore filters and counted in the scintillation counter.[10]

Materials and solutions: SDS buffer is 0.1 *M* NaCl, 0.01 Tris, pH 7.4, 0.001 *M* EDTA, 0.5% SDS (in certain experiments it is noted that the SDS buffer contained only 0.1% SDS). Acetate buffer is 0.05 *M* sodium acetate, pH 5.1, 0.01 *M* EDTA. SSC is 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7.0. Crystalline pancreatic ribonuclease was purchased from Worthington, Freehold, N. J. Cesium sulfate was a product of Gallard Schlesinger, N. Y. H^3-uridine was purchased from Nuclear-Chicago, Des Plaines, Ill., and had a specific activity of 13–20 c/mmole.

Results.—After exposure of infected, actinomycin-treated HeLa cells to H^3-uridine for 15 min or longer, the predominant species of labeled RNA is single-stranded viral RNA which has a sedimentation coefficient in a sucrose gradient of about 35*S* (Fig. 1*A*).[12] Treatment of fractions of the gradient with RNase reveals that there is also a labeled species of RNA with a sedimentation coefficient of 18*S* which is not digested by RNase and which has been shown to be double-stranded (blackened region of lower curve, Fig. 1*A*).[2, 3] However, after exposure of infected cells to H^3-uridine for 2.5 min ("pulse-label"), the distribution of the acid-insoluble radioactivity in a sucrose gradient is quite different (Fig. 1*B*). While there is a discernible peak of 35*S* RNA, the bulk of the labeled RNA is heterogeneous, sedimenting from 15*S* to 40*S*. Furthermore, when aliquots of each sample of such a sucrose gradient are treated with RNase, a heterogeneous RNase-

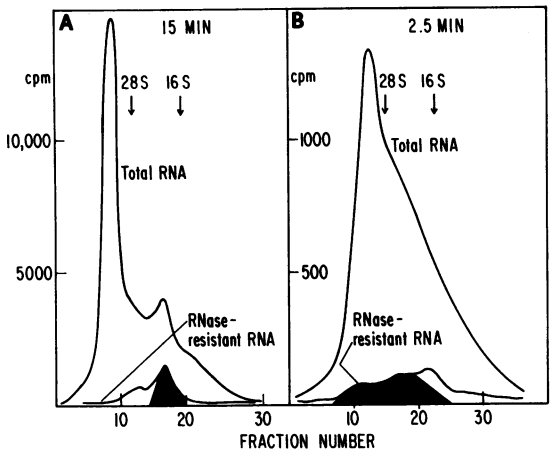

FIG. 1.—Sucrose gradient patterns of the RNA from cells labeled for 15 min (*A*) and 2.5 min (*B*). RNA was prepared as described in *Materials and Methods* (pulse-labeling of infected cells). The lower curves in each panel represent the RNase-resistant portion of each fraction. The blackened section of the lower curve of (*A*) represents the double-stranded RNA. The blackened section in (*B*) represents the HRR.

resistant RNA (HRR) is revealed, most of which sediments between 20*S* and 35*S* (blackened region of lower curve, Fig. 1*B*); especially notable is the lack of a definite peak at 18*S*. When cells are exposed to H^3-uridine during the linear phase of viral RNA synthesis,[7] HRR accounts for 10–25 per cent of the labeled RNA if the period of exposure is less than 3 min. After 15 min of labeling, HRR forms 3 per cent or less of the labeled RNA.

When RNA from cells labeled for 0.5–3 min is analyzed, both the RNase-sensitive and RNase-resistant fractions display heterogeneity as seen in Figure 1*B*. However, the exact profile of the pulse-labeled RNA is not reproducible from one experiment to another.

Properties of the HRR fraction: The following experiments were designed to test the hypothesis that the HRR is the double-stranded portion of the RI. The RI is postulated to have both single- and double-stranded RNA. If the HRR is the double-stranded portion, the attached single-stranded RNA should cause the HRR to behave as if it were single-stranded by certain criteria. However, after RNase digestion, the RNase-resistant portion should be indistinguishable from double-stranded RNA.

(1) *Precipitability in LiCl:* It has previously been shown that true double-stranded poliovirus RNA is soluble in 2 *M* LiCl, while single-stranded RNA is completely precipitated.[3] If the HRR is attached to single-stranded RNA, then it might be precipitated in 2 *M* LiCl. A sample of pulse-labeled RNA containing HRR was submitted to precipitation in 2 *M* LiCl, and the precipitated portion was analyzed on a sucrose gradient in parallel with an equivalent sample of nonprecipitated RNA. The two patterns are very similar (Fig. 2), showing that none of the HRR was lost during precipitation. This behavior of the HRR distinguishes it from true double-stranded RNA, which is soluble in 2 *M* LiCl, and suggests that the HRR is attached to single-stranded RNA.

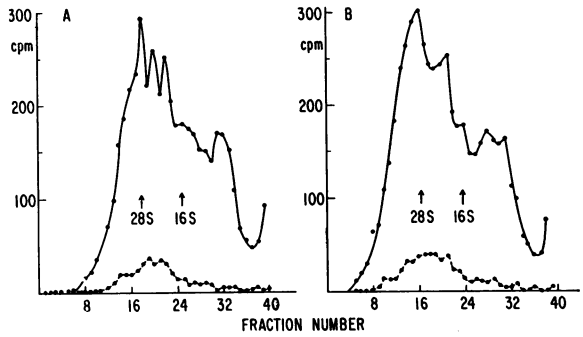

FIG. 2.—Precipitation of HRR by 2 *M* LiCl. The RNA heavier than about 25*S* from a sucrose gradient similar to that shown in Fig. 1*B* was pooled and ethanol-precipitated. The RNA was redissolved in 2 ml SDS buffer with 0.1% SDS, and 1 ml of 4 *M* LiCl was added to 1 ml of the RNA. After incubating at 4° for 16 hr, the precipitate was collected by centrifugation, redissolved in SDS buffer, and analyzed on a sucrose gradient in parallel with the untreated portion of the RNA. (*A*) Untreated; (*B*) 2 *M* LiCl precipitate.

(2) *Treatment with RNase:* When RNase digestion is performed before sucrose gradient analysis, most of the HRR is converted to 18*S* RNA which is identical to double-stranded RNA. To demonstrate this fact, cells were labeled with H^3-uridine from 1 to 3.25 hr of infection, and RNA was purified by LiCl precipitation followed by sucrose gradient fractionation. The RNA heavier than 25*S* was mixed with P^{32}-labeled double-stranded RNA, and aliquots were digested with various concentrations of RNase. Sucrose gradient analysis of a sample treated with 40 μg/ml of RNase shows a sharp peak of resistant RNA sedimenting identically to the added P^{32}-labeled double-stranded RNA (Fig. 3*A*). After treatment of the RNA with 200 μg/ml (Fig. 3*B*), the peak of resistant RNA is broader, but the P^{32}-labeled RNA shows a similar broadening. Between 40 and 200 μg/ml there has been a loss of no more than 2 per cent of the acid-insoluble radioactivity of the peak. HRR prepared from pulse-labeled RNA also yields 18*S* double-stranded RNA. Thus, the RNase treatment shows that the HRR is largely double-stranded RNA which is indistinguishable from authentic double-stranded RNA in its sensitivity to RNase. In both of the gradients there is some RNase-resistant RNA sedimenting more slowly than the 18*S* peak. Some of it, especially at the lower RNase concentration, is certainly undigested single-stranded RNA. The nature of this material, however, has not been investigated.

(3) *Density of RI in cesium sulfate:* When a sample of pulse-labeled RNA was centrifuged to equilibrium in a gradient of cesium sulfate, the RNase-resistant RNA was found entirely in the band of single-stranded RNA (Fig. 4*A*). Authentic P^{32}-labeled double-stranded RNA, which had been added to the same gradient, was found at a lighter density in the gradient.[2] If the pulse-labeled RNA was treated with RNase before centrifugation, the resistant portion had the same density as added double-stranded RNA (Fig. 4*B*).

This experiment shows quite clearly the dual nature of the RI, as detected by its content of HRR: before RNase treatment it behaves like single-stranded RNA, while after digestion it has the same density as double-stranded RNA. The finding that the density of the RI is identical to that of single-stranded RNA, and

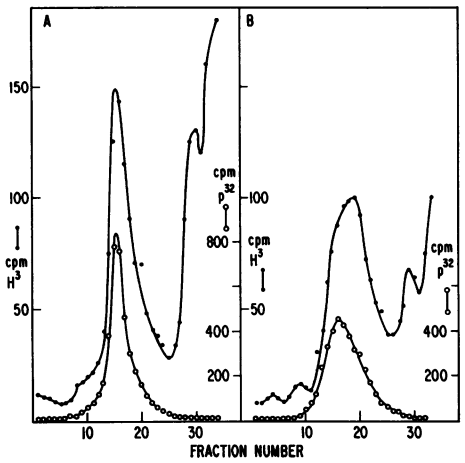

FIG. 3.—Ribonuclease treatment of HRR. RNA from cells labeled with H^3-uridine 1–3.25 hr after infection was precipitated with 2 *M* LiCl and then submitted to sucrose gradient centrifugation. The RNA heavier than 25*S* was collected by ethanol precipitation, dissolved in 1 ml 2 × SSC, mixed with P^{32}-labeled, double-stranded RNA, and digested with either 40 μg or 200 μg of pancreatic RNase. After 20 min at 22°, 0.1 ml of 10% SDS was added, and the samples were immediately layered onto sucrose gradients. (*A*) 40 μg RNase; (*B*) 200 μg RNase.

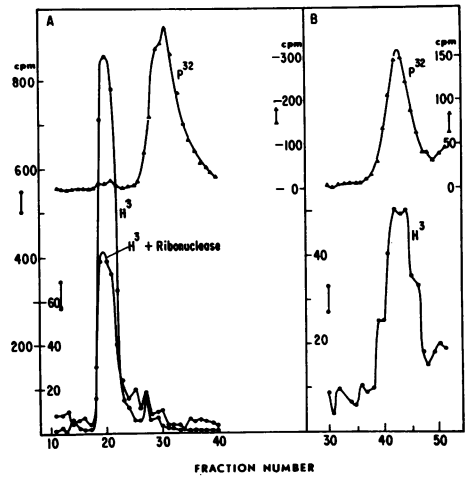

FIG. 4.—Equilibrium sedimentation in cesium sulfate of pulse-labeled RNA. (*A*) Untreated sample; (*B*) sample treated with RNase before addition of cesium sulfate.

not intermediate between double- and single-stranded RNA, may be a consequence of the fact that the single-stranded ribosomal RNA in the gradient forms a visible precipitate which could trap all single-stranded RNA in the gradient. The double-stranded RNA is not similarly caught in the precipitate.

Kinetics of accumulation of RNase-resistant RNA's: The results presented above, coupled with earlier results,[3] provide a method for distinguishing two classes of RNase-resistant RNA's. Since the double-stranded RNA is soluble in 2 *M* LiCl, whereas the HRR is precipitated, fractionation by LiCl precipitation will separate the two types of RNA. After treating the LiCl-precipitable RNA with RNase, the amounts of each type of RNA can be determined by sucrose gradient fractionation. Thus, to determine the kinetics of accumulation of the two classes of RNase-resistant RNA, cells were exposed to H^3-uridine soon after infection, and aliquots were harvested at various times. The phenol-extracted RNA was submitted to LiCl precipitation, the precipitated portion was digested with RNase, and the 18*S* RNase-resistant RNA was determined in the soluble and precipitated fractions by sucrose gradient fractionation (Fig. 5).

This experiment shows that the over-all kinetics of synthesis of RNase-resistant and 35*S* viral RNA are similar. Both the HRR and the double-stranded RNA are evident as early as 2.25 hr after infection (when the first sample was taken). HRR is the predominant species of RNase-resistant RNA during the exponential period of RNA synthesis,[7] but later the double-stranded RNA is the majority component.

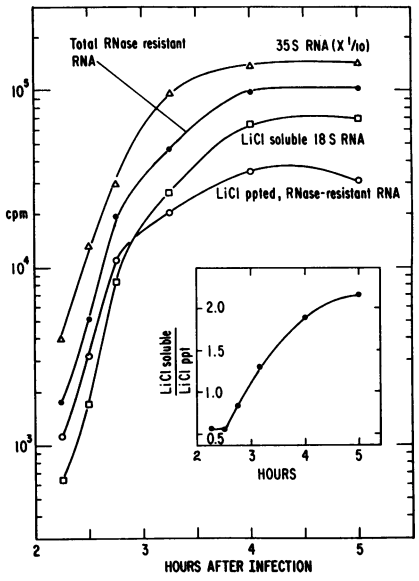

FIG. 5.—Kinetics of accumulation of various classes of RNA. At 30 min after infection, 2.2 μmoles of H^3-uridine (320 μc/μmole) were added to a 70-ml culture of infected cells (4×10^6 cells per ml). At various times 11-ml samples were harvested by centrifugation, suspended in 12 ml acetate buffer plus 1 ml 10% SDS, and extracted 2 times with an equal volume of buffer-saturated phenol at 60°.[9] The extracted RNA was precipitated with ethanol, redissolved in 1 ml SDS buffer lacking SDS, and 1 ml of 4 *M* LiCl was added. The RNA precipitated after 16 hr at 4° was redissolved in 1 ml 2 × SSC and treated with 100 μg pancreatic RNase for 30 min at 22°. The RNase-treated, precipitated RNA and the LiCl-soluble RNA were fractionated by sucrose gradient sedimentation and the cpm in the 18*S* peak of the gradients were determined. Total 35*S* viral RNA was determined on an aliquot of the LiCl-precipitated RNA. Results were corrected for recovery of RNA after phenol extraction, as determined by the absorbance at 260 mμ of the 2 *M* LiCl precipitate. □-□-□, Double-stranded RNA; ○-○-○, HRR; ●-●-●, sum of double-stranded RNA and HRR; △-△-△, 35*S* viral RNA (multiplied by 0.1). The inset shows the ratio of double-stranded RNA to HRR at various times after infection.

The ratio of the two classes of RNase-resistant RNA (inset in Fig. 5) is constant through the exponential phase but then increases continually to the end of infection.

Discussion.—The occurrence of RNase-resistant viral RNA which is heavier than double-stranded RNA (HRR) raises two distinct questions: What is the nature of this RNA? Is this RNA an intermediate in viral RNA synthesis? The experiments reported here bear on both questions.

The analysis of the HRR by a number of methods suggests that it is double-stranded RNA, complexed with single-stranded RNA. This conclusion is a result of the fact that prior to nuclease digestion the HRR is precipitable by 2 *M* LiCl and bands in cesium sulfate like viral RNA. After digestion, however, both by velocity sedimentation in sucrose gradients and by equilibrium sedimentation in cesium sulfate, the HRR is indistinguishable from double-stranded RNA.

Such a complex of double- and single-stranded RNA is exactly the structure which would be expected for growing chains of viral RNA attached to a complementary strand of RNA. This complex has been called the replicative intermediate.[6] The molecular architecture of the intermediate in viral RNA synthesis has been a matter of some speculation,[5, 13] but no critical evidence has yet been adduced for any one specific model. The complex of DNA and RNA, which Hayashi[14] has shown to be an intermediate in the DNA-dependent synthesis of RNA, is the only other such structure which has been identified.

The preferential labeling of HRR after short periods of exposure to H^3-uridine is consistent with its postulated role as an intermediate in viral RNA synthesis. The fact that no more than 25 per cent of the RNA is RNase-resistant, even after 30 sec of labeling, may be due to partial disintegration of the RI during extraction. If this is true, the high proportion of molecules sedimenting slower than 35*S* after a pulse label might be mainly chains of viral RNA which were removed from their template by the phenol extraction procedure. Some of these molecules are still

attached to the template, but the proportion of such molecules is impossible to estimate from the experiments described here.

We have previously shown that viral RNA synthesis can be demonstrated as early as 1.25 hr after infection.[7] Synthesis occurs at an exponentially increasing rate until about 3 hr, the rate then becoming constant for 1 hr before synthesis ceases. The present study shows that RNase-resistant RNA is demonstrable at 2.25 hr postinfection and increases in parallel with the 35*S* viral RNA. During the exponential phase of synthesis, about 70 per cent of the total RNase-resistant RNA can be accounted for by LiCl-precipitable HRR while the rest is double-stranded RNA. As viral RNA synthesis slows to a linear rate, the ratio of LiCl-precipitable to LiCl-soluble RNase-resistant RNA begins to increase, and by 5 hr there is at least twice as much double-stranded RNA as HRR. This kinetic course leads to the suggestion that the double-stranded RNA is a product of the RI. This possibility is under study at present.

The evidence presented here can only show that the RI *might* be an intermediate in the synthesis of viral RNA. To prove this, it would be necessary to show that chains of partially finished RNA are found in the RI and that these are finished and released as viral RNA molecules. The best method to show this would be to provide cells with a radioactive precursor for a short period of time and then allow further growth on nonradioactive precursors (pulse-chase experiment). This is impossible with HeLa cells because their internal nucleotide pool is large and does not exchange with external nucleosides. However, in bacteria, where the pool is small, such an experiment is possible. Two laboratories have reported that after a short pulse of C^{14}-uracil some of the isotope in double-stranded RNA can be displaced from the double-stranded state by growth in nonradioactive medium.[5, 15] Using a temperature-sensitive mutant which makes no stable double-stranded RNA at high temperature, Lodish and Zinder[15] were able to show complete chase of the isotope in double-stranded RNA. However, because there was a substantial increase in acid-insoluble isotope after the "chase" in these experiments, it was impossible to observe the very same molecules moving from the template to the final, completed state. Thus, the proof that the RI, or any double-stranded RNA, is an intermediate in the synthesis of viral RNA is incomplete. It is hoped that an *in vitro* system, where true chase conditions can be obtained, will provide the proof.

Summary.—A complex of double-stranded and single-stranded RNA has been identified in poliovirus-infected HeLa cells. It may be an intermediate in the synthesis of viral RNA.

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The following abbreviations were used: RNase, ribonuclease; SDS, sodium dodecyl sulfate; HRR, heterogeneous ribonuclease-resistant RNA; RI, replicative intermediate.

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