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Replicative Intermediate of an Arbovirus

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One hour after infection of chick fibroblasts with Semliki Forest virus (SFV), a viral ribonucleic acid (RNA) structure is present which has many of the properties described for the replicative intermediate of several RNA bacteriophages. These properties include a polydisperse nature on sucrose density gradient analysis, ribonuclease resistance, a variation in sedimentation pattern associated with changes in salt concentration, and recovery of infectious viral RNA upon denaturation. Most of the replicative intermediate present in SFV infection appears to be membrane-associated.

Three forms of viral ribonucleic acid (RNA) have been described in cells infected with Semliki Forest virus (SFV): a ribonuclease-sensitive 42*S* RNA identical to the RNA of the virion, a ribonuclease-sensitive 26*S* RNA which has the same base composition as the 42*S* form, and a 20*S* ribonuclease-resistant form. Only the 42*S* RNA is infectious (8, 17).

In the present study, viral RNA was labeled with radioisotope very early in infection. The radioactive RNA proved to be polydisperse on sucrose density gradient analysis and had properties of both the single- and the double-stranded forms of the viral RNA. This early-labeled viral RNA, therefore, had characteristics of the replicative intermediate (RI) originally described for the RNA bacteriophages MS2 and R17 (4, 10).

MATERIALS AND METHODS

Cell cultures, virus pools, and sucrose density gradient analysis. The methods employed in preparing pools of SFV and monolayers of primary chick fibroblasts have been described previously (8). Sucrose density gradient analysis and the method for determining acid precipitable radioactivity in gradient fractions were also performed by previously described methods (8).

Preparations of RI. Monolayers were infected at a virus plaque-forming unit (PFU)-cell multiplicity of 80:1. The virus was diluted into Eagle's medium containing 10% fetal calf serum and 2 μg of actinomycin D per ml. After 1 hr, this medium was removed, and 1 ml of Eagle's medium with 100 μc of uridine-5-^3H was added for 20 min. The monolayers were then drained and washed three times with cold 0.85% NaCl and twice with acetate buffer (0.1 M NaCl, 0.01 M sodium acetate, *p*H 5.1). The cells were scraped into 1 ml of acetate buffer, and 0.1 ml of 10% sodium dodecyl sulfate (SDS) was added. The RNA was then extracted twice with phenol at 60 C (15). (Extraction at room temperature gave results identical to those reported here.) The phenol was extracted with ether which was removed in turn by bubbling nitrogen through the extract. In some preparations, the RNA was precipitated from the extract by addition of 2 volumes of alcohol after the phenol extraction step.

Base ratio analysis. Phenol-SDS extracts were prepared from infected cells incubated with carrier-free $H_3{}^{32}PO_4$ (100 μc/ml) from 0 to 2 hr after infection (7). The ^{32}P-labeled extracts were then alcohol-precipitated, and the precipitate was dissolved in 0.1 M KCl, 0.01 M tris(hydroxymethyl)aminomethane (Tris), and 0.001 M ethylenediaminetetraacetate (EDTA), *p*H 7.1. This solution was treated with 2 μg of ribonuclease per ml at 37 C for 30 min and then was passed through a Sephadex G-25 column. The RNA was precipitated from the first 6 ml of the effluent following the void volume of the column. The precipitated RNA was further purified by banding twice in sucrose density gradients. The 16S RNA from the second gradient was hydrolyzed by overnight treatment with 0.3 N KOH and was subjected to high-voltage electrophoresis to determine the mole fractions of the nucleotides present (16).

Denaturation in dimethyl sulfoxide (DMSO). Pooled sucrose density gradient fractions were dialyzed overnight against 3,000 volumes of 0.003 M NaCl-3 × 10^{-4} M sodium citrate, and 0.5 ml of the dialysate was added to 4.5 ml of DMSO (9). The mixture was incubated at 37 C for 30 min and then rapidly cooled. The salt concentration was adjusted to 0.1 M by addition of 10 μliters of 5 M NaCl, and 10 ml of alcohol was then added. The viral RNA was precipitated by addition of ribosomal RNA or of polyadenylic acid and overnight incubation at −20 C. The precipitate was dissolved in 0.3 M NaCl, 0.03 M sodium citrate. Controls were treated in the same manner except that exposure to DMSO was omitted.

Assay for infectious RNA. Infectivity of RNA preparations was determined by the method of Richter and Wecker (14).

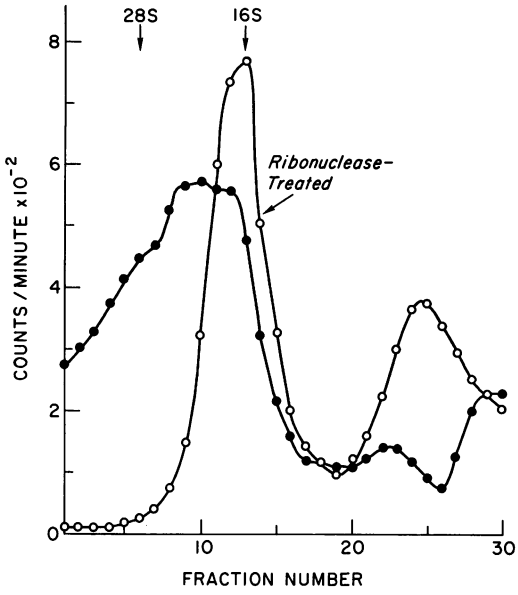

FIG. 1. *Early labeled RNA. Chick embryo fibroblasts (10^8) were infected with Semliki Forest virus in the presence of 2 μg/ml of actinomycin D. After 1 hr, 100 μc of uridine-5-^3H per ml was added for 20 min. RNA was extracted by the phenol-SDS method, and 0.1 ml of the extract was sedimented in a 6 to 30% sucrose gradient for 3 hr at 200,000 × g. An additional 0.1-ml portion was treated with ribonuclease (2 μg/ml, 37 C, 10 min) before sedimentation. Fractions were collected and analyzed for optical density at 260 mμ and for acid-precipitable radioactivity. The optical density peaks of the ribosomal RNA present in the extract are designated 28S and 16S. The bottom of the gradient is to the left in this and other figures.*

Reagents. Actinomycin D was donated by Merck, Sharp and Dohme, Rahway, N.J. Uridine-5-^3H (2 c/mmole) was purchased from Schwarz Bio Research, Orangeburg, N.Y.; pancreatic ribonuclease, from Worthington Biochemical Corp., Freehold, N.J.; and carrier-free $H_3{}^{32}PO_4$, from New England Nuclear Corp., Boston, Mass.

RESULTS

Pulse-labeled viral ribonucleic acid early in infection (early labeled RNA). Chick fibroblasts were infected with SFV in the presence of 2 μg of actinomycin D per ml (8, 17). After 1 hr, 100 μc of uridine-5-^3H per ml was added for 20 additional min. The RNA was extracted with SDS and phenol, and the extract was layered over a 6 to 30% sucrose gradient and sedimented at 200,000 × *g* for 3 hr. Gradient fractions were collected and analyzed for acid-precipitable counts or for optical density at 260 mμ (Fig. 1).

The tritium-labeled RNA was polydisperse, running from about 14*S* to at least 30*S*. Some variability was noted in the distribution of radioactivity in different preparations of early labeled RNA (Fig. 1, 2, and 3). Ribonuclease treatment (2 μg/ml, 37 C, 0.1 M NaCl, 10 min) *before sedimentation* gave rise to a structure sedimenting with a sharp peak at 16*S* (Fig. 1).

The experiment was repeated with another preparation of early labeled RNA, but in this instance one-half of each of the gradient fractions collected *after sedimentation* was treated with ribonuclease. The acid-precipitable counts before and after ribonuclease treatment are shown in Fig. 2. The ribonuclease-resistant RNA had a polydisperse pattern, with lower levels of acid-precipitable radioactivity in each fraction than those seen in the pattern for the total acid-precipitable radioactivity in each fraction.

The results suggested that the early labeled RNA was a RI form of SFV, that is, a base-paired structure with attached nascent viral RNA. The 16*S* form appeared to be the "core" of the RI consisting of a base-paired structure with intact positive and negative (complementary) strands or an intact negative strand base-paired with fragments of the nascent positive strand (4, 10, 11).

Base ratio analysis of 16S RNA. Viral RNA produced from 0 to 2 hr after infection was

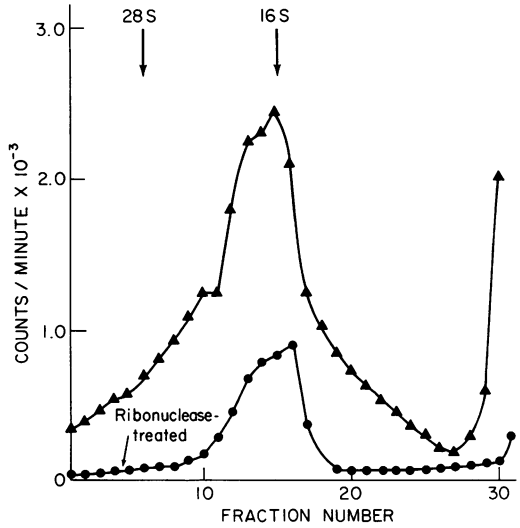

FIG. 2. *Early labeled RNA. Chick cells were infected and were labeled as described in the legend to Fig. 1. RNA was extracted and sedimented. The gradient fractions collected after sedimentation were assayed for acid-precipitable radioactivity before and after treatment of samples from each fraction with 2 μg of ribonuclease per ml. The designations 28S and 16S indicate the optical density peaks of the ribosomal RNA present.*

labeled with ^{32}P. The extracted RNA was purified and treated with ribonuclease. The 16S RNA fraction was hydrolyzed and subjected to base ratio analysis. The values obtained are shown in Table 1, where they are compared with the values previously determined in my laboratory for 42S or 26S viral RNA and with the values calculated for a base-paired duplex containing one 42S or 26S (positive) strand together with one complementary (negative) strand (7). The base composition of the 16S RNA "core" resembled that predicted for the base-paired duplex.

Sedimentation in salt media of various ionic concentrations. The sedimentation pattern of base-paired RNA structures is only slightly affected by the salt concentration of the sedimenting medium, but the sedimentation pattern of single-stranded RNA is markedly altered by changes in ionic strength (3). To determine whether early labeled RNA resembled single-stranded RNA or base-paired RNA, early pulse-labeled viral RNA was sedimented in sucrose gradients in 0.5, 0.1, or 0.001 M NaCl. These gradient patterns are compared in Fig. 3 to those of samples of the same early pulse-labeled RNA preparation which had been treated with ribonuclease before sedimentation in various salt concentrations.

The sedimentation value of the ribonuclease-treated RNA "core" in 0.5 or 0.1 M NaCl was 16S; in 0.001 M NaCl, the value was somewhat decreased to about 12S (Fig. 3B). In all three salt concentrations, a single, homogenous peak of radioactivity was found. On the other hand, the sedimentation pattern of the early labeled RNA varied with changes in the ionic concentration (Fig. 3A). Sedimentation in 0.1 M NaCl was similar to the pattern in Fig. 1 and 2; however, in 0.5 M NaCl a larger fraction of the radioactivity sedimented more rapidly than did the 16S peak. In 0.001 M NaCl, very little radioactivity was found sedimenting at values greater than 16S.

Thus, the sedimentation value of the ribonuclease-resistant base-paired "core" was only slightly affected by variations in the salt concentration. The sedimentation pattern of the early labeled viral RNA, on the other hand, was similar to that of single-stranded RNA, in that it varied greatly with changes in the salt concentration.

Denaturation of the early labeled RNA. Several attempts to recover intact single-stranded RNA from the early labeled RNA by heat denaturation were unsuccessful. The T_m of this structure (102 C in 0.01 M Tris-0.003 M EDTA) is very close to the temperature at which thermal disintegration of the macromolecules takes place (7). Attempts at thermal denaturation in the presence of DMSO in 0.1 M NaCl (9) were also unsuccessful. At lower salt concentrations (0.003 M NaCl), however, denaturation could be obtained (Table 2). The RNA was completely hydrolyzed by ribonuclease after exposure to DMSO at 37 C. Both the more rapidly sedimenting RNA and the 16S peak could be denatured by DMSO. In this experiment, the 16S RNA contained a smaller fraction of ribonuclease-resistant RNA and resisted denaturation somewhat more than did the more rapidly sedimenting RNA, but this was not regularly observed (*see* Fig. 2).

Sedimentation analysis of the RNA before and after DMSO treatment is shown in Fig. 4, where the DMSO-treated portion (Fig. 4B) is compared with an untreated portion from the same sample (Fig. 4A) and with RNA extracted from the SFV virion (Fig. 4C). The sedimentation pattern of the untreated sample (Fig. 4A) was similar to that observed in Fig. 1 and 2. The pattern seen for the DMSO-treated specimen (Fig. 4B) contained a ribonuclease-sensitive peak at 42S like that of the virion RNA (Fig. 4C) which was previously shown to be ribonuclease-sensitive (8, 17). The 42S RNA peak of the denatured sample (Fig. 4B), however, was somewhat more asymmetric than was that of the RNA of the virion.

TABLE 1. *Mole fractions of bases present in Semliki Forest virus ribonucleic acid forms*

RNA form[a]	Base			
	Guanine	Cytosine	Adenine	Uridine
42S or 26S	27.0 ± 0.2	26.2 ± 0.3	27.0 ± 0.3	19.8 ± 0.2
16S	26.7 ± 0.2	26.5 ± 0.2	24.0 ± 0.3	22.8 ± 0.3
Calculated duplex	26.6	26.6	23.4	23.4

[a] The values for the mole fractions of the bases in the single-stranded 42S or 26S RNA forms are taken from reference 7. The 16S RNA was prepared and analyzed as described in Materials and Methods. The values for the calculated duplex are those of a structure containing one strand of 42S or 26S RNA (positive strand) together with a strand of complementary RNA (negative strand). All values are the mean and standard deviation of the mean on a total of at least eight determinations from at least two preparations.

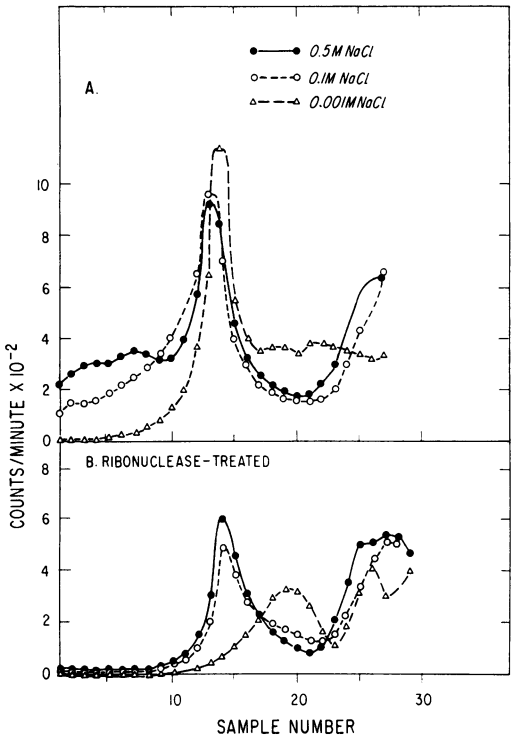

FIG. 3. *Sedimentation of early labeled RNA in various salt concentrations. RNA extracts in 0.1 M NaCl were prepared from Semliki Forest virus infected and radioactively labeled cells. One half of the extract was treated with 0.2 μg of ribonuclease per ml and then with 50 μg of Pronase per ml at 37 C to destroy the ribonuclease. The untreated (A) and ribonuclease-treated (B) portions of the extract were each further divided into three equal parts. To one-third of each portion, NaCl to 0.5 M was added; another one-third was dialyzed against 0.001 M NaCl. The samples were then sedimented (200,000 × g, 3 hr) in gradients of 6 to 30% sucrose in the salt concentrations to which they had been adjusted (0.5, 0.1, or 0.001 M NaCl). Gradient fractions were analyzed for acid-precipitable radioactivity.*

Infectivity of denatured and undenatured early labeled RNA. Portions of early labeled RNA fractions employed in denaturation studies were tested for infectivity. As previously shown by Mecs et al. (13), the undenatured RNA in the 14 to 30S region of the gradient had little or no infectivity (Table 3). After denaturation in DMSO, however, both the rapidly sedimenting RNA and the 16S RNA peak became infectious. The early labeled RNA must, therefore, include intact 42S viral RNA which can be released by denaturation.

Cytoplasmic location of early labeled RNA. Virus-infected cells were pulse-labeled with uridine-5-^3H after 1 hr of infection, as previously described (Fig. 1). The cells were disrupted with a Dounce homogenizer, and the nuclei were removed by sedimentation at 800 × *g*. The cytoplasmic extract was divided into two equal portions, one of which was treated with 1% deoxycholate. Both extracts were then layered over a sucrose gradient and sedimented.

As shown in Table 4, the untreated sample contained 78% of its total acid-precipitable radioactivity in the material in the pellet, whereas the detergent-treated sample contained only 25% of its radioactivity in the pellet. These results suggested that the early labeled RNA is, for the most part, membrane-associated.

TABLE 2. *Denaturation of early labeled viral RNA*

Treatment[a]	Acid-precipitable counts/ml	Percentage of counts ribonuclease resistant
Fractions 3–7		
None	13,900	—
Ribonuclease	10,000	72
DMSO	14,950	—
DMSO + ribonuclease	299	2
Fractions 10 and 11		
None	17,850	—
Ribonuclease	6,200	36
DMSO	14,250	—
DMSO + ribonuclease	1,290	9

[a] Fractions 3–7 or 10 and 11 (Fig. 1, untreated with ribonuclease) were pooled, dialyzed, and denatured by dimethyl sulfoxide treatment (DMSO). Portions were dissolved in 0.3 M NaCl, 0.03 M sodium citrate, and were treated with ribonuclease (2 μg/ml, 37 C, 30 min). Radioactivity was precipitated with 2.5% perchloric acid on membrane filters which were assayed in a liquid scintillation spectrometer.

DISCUSSION

RI forms have been described for RNA bacteriophages (4, 10), and a structure possessing some of the characteristics of an RI has also been described for poliovirus (1). The RI is polydisperse on sucrose density gradient analysis and contains a "core" resistant to ribonuclease treatment. Upon denaturation, infectious viral RNA is recovered from the noninfectious RI of the bacteriophages (5). The structure of the purified RI may not reflect an in vivo structure (2).

The early labeled RNA in SFV infection has many of the properties described for the bacterio-

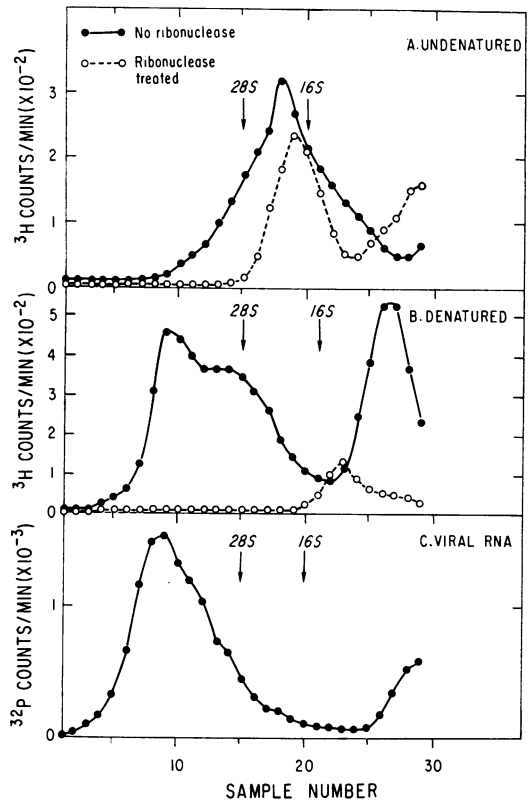

FIG. 4. *Denaturation of early labeled RNA. RNA from pooled fractions 5 to 11 in Fig. 1 was dialyzed against 0.003 M NaCl, 3 × 10^{-4} M sodium citrate, and 0.5 ml was denatured by DMSO. The precipitated RNA was resuspended in 0.3 M NaCl, 0.03 M sodium citrate and was sedimented in a 6 to 30% sucrose gradient at 100,000 × g for 3 hr. Fractions were analyzed for optical density and acid-precipitable radioactivity. (A) Pooled RNA fractions (0.1 ml), no DMSO treatment; 0.1 ml was treated with 2 μg of ribonuclease per ml before sedimentaion. (B) Pooled RNA (as in A) treated with DMSO; ribonuclease treatment was before sedimentation. (C) RNA extracted from purified Semliki Forest virus labeled with ^{32}P.*

phage RI. These include: a polydisperse nature on sucrose density gradient analysis, each radioactive fraction containing some ribonuclease-resistant counts; a 16S "core" seen after ribonuclease treatment of early labeled RNA before sedimentation analysis, the 16S "core" being a base-paired structure with sedimentation properties independent of the salt concentration of the sedimenting medium; a marked variation in sedimentation pattern associated with changes in the salt concentration; and recovery of 42S infectious RNA upon denaturation in DMSO.

TABLE 3. *Infectivity of early labeled viral RNA before and after denaturation*

Fractions	Treatment[a]	Plaque-forming units/ml
3–7	None	0
	DMSO	520
10 and 11	None	0
	DMSO	660

[a] Fractions 3–10 or 10 and 11 (Fig. 1, untreated with ribonuclease) were pooled, dialyzed, and denatured by treatment with dimethyl sulfoxide (DMSO). The RNA was dissolved in 1 M NaCl–0.1 M Tris (*p*H 8.2) and assayed for infectivity.

TABLE 4. *Cytoplasmic location of early labeled RNA*

Treatment	Acid-precipitable counts/min		Total
	In pellet	In supernatant fluid	
Untreated[a]	41,816	11,757	53,573
DOC-treated	12,872	38,712	51,584

[a] Cells (5×10^7) were pulse-labeled with 100 μc of uridine-5-^{3}H per ml for 20 min, 1 hr after virus infection. The cells were washed and disrupted with a Dounce homogenizer, and the nuclei were removed by sedimentation at 800 × *g*. The cytoplasmic extract was divided into two equal portions, one of which was treated with 1% deoxycholate (DOC). Both portions were then sedimented at 20,000 × *g* for 30 min in a 15 to 30% sucrose gradient in 0.01 M NaCl, 1.5×10^{-3} M $MgSO_4$. The supernatant fluid was collected in fractions and assayed for radioactivity as previously described. The sediment was solubilized with 1% SDS and was also assayed for radioactivity.

The 42S RNA recovered by denaturation has an asymmetric peak on sucrose density gradient analysis, as does infectious RNA recovered from other RI structures (5). The more rapidly sedimenting species of RNA present in the early labeled RNA of SFV may be separated from the ribonuclease-resistant "core" on benzoylated diethylaminoethyl cellulose columns (11), which fractionate nucleic acids on the basis of the extent of their double-stranded character (R. Stern and R. M. Friedman, *unpublished data*). Therefore, the early labeled RNA, like the RI, possesses properties of both single- and double-stranded viral RNA. These data indicate that the early labeled RNA of SFV contains an RI form.

The RI is the only structure which is pulse-

labeled early in SFV infection. Later in infection, it is the first RNA form to become labeled following very short radioactive uridine pulses (8). As in the case of RNA bacteriophages, it is likely that the RI of SFV is a structure associated with RNA replication. In addition, the RI of SFV appears to be associated with protein synthesis, as it can be extracted from polyribosome structures which are producing viral proteins (6). The RI of SFV is found in the membrane fraction of the cell cytoplasm, as is the SFV RNA polymerase (12). It is of interest that the RI of SFV, unlike that of poliovirus, is not infectious before denaturation.

Previous work has indicated that the ribonuclease-resistant "core" of SFV is a 20*S* structure (8, 17). These determinations were performed on RNA extracted by SDS alone. Phenol-SDS extraction, as employed in this study, is a more efficient deproteinization process than SDS extraction. The presence of residual protein may make nascent RNA strands more resistant to ribonuclease treatment. The recovery of the 20*S* structure may have been due to the persistence of a small, single-stranded tail on the "core" structure.

LITERATURE CITED

1. Baltimore, D., and M. Girard. 1966. An intermediate in the synthesis of poliovirus RNA. Proc. Natl. Acad. Sci. U.S. **56**:741–748.
2. Borst, P., and C. Weissman. 1965. Replication of viral RNA. VIII. Studies on the enzymatic mechanism of replication of MS2 RNA. Proc. Natl. Acad. Sci. U.S. **54**:982–987.
3. Duesberg, P. H., and W. S. Robinson. 1967. On the structure and replication of influenza virus. J. Mol. Biol. **25**:383–406.
4. Fenwick, M. L., R. L. Erickson, and R. M. Franklin. 1964. Replication of the RNA of bacteriophage R 17. Science **146**:527–530.
5. Franke, B., and P. H. Hofschneider. 1966. Infectious nucleic acids of *E. coli* bacteriophages. IX. Sedimentation constants and strand integrity of infectious M12 phage replicative form RNA. Proc. Natl. Acad. Sci. U.S. **56**: 1883–1890.
6. Friedman, R. M. 1968. Protein synthesis directed by an arbovirus. J. Virol. **2**:26–32.
7. Friedman, R. M., and I. Berezesky. 1967. Cytoplasmic fractions associated with Semliki Forest virus ribonucleic acid replication. J. Virol. **1**:374–383.
8. Friedman, R. M., H. B. Levy, and W. B. Carter. 1966. Replication of Semliki Forest virus. Three forms of viral RNA produced during infection. Proc. Natl. Acad. Sci. U.S. **56**:440–446.
9. Katz, L., and S. Penman. 1966. The solvent denaturation of double-stranded RNA from poliovirus infected HeLa cells. Biochem. Biophys. Res. Commun. **23**:557–566.
10. Kelly, R. B., and R. L. Sinsheimer. 1964. A new RNA component in MS2-infected cells. J. Mol. Biol. **8**:602–605.
11. Kelly, R. B., and R. L. Sinsheimer. 1967. The replication of bacteriophage MS2. IX. Structure and replication of the replicative intermediate. J. Mol. Biol. **29**:237–249.
12. Martin, E. M., and J. A. Sonnabend. 1967. Ribonucleic acid polymerase catalyzing synthesis of double-stranded arbovirus ribonucleic acid. J. Virol. **1**:97–109.
13. Mecs, E., J. A. Sonnabend, E. M. Martin, and K. H. Fantes. 1967. The effect of interferon on the synthesis of RNA in chick cells infected with Semliki Forest virus. J. Gen. Virol. **1**:25–40.
14. Richter, A., and E. Wecker. 1963. The reaction of EEE virus preparations with sodium deoxycholate Virology **20**:263–268.
15. Scherrer, K., and J. E. Darnell. 1962. Sedimentation characteristics of rapidly labelled RNA from HeLa cells. Biochem. Biophys. Res. Commun. **7**:486–490.
16. Sebring, E. D., and N. P. Salzman. 1964. An improved procedure for measuring the distribution of $P^{32}O_4^{---}$ among the nucleotides of ribonucleic acid. Anal. Biochem. **8**:126–129.
17. Sonnabend, J. A., E. M. Martin, and E. Mecs. 1967. Virus specific RNAs in infected cells. Nature **213**:365–367.