

# Structure and Function of Bacteriophage R17 Replicative Intermediate Ribonucleic Acid

## II. Properties of the Parental Labeled Molecule

R. L. ERIKSON AND E. ERIKSON

*Department of Pathology, University of Colorado Medical School, Denver, Colorado 80220*

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Replicative intermediate ribonucleic acid (RNA), designated RI, which contained parental RNA labeled with  $^{32}\text{P}$  was separated by filtration through agarose from the nucleic acids prepared from  $^{32}\text{P}$ -labeled RNA phage-infected *Escherichia coli*. A larger amount of ribonuclease-sensitive parental label was found in the rapidly sedimenting forms of RI than in the slower sedimenting forms, indicating that parental RNA is displaced to form a single-stranded tail. This result indicates that some phage RNA is generated by asymmetric semiconservative replication of RI, but it does not mean that a portion of the RI duplexes cannot be conserved during generation of phage RNA. Parental RNA was also found in double-stranded RNA with no apparent tails which sedimented with an *S* value of 13. This RNA was soluble in 2 M NaCl, and its sedimentation rate was unaffected by ribonuclease; nevertheless, single-strand scissions were produced by ribonuclease and were detected after the duplex was converted to its component single strands.

In cells infected with small ribonucleic acid (RNA)-containing viruses, a virus-specific double-stranded RNA molecule termed replicative form (RF) has been found (17, 19, 20). This molecule has been isolated by virtue of its solubility in solutions of high ionic strength (1, 2) and has been shown by Ammann, Delius, and Hofschneider (1) to contain a biologically active strand of viral RNA. Replicative form RNA is highly resistant to ribonuclease. By the use of very short pulses of  $^3\text{H}$ -uridine, a second, rapidly labeled, virus-specific RNA component which is only partially resistant to ribonuclease has been identified in infected cells (13), and it has been termed replicative intermediate RNA (RI). RI, in contrast to RF, is precipitated by 1 M NaCl (3, 7, 14) and appears to have nascent strands of viral RNA attached to a double-stranded RNA core. Since after short pulses of RNA precursors RI is labeled more rapidly than is viral RNA or RF (3, 7, 13), it may be the precursor to viral RNA.

Without resort to enzymatic treatment, the RI of R17 bacteriophage can be separated from viral and cellular RNA in an undegraded form (12, 14). Purified RI was found to contain RNA strands identical to those isolated from intact phage, and native RI was also found to be biologically active (8). This strengthens the claim that RI does

indeed serve as an intermediate in bacteriophage RNA biosynthesis.

In this communication, we describe some of the properties of RI in which the parental RNA is labeled with  $^{32}\text{P}$ . The results support those recently reported by Lodish and Zinder (18) on the mechanism of replication of f2 RNA and support the earlier suggestions that at least a portion of bacteriophage RNA is synthesized in an asymmetric semiconservative manner (13, 21). A preliminary account of the present report has appeared (R. L. Erikson, Abstr. Ann. Meeting Biophys. Soc., 11th, 1967).

### MATERIALS AND METHODS

The media and strains of bacteria and phage and the methods of infection of bacteria and spheroplasts have been described previously (8). This earlier communication also listed the techniques of ultracentrifugation and radioactivity assay as well as those concerning the purification of RI. The preparation of  $^{32}\text{P}$ -labeled R17 bacteriophage was carried out as described previously (9).

### RESULTS AND DISCUSSION

*Experimental rationale.* Our current concept of the relationship between the sedimentation behavior of RI and two of its possible structures may be illustrated as in Fig. 1 (8, 11, 13). In model *a*, the plus (phage-like) and minus (com-

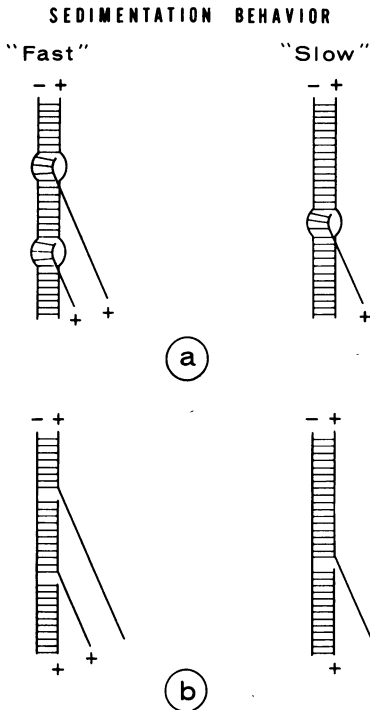


FIG. 1. Two of the possible mechanisms for the generation of phage RNA (+) strands from complementary (-) strands. Sedimentation behavior refers to the fact that RI apparently moves more rapidly through a sucrose gradient when it consists of a greater amount of single-stranded RNA.

plementary) strands in the duplex are conserved, and a new plus strand is generated in a manner that is presumably analogous to that of deoxyribonucleic acid (DNA)-dependent RNA synthesis. In contrast, operation of model *b* results in the displacement of the plus strand during the synthesis of another plus strand. One method of specifically tagging only a plus strand and no other RNA consists of the infection of bacteria with bacteriophage in which the RNA is labeled with  $^{32}\text{P}$ . Then, at a suitable time after infection, the RI formed in these infected cells is purified. When such RI is fractionated on the basis of its sedimentation behavior and when the parental strand is examined for ribonuclease sensitivity, we would expect that, if the mechanism depicted by model *a* were operative, both fast and slow RI would contain parental label equally resistant to ribonuclease. According to model *b*, however, the parental-labeled RNA in fast RI should be more sensitive to ribonuclease than it would be in slow RI, because the plus strand with the longest region exposed to ribonuclease action would be the one containing parental label.

All of these considerations also hold if the

minus strand were in a ring form as proposed by Brown and Martin (4). Although the electron micrographs published by Granboulan and Franklin (15) do not show any circular molecules, they do demonstrate the presence of noncircular branched molecules.

*Replicative intermediate in which the parental strand is labeled with  $^{32}\text{P}$ .* A few minutes after the infection of *Escherichia coli* with  $^{32}\text{P}$ -labeled bacteriophage, the parental RNA can be detected in RI (9, 17, 21). Pure parental-labeled RI cannot be obtained from these cells without additional fractionation of the nucleic acids, because, as shown by the above investigations, the conversion of parental RNA to RI is never complete, and, therefore, intact single-stranded labeled parental RNA is detectable throughout infection. A further complication of these studies arises when, apparently owing to nuclease action or to changes in RNA structure, single-stranded parental RNA is detected as slower sedimenting forms in sucrose gradients (10, 16).

Nucleic acids were prepared by phenol-sodium lauryl sulfate extraction of infected cells. Ribosomal RNA, phage RNA, and RI were precipitated with 2 M NaCl, whereas DNA, 4S host-soluble RNA, and RF (double-stranded RNA) remained in the 2 M NaCl supernatant fluid. The nucleic acids precipitated by 2 M NaCl are then passed through columns of 4% agarose granules (Mann Research Laboratories, Inc., New York, N.Y.); in STE buffer [0.15 M NaCl; 0.05 M tris-(hydroxymethyl)aminomethane (Tris), pH 7.2;  $10^{-3}$  M ethylenediaminetetraacetic acid (EDTA)]. The RI is excluded by 4% agarose, and elutes in the void volume, whereas the single-stranded RNA is retarded. In this way, after three passages through such a column, the RI is freed of all single-stranded cellular and phage RNA.

Fig. 2a illustrates the distribution of purified RI after sedimentation through a sucrose gradient with purified 27S R17 RNA added for a sedimentation rate marker. When a similar mixture in  $10^{-3}$  M EDTA plus  $10^{-2}$  M Tris (pH 7.2) was heated to 95 C for 3 min, quickly chilled, and then centrifuged, the  $^{32}\text{P}$ -labeled RNA sedimented with the optical density (OD) marker of R17 RNA (Fig. 2b). Before heat denaturation, the labeled RI had a broad distribution of *S* values (from 15 to 35) and was partially resistant to ribonuclease (Pancreatic ribonuclease A; Worthington Biochemical Corp., Freehold, N.J.), whereas after denaturation the label behaved like phage RNA in both respects. No  $^{32}\text{P}$  labeled R17 RNA was detected in RI when it was simply added to an infected cell lysate prior to phenol treatment. Excluding the unlikely possibility of breakdown and resynthesis, this sedimentation behavior of the  $^{32}\text{P}$ -labeled RNA indicates that

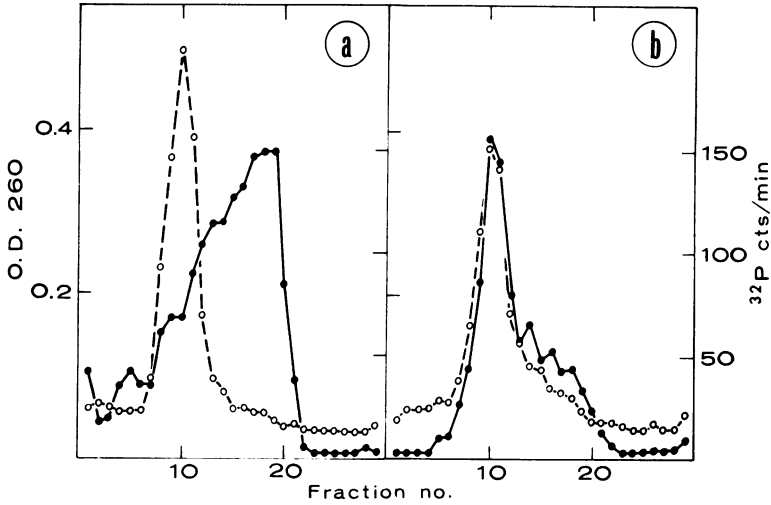


FIG. 2. Sedimentation analysis of RI in which the parental RNA strand is  $^{32}\text{P}$ -labeled. As for the rest of the figures, the sedimentation was through a 4.4-ml 7 to 20% sucrose gradient (w/v) containing 0.1 M NaCl (45,000 rev/min), at 10 C, by use of a Beckman L2 ultracentrifuge. (a) Native RI ( $^{32}\text{P}$ ) and 27S R17 RNA (OD). (b) Same as (a), but denatured for 3 min at 95 C in  $10^{-3}$  M EDTA plus  $10^{-2}$  M Tris. Centrifugation time was 150 min. Symbols:  $\circ$ , OD<sub>260</sub>;  $\bullet$ ,  $^{32}\text{P}$  radioactivity.

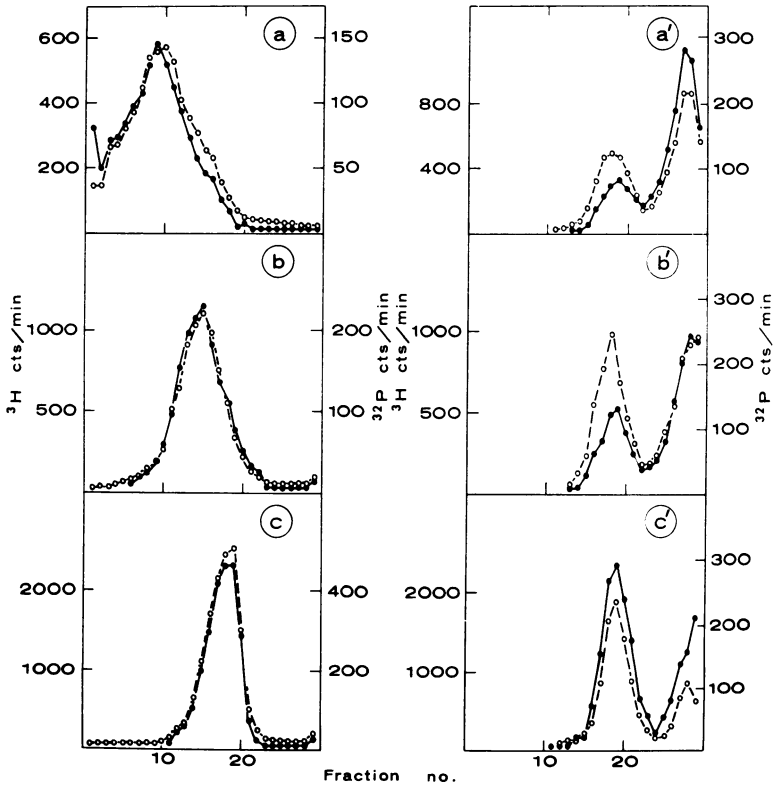


FIG. 3. Sedimentation analysis (see Fig. 2 legend) of treated and untreated RI preparations. Ribonuclease treatment: 0.1  $\mu\text{g}$  of ribonuclease per ml, 10 min at 37 C in 0.15 M NaCl, 0.05 M Tris (pH 7.2), and  $10^{-3}$  M EDTA. Immediately after treatment, the preparations were chilled, layered on sucrose gradients, and centrifuged. (a), (b), and (c) Untreated preparations, centrifuged 150 min; (a'), (b'), and (c') treated preparations, centrifuged 180 min. Symbols:  $\bullet$ ,  $^{32}\text{P}$  radioactivity (parental RNA);  $\circ$ ,  $^3\text{H}$  radioactivity (newly synthesized RNA).

the parental strand is still intact. The RI described in Fig. 2 and 3 was obtained 30 min after infection, a time when phage RNA synthesis was proceeding rapidly, from cells which had been infected at a multiplicity of 10. However, similar results were obtained with RI prepared 10 or 20 min after infection from cells infected at a multiplicity of 1.

Other infections were performed in the presence of uridine-5-<sup>3</sup>H to label uniformly all RNA synthesized after infection. Therefore, the purified RI was doubly labeled; the parental strand was labeled with <sup>32</sup>P, and the rest of the molecule and all other RI molecules not containing parental RNA, with <sup>3</sup>H. This RI preparation was separated, on the basis of sedimentation rate, into the three arbitrarily selected fractions shown in Fig. 3a, b, and c. The *S* values of these preparations as determined by the positions of the peaks were 32, 24, and 17. When a sample from each of these preparations was treated with 0.1 μg of ribonu-

lease per ml for 10 min at 37 C and recentrifuged, the results (Fig 3a', b', and c') demonstrate that each preparation yielded a ribonuclease-resistant fraction (*S* = 13) and nonsedimentable fragments. The values for the fraction of ribonuclease-resistant RNA in this experiment are given in Table 1. These results indicate that some of the RI has the structure illustrated by model *b* in Fig. 1, but they do not rule out that RI also exists in the form shown by model *a*.

The values for the resistance of the <sup>3</sup>H label in RI are similar to those described previously (8), but in themselves they yield no information on structure other than the fact that strands of ribonuclease-sensitive RNA are a part of the RI structure. Asymmetric semiconservative replication of a portion of phage RNA is implied by the resistance of the parental label in RI and by the experiments of Lodish and Zinder (18) which demonstrate that all phage f2 parental RNA is chased out of a double-stranded form during the synthesis of single-stranded RNA in the absence of synthesis of double-stranded RNA. These authors also point out that the time required to chase parental RNA out of a double-stranded form may mean both mechanisms operate (Fig. 1); that is, there is a possibility at the initiation of each new strand that the duplex is conserved. Previous experiments (5, 6) have established that parental phage RNA is not transferred to progeny phage. Therefore, some mechanism must exist to ensure that parental RNA is recycled into RI and not into phage. However, no explanation as to the nature of this mechanism is available at this time.

*Replicative form in which the parental strand is*

TABLE 1. Ribonuclease digestion of parental-labeled RI

Avg <i>S</i> prior to digestion	Resistance (%) <sup>a</sup>	
	<sup>3</sup> H	<sup>32</sup> P
32	43	28
24	52	42
17	73	68

<sup>a</sup> Determined as the percentage of radioactive RNA which sediments with an *S* value of 13 after ribonuclease treatment, as described in legend to Fig. 3.

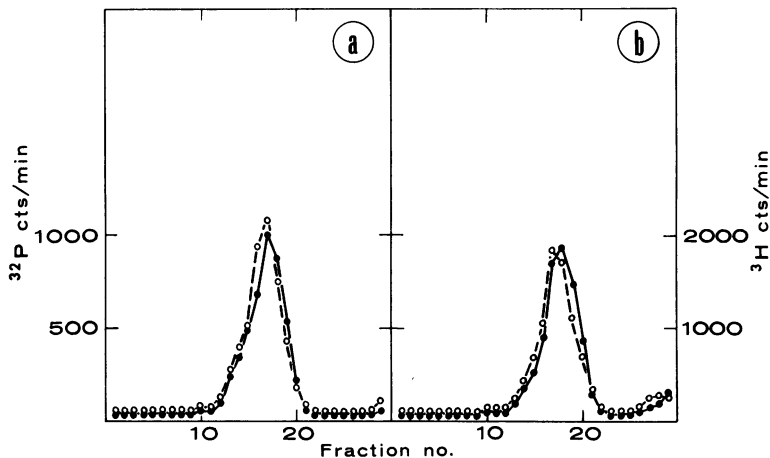


FIG. 4. Sedimentation analysis, as in legend to Fig. 2, of replicative form RNA, treated and untreated with ribonuclease. (a) Untreated; (b) treated as described in the legend to Fig. 3. Centrifugation time, 180 min. Symbols: ●, <sup>32</sup>P radioactivity (parental RNA); ○, <sup>3</sup>H radioactivity (newly synthesized RNA).

labeled with  $^{32}\text{P}$ . As mentioned in the previous section, RF, the molecule which is apparently completely double-stranded, is soluble in 2 M NaCl. According to the reasoning in the experimental rationale discussed above, the parental strand in this molecule should be completely resistant to the action of ribonuclease. The nucleic acids soluble in 2 M NaCl were centrifuged through a sucrose gradient, RNA from appropriate fractions was collected, and purified RF was obtained from this RNA by filtration through agarose. RF displayed the same sedimentation characteristics before or after ribonuclease treatment (Fig. 4a and b), and was greater than 95% resistant by this test to ribonuclease. This behavior is in marked contrast to even slowly sedimenting forms of RI, the sedimentation characteristics of which are altered by treatment with ribonuclease. This result satisfies the expectation that the parental RNA molecule hydrogen-bonded to the complementary (minus) strand over its entire length is not degraded by pancreatic ribonuclease, with the qualifications presented in the following section.

**Effect of ribonuclease on double-stranded RNA.** RF is not affected by ribonuclease treatment as measured by sedimentation behavior or by release of nonsedimentable fragments. However, this result does not exclude the possibility that phosphate diester bonds are hydrolyzed, because this hydrolysis may go undetected in the double-stranded molecule. To test for this possibility, RF (about 10  $\mu\text{g}/\text{ml}$ ) in which the parental strand was labeled with  $^{32}\text{P}$  was treated with 1  $\mu\text{g}$  of ribonuclease per ml for 10 min at 37 C, chilled, and extracted three times with cold phenol to remove the ribonuclease. This preparation was then mixed with 27S R17 RNA in  $10^{-3}$  M EDTA plus  $10^{-2}$  M Tris (pH 7.2) and was heat-denatured (3 min, at 95 C).

The RF which had been ribonuclease-treated prior to denaturation yielded almost no 27S  $^{32}\text{P}$ -labeled RNA (Fig. 5c), whereas the denatured control (Fig. 5b) yielded a significant fraction of RNA with sedimentation behavior similar to that of R17 RNA. Prior to denaturation, both preparations sedimented exactly as shown by the undenatured control (Fig. 5a). Such a result establishes that pancreatic ribonuclease hydrolyzes bonds in the component single strands of the double-stranded RNA molecule without affecting the sedimentation properties of the native double-stranded molecule. In addition, unpublished results of R. L. Erikson and R. M. Franklin show that treatment of RF with higher ribonuclease concentration does reduce its S value without the release of nonsedimentable fragments, indicating that enough single-strand

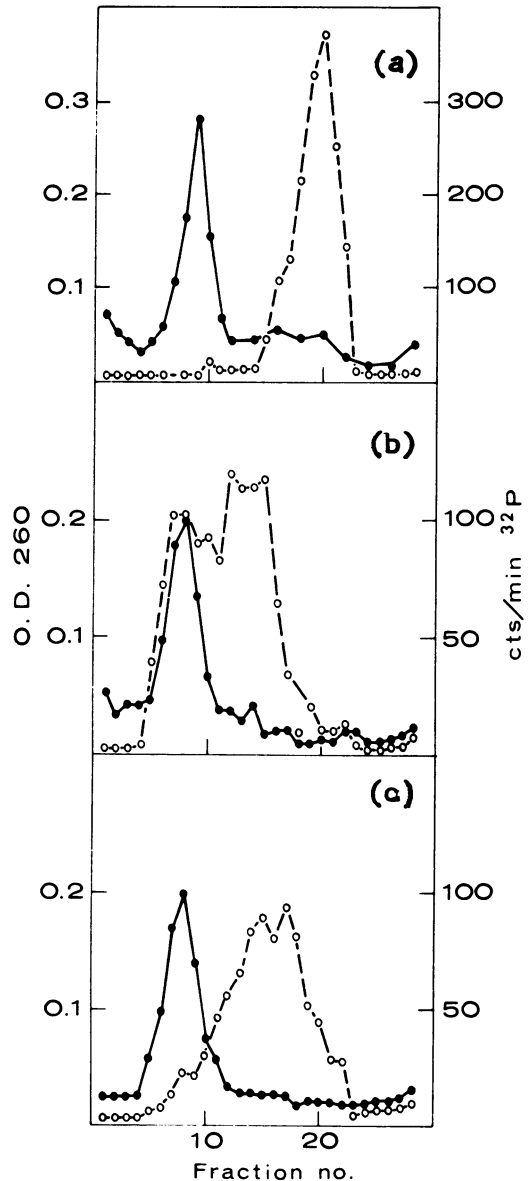


FIG. 5. Effect of ribonuclease on double-stranded RNA. (a) Replicative form RNA plus R17 RNA; (b) same as (a), but denatured at 95 C for 3 min in  $10^{-3}$  M EDTA plus  $10^{-2}$  M Tris; (c) replicative form RNA, ribonuclease-treated and phenol-extracted prior to the addition of R17 RNA and denaturation. Symbols: ●, OD<sub>260</sub>; ○,  $^{32}\text{P}$  radioactivity. Centrifugation time, 150 min.

breaks can be obtained to result in scission of the double-stranded molecule. It should be noted that, since the R17 RNA exhibited the same profile in all three instances, the heat denaturation did not cause significant breakdown and that an

appreciable amount of ribonuclease which could have hydrolyzed the RNA after heat denaturation was not carried through.

An unexplained result of these experiments is the appearance of a second, slower-moving peak of single-stranded RNA in Fig. 5b. The result expected, as in Fig. 2b, was that all parental label would appear at 27S after denaturation. However, six preparations of RF each yielded a second, slower moving peak (about 19S), as shown in Fig. 5b, whereas an equal number of denatured RI preparations yielded in addition to phage-like RNA only an ill-defined trail of slowly sedimenting material. This result may indicate a real difference between RI and RF molecules.

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#### LITERATURE CITED

- AMMANN, J., H. DELIUS, AND P. H. HOFSCHEIDER. 1964. Isolation and properties of an intact phage-specific replicative form of RNA phage M12. *J. Mol. Biol.* **10**:557-561.
- BALTIMORE, D. 1966. Purification and properties of poliovirus double-stranded ribonucleic acid. *J. Mol. Biol.* **18**:421-428.
- BALTIMORE, D., AND M. GIRARD. 1966. An intermediate in the synthesis of poliovirus RNA. *Proc. Natl. Acad. Sci. U.S.A.* **56**:741-748.
- BROWN, F., AND S. J. MARTIN. 1965. A new model for virus ribonucleic acid replication. *Nature* **208**:861-863.
- DAVIS, J. E., AND R. L. SINSHEIMER. 1963. The replication of bacteriophage MS2. I. Transfer of parental nucleic acid to progeny phage. *J. Mol. Biol.* **6**:203-207.
- DOI, R. H., AND S. SPIEGELMAN. 1963. Conservation of a viral RNA genome during replication and translation. *Proc. Natl. Acad. Sci. U.S.A.* **49**:353-360.
- ERIKSON, R. L. 1966. Fractionation of viral-specific ribonucleic acid by cesium sulfate equilibrium density-gradient centrifugation. *J. Mol. Biol.* **18**:372-381.
- ERIKSON, R. L., E. ERIKSON, AND J. A. GORDON. 1966. Structure and function of bacteriophage R17 replicative intermediate RNA. I. Studies on sedimentation and infectivity. *J. Mol. Biol.* **22**:257-268.
- ERIKSON, R. L., M. L. FENWICK, AND R. M. FRANKLIN. 1964. Replication of bacteriophage RNA: Studies on the fate of parental RNA. *J. Mol. Biol.* **10**:519-529.
- ERIKSON, R. L., M. L. FENWICK, AND R. M. FRANKLIN. 1965. Replication of bacteriophage RNA: Some properties of the parental-labeled replicative intermediate. *J. Mol. Biol.* **13**:399-406.
- ERIKSON, R. L., AND R. M. FRANKLIN. 1966. Symposium on replication of viral nucleic acids. I. Formation and properties of a replicative intermediate in the biosynthesis of viral ribonucleic acid. *Bacteriol. Rev.* **30**:267-278.
- ERIKSON, R. L., AND J. A. GORDON. 1966. Replication of bacteriophage RNA: Purification of the replicative intermediate by agarose column chromatography. *Biochem. Biophys. Res. Commun.* **23**:422-428.
- FENWICK, M. L., R. L. ERIKSON, AND R. M. FRANKLIN. 1964. Replication of the RNA of bacteriophage R 17. *Science* **146**:527-530.
- FRANKLIN, R. M. 1966. Purification and properties of the replicative intermediate of the RNA bacteriophage R17. *Proc. Natl. Acad. Sci. U.S.A.* **55**:1504-1511.
- GRANBOULAN, N., AND R. M. FRANKLIN. 1966. Electron microscopy of viral RNA, replicative form and replicative intermediate of the bacteriophage R17. *J. Mol. Biol.* **22**:173-177.
- KELLY, R. B., J. L. GOULD, AND R. L. SINSHEIMER. 1965. The replication of bacteriophage MS2. IV. RNA components specifically associated with infection. *J. Mol. Biol.* **11**:562-575.
- KELLY, R. B., AND R. L. SINSHEIMER. 1964. A new component in MS2-infected cells. *J. Mol. Biol.* **8**:602-605.
- LODISH, H. F., AND N. D. ZINDER. 1966. Semi-conservative replication of bacteriophage f2 RNA. *J. Mol. Biol.* **21**:207-209.
- MONTAGNIER, L., AND F. K. SANDERS. 1963. Replicative form of encephalomyocarditis virus nucleic acid. *Nature* **199**:664-667.
- WEISSMANN, C., AND P. BORST. 1963. Double-stranded ribonucleic acid formation *in vitro* by MS2 phage-induced RNA synthetase. *Science* **142**:1188-1191.
- WEISSMANN, C., P. BORST, R. H. BURDON, M. A. BILLETER, AND S. OCHOA. 1964. Replication of viral RNA. IV. Properties of RNA synthetase and enzymatic synthesis of MS2 phage RNA. *Proc. Natl. Acad. Sci. U.S.A.* **51**:890-897.