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The Replication of Bacteriophage MS2

IX. †Structure and Replication of the Replicative Intermediate

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Experiments are described to determine whether the ribonuclease-sensitive component of the replicative intermediate formed during RNA bacteriophage infection is the nascent strand (conservative replication), or is a displaced portion of the viral strand of the RNA duplex (semi-conservative replication). Appropriate labeling experiments indicate that this component arises in each replicative intermediate molecule, with equal probability, from either origin. Those replicative intermediate molecules in which the nascent strand is ribonuclease-sensitive are more readily denatured than those in which the viral strand is partially displaced. The two types of replicative intermediate can be distinguished on this basis. It is proposed that each replicative intermediate molecule replicates both conservatively and semi-conservatively, equally often.

1. Introduction

In order to clarify some of the unsolved problems of nucleic acid replication, extensive study has been made in recent years of the mechanism of replication of the single-stranded ribonucleic acid bacteriophages. Evidence has accumulated that a double-stranded RNA molecule appears during the replication cycle (Kelly & Sinsheimer, 1964; Weissmann, Borst, Burdon, Billeter & Ochoa, 1964) and has a direct role in the production of progeny single-stranded molecules (Fenwick, Erikson & Franklin, 1964; Ammann, Delius & Hofschneider, 1964; Mills, Pace & Spiegelman, 1966). Thus the principle that nucleic acid replication requires the synthesis of a complementary strand seems to be valid also for the replication of single-stranded RNA.

It is not yet known how single-stranded RNA molecules are produced from the double-stranded intermediate. Two mechanisms have been described for the *in vitro* production of single-stranded RNA from double-stranded templates using the DNA-dependent RNA polymerase. If the template is a synthetic DNA–RNA hybrid, the pre-existing RNA is displaced, at least part of the time, by the newly synthesized RNA strand (Sinsheimer & Lawrence, 1964; Chamberlin & Berg, 1964). This mode of transcription has been called semi-conservative replication. If, however, the template is double-stranded DNA (ϕX replicative form) the newly synthesized RNA is displaced from the duplex in a conservative mode of replication (Hayashi, 1965). There is no basis for predicting which, if any, of these mechanisms will be used when the template is a double-stranded RNA.

† The previous paper in this series is Kelly & Sinsheimer (1967*b*).

From a detailed knowledge of the structure of the replicative intermediate, as the *in vivo* precursor to single-stranded RNA is often called, it should be possible to distinguish between a semi-conservative and a conservative mode of replication. The present evidence supports a structure for the RI† consisting of an RNA duplex of constant size (Erikson, Fenwick & Franklin, 1965) to which is attached one single-stranded RNA chain of length less than that of the viral RNA (Kelly & Sinsheimer, 1967*b*). A structure of this type would be expected if the growing strand does not dissociate from the duplex during the extraction procedure, and can be explained either by a conservative or a semi-conservative replication mechanism (Fig. 1). If the duplex is transcribed semi-conservatively, it is obvious from Fig. 1 that the nascent strand displaces the viral strand. Conversely, if the transcription is conservative, the nascent strand, except for a short region at the growing point, is displaced from the duplex. The experiments described below have been designed to determine whether the nascent or the viral strand is so displaced, and on this basis to decide whether replication is conservative or semi-conservative. The results suggest that both types of replication occur with equal probability.

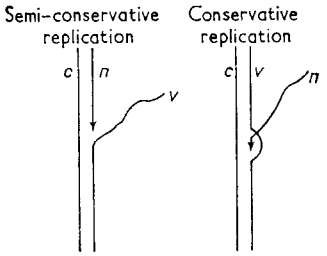

FIG. 1. A schematic diagram of possible structures for the MS2 replicative intermediate.

v denotes the viral strand, identical to that in the phage. *c* is the strand complementary to the viral strand. *n* is the nascent strand, complementary to *c*, with the growing point indicated by an arrowhead. The single-stranded portion of the RI is referred to as the tail, and the double-stranded portion as the core or RNase-resistant duplex. The tail can be removed from the core by digestion with ribonuclease.

2. Materials and Methods

Many of the techniques used in this paper have been previously described. Information on growth media, and on the methods of lysing the cells, and extracting and precipitating the nucleic acids were described by Kelly, Gould & Sinsheimer (1965). The preparation of [$^{12}C^{14}N^{32}P$]MS2 and of [$^{13}C^{15}N^{32}P$]MS2 and the procedures for determining the buoyant density of RNA in cesium chloride density-gradients at 65°C were outlined by Kelly & Sinsheimer (1967*a*). Finally, ethidium bromide–cesium chloride density-gradient centrifugation, fractionation of nucleic acids on BNC columns, and ribonuclease (RNase) digestion have been discussed by Kelly & Sinsheimer (1967*b*).

(a) *Pulse-labeling*

Escherichia coli grown at 37°C (but not at 25°C) can be infected at 25°C by MS2 phage and will produce infective RNA. In order to label infected cells for periods which were short compared to the time of transcription of a viral strand, label was added for a few seconds at 25°C. Cells were grown in TPA medium at 37°C to 2×10^8 cells/ml. and infected with MS2 phage at a multiplicity of 5. After 20 min incubation at 37°C the cells were

† Abbreviations used: RI, replicative intermediate; BNC, benzoylated-naphthoylated cellulose.

chilled to 25°C for 15 min. [^{3}H]Uracil (2 μc/ml., 15 c/m-mole) was added to the culture, with vigorous stirring. After several seconds, the incubation was stopped by addition of excess crushed ice and sodium azide to a final concentration of 0·01 M. The chilled culture was immediately filtered (Millipore HA, 142 mm) and washed with cold 0·1 M-Tris (pH 7·4) and 0·01 M-sodium azide before resuspending in the same buffer. The cells were concentrated by sedimentation, lysed with sodium dodecyl sulfate and extracted with phenol.

(b) *Sucrose gradient sedimentation*

Samples to be analyzed by preparative sedimentation were centrifuged in 5-ml. Polyallomer tubes containing a 5 to 20% sucrose density-gradient. For non-denaturing gradients, 0·1 M-Tris (pH 7·4) was used as the solvent, and the samples were sedimented in a Spinco SW65 for 2 hr at 65,000 rev./min (5°C). For partial denaturing gradients, 60% dimethyl sulfoxide and 10^{-3} M-EDTA were used as solvent. Samples were centrifuged 10 hr at 65,000 rev./min and at 25°C. For fully denaturing gradients, the sucrose was dissolved in 99% dimethyl sulfoxide, 10^{-3} M-EDTA and the samples centrifuged 12 hr at 65,000 rev./min and at 25°C. In the latter case, 50 μl. of the sample to be analyzed were mixed with 100 μl. dimethyl formamide and 50 μl. dimethyl sulfoxide before layering on the gradient. Under these conditions, a very stable layer is formed above the 99% dimethyl sulfoxide sucrose density-gradient, and no convection occurs. Further details of this procedure have been described by Strauss (1966).

3. Results

(a) *RNase-sensitivity of the nascent strand*

From Fig. 1 it is apparent that—by definition—if the nascent strand is displaced from the duplex the replication is conservative, and if it is not the replication is semi-conservative. If infected cells are labeled for short periods, the majority of the phage-specific radioactivity appears in the RI. On denaturing the RI, this radioactivity can be shown to be associated with the nascent strand (*vide infra*). If, in the RI, the nascent strand is displaced from the duplex, it will be sensitive to RNase digestion; if not, it will be resistant. Consequently, by labeling the nascent strand with a brief pulse of radioactivity and determining the sensitivity of the radioactivity to RNase, a simple test of the mechanism of replication should be possible.

Cells infected with MS2 for 30 minutes at 37°C were cooled to 25°C and labeled for 10 seconds with [^{3}H]uracil (2 μc/ml., 15 c/m-mole). The cells were lysed and their nucleic acids extracted with phenol and precipitated with alcohol. After resuspending the nucleic acids in 0·3 M-NaCl, [^{32}P]MS2 RNA was added as marker and the material chromatographed on a BNC column. Each fraction was assayed for total and RNase-resistant radioactivity (Fig. 2).

The RI radioactivity (fractions 27 to 31) is neither fully resistant to RNase, as a semi-conservative mode of replication predicts, nor fully sensitive, as is predicted by a conservative mode, but is 40 to 50% resistant. The small shoulder (fraction 34) on the peak of 16 s ribosomal RNA, coincident with the MS2 RNA marker, perhaps represents some completion and release of MS2 RNA. Fractions 23 to 26 consist largely of completed duplexes (*vide infra*) and thus are highly resistant to RNase.

There are several possible interpretations of a pulse label which is 40 to 50% RNase-resistant. Even if the replication is conservative, the segment of the nascent strand at the growing point (Fig. 1) might be insusceptible to RNase. Hayashi (1965) has shown that, during the *in vitro* synthesis of RNA on a double-stranded DNA template, about 50 nucleotides at the growing point of the nascent strand are hydrogen-

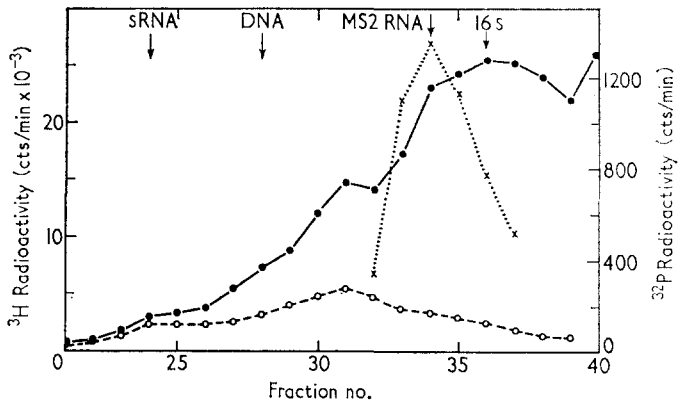

FIG. 2. Fractionation of pulse-labeled replicative intermediate on a BNC column.

The nucleic acids from MS2-infected cells labeled at 25°C with [^{3}H]uracil for 10 sec just prior to lysis were applied to a BNC column and eluted with a 0·3 to 1·0 M linear gradient of NaCl (buffered with 0·01 M-Tris, pH 7·4). 40 fractions of 2·5 ml. were obtained. Samples were assayed for absorbance (peaks indicated by arrows) and for trichloroacetic acid-precipitable ^{3}H-radioactivity both before (—●—●—) and after (--○--○--) RNase digestion. ·· × ·· × ·· indicates the position of a single-stranded [^{32}P]MS2 RNA marker.

bonded to the template and thus resistant to RNase. The remainder of the nascent strand is sensitive to RNase. If, during a conservative replication, a similar number of nucleotides were required to bind the nascent strand to the duplex in the MS2 RI, the pulse label would only become sensitive to RNase when the labeling time exceeded about 2% of the transcription time. (The transcription time is the time for a replication point to travel from one end of the template to the other, incorporating 3300 nucleotides.) A 50% resistance to RNase could arise if the labeling time (10 seconds at 25°C) was about 4% of the transcription time. This is unlikely, however, since the pulse-label in the various RI fractions remained 50% resistant to RNase when the labeling time was varied from 5 to 30 seconds. It is concluded that significantly more than 100 nucleotides are added to the nascent strand during 10 seconds at 25°C, and that another explanation of the resistant fraction must be sought.

If, alternatively, replication is semi-conservative and the viral strand is displaced by the nascent strand, it can be concluded from Fig. 1 that the pulse label in RI will only begin to be RNase-sensitive when the labeling time exceeds the transcription time. Hence, to account for the observed 50% or more of RNase-sensitive label in RI, one must postulate that the 10-second pulse at 25°C is equivalent to two or more transcription times (and then this result could be obtained only for the RI with long-tails: Table 1). As can also be seen from Table 1, however, a more sensitive assay than RNase-resistance for determining if several cycles of replication have occurred is to measure the amount of label in RI present as full-length strands. In Fig. 3(b) it is apparent that, after denaturation, the great majority of the radioactive material, initially present in a RI fraction with relatively long tails, sediments more slowly than does MS2 RNA, and is thus in incomplete nascent strands. Thus it is not possible to explain the RNase-sensitivity by any wholly semi-conservative replication process.

A third and trivial explanation would be that the 50 to 60% of the label in the column fractions which is sensitive to RNase is not a part of the RI but corresponds

TABLE 1

Semi-conservative replication

Length of tail	0·1		0·5		0·9	
Number of replication cycles	1	⩾ 2	1	⩾ 2	1	⩾ 2
% of label in full-length strands	90	91	50	66⅔	10	53
% of label in nascent strands	10	9	50	33⅓	90	47
% of label which is RNase-sensitive	0	9	0	33⅓	0	47

Proportion of label expected to remain in various components of replicative intermediate of various tail lengths, assuming exclusively semi-conservative replication and labeling for the indicated number of replication cycles.

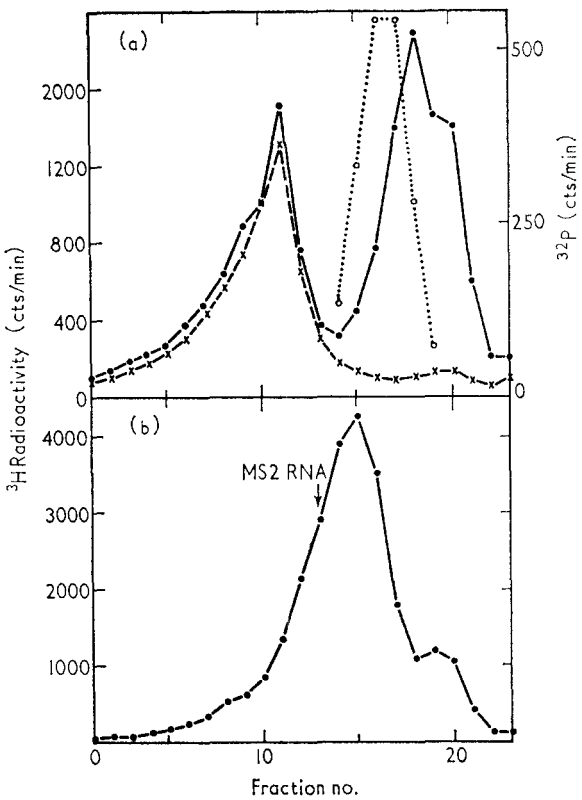

FIG. 3. Centrifugation of pulse-labeled RI under partial and complete denaturing conditions.

(a) A sample of fraction 29, Fig. 2, was centrifuged in a 5 to 20% sucrose density-gradient containing 60% dimethyl sulfoxide, 10^{-3} M-EDTA (pH 7·1) for 12 hr at 60,000 rev./min in a Spinco SW65 at 25°C.

(b) A sample of fraction 30, Fig. 2, was centrifuged in a 5 to 20% sucrose density-gradient containing 99% dimethyl sulfoxide, 10^{-3} M-EDTA for 12 hr at 63,000 rev./min in a Spinco SW65 at 25°C.

^{3}H radioactivity before (—●—●—) and after (– – × – – × – –) RNase digestion. A single-stranded [^{32}P]MS2 RNA marker (· · ○ · · ○ · ·) is included in (a). The arrow in (b) indicates the distance traveled by an external marker under identical conditions.

to single-stranded RNA fragments. Indeed, when one of the column fractions is sedimented under fully denaturing conditions (Fig. 3(b)), a small peak (fraction 19) of labeled RNA is observed which sediments much more slowly than the nascent strand; but this corresponds to only 15% of the total radioactivity of the fraction. The remaining 45% of the label which was RNase-sensitive and the 40% of the label which was RNase-resistant must, after denaturation, have approximately the same sedimentation rate and therefore, under these conditions, the same molecular weight (Strauss, Kelly & Sinsheimer, in preparation). Also, on centrifuging the pulse-labeled RI to equilibrium in an ethidium bromide–cesium chloride density-gradient at 25°C, only one peak of RNA (partially RNase-sensitive) is observed, i.e. the RNase-resistant and RNase-sensitive material are found at the same density. (The small 15% component is not resolved from the RI in such experiments.)

Thus the RNase-sensitive and the RNase-resistant radioactive material are eluted together from a BNC column, have the same buoyant density and, after denaturation, approximately the same molecular weight. It is evident that these results cannot be explained by either of the postulated replication mechanisms. They are understandable if, in fact, both modes of replication are used. More direct evidence indicative of the presence of both types of RI will now be presented.

(b) *Partial denaturation of RI*

The melting temperature of double-stranded molecules is a function of the length of the polynucleotide chain (Steiner & Beers, 1961). If the nascent strand is held to the duplex by only a small number of nucleotides (conservative replication), it should be possible by appropriate choice of denaturing conditions to remove the nascent strand from the duplex without denaturing the duplex. Alternatively, if the viral strand is displaced during replication, it should not be possible to remove the nascent strand in this way. The results presented below suggest that in fact the nascent strand can be dissociated from a portion of the RI molecules by centrifugation in sucrose density-gradients containing 60% dimethyl sulfoxide.

It has been observed (Kelly & Sinsheimer, 1967*b*) that a double-stranded RNA sediments faster than its single-strand components in concentrations of dimethylsulfoxide which denature single- but not double-stranded RNA. For example, when ^{3}H-labeled RNase-resistant core material was sedimented with ^{32}P-labeled MS2 RNA in a partially denaturing sucrose gradient (Fig. 4), the core sedimented 3·5 times faster than the single strand. When fraction 29 (Fig. 2) was sedimented under identical conditions, two peaks of radioactivity were observed (Fig. 3(a)); one component is completely resistant to RNase and is faster than MS2 RNA, whereas the other is completely sensitive and slower.

The explanation proposed for this result is that the fast peak of radioactive material corresponds to a fraction of RI molecules in which the unlabeled viral strand has been partially displaced and, in consequence, the labeled nascent strand is fully resistant to RNase. The other peak of label arises from a RI fraction in which the nascent strands have been displaced. This fraction of RI molecules has dissociated, under the conditions chosen, to an unlabeled duplex and a RNase-sensitive nascent strand, which sediments more slowly than MS2 RNA. From the areas of the peaks, 46% of the radioactivity is in the former type of RI molecule.

If this interpretation is correct, the sedimentation rates of the "semi-conservative" RI molecules (the fast peak) and the dissociated nascent strands should reflect the

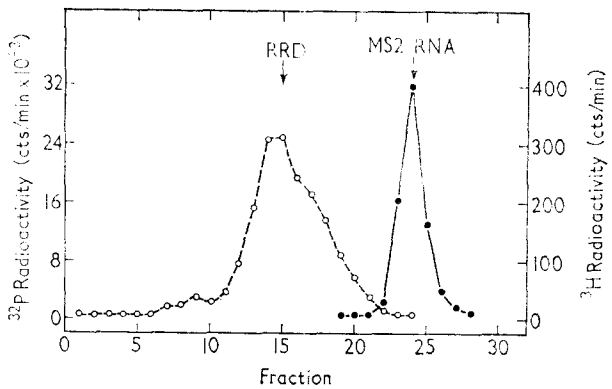

FIG. 4. Comparison of the sedimentation rates of double- and single-stranded RNA under partial denaturing conditions, 60% dimethyl sulfoxide, 10^{-3} M-EDTA, 5 to 20% sucrose.

^{3}H-labeled double-stranded RNA was isolated from the nucleic acids of infected cells as a 14 S peak after RNase digestion. It was mixed with [^{32}P]MS2 RNA and centrifuged, as in Fig. 3(a), for 4 hr at 65,000 rev./min. --○--○--, ^{3}H in ribonuclease-resistant duplex (RRD); —●—●—, ^{32}P in MS2 RNA.

length of tail present in the original RI molecules. Fractions 23, 27 and 31 (Fig. 2) were precipitated and centrifuged under partially denaturing conditions. Each fraction resolved into two peaks (Fig. 5), a fast peak, presumably RI, and a slow peak, presumably nascent strands. The smaller the tail on the original "semi-conservative" RI molecule, the faster it sediments in 60% dimethyl sulfoxide, implying that the tail exerts some drag on the duplex. Conversely, the smaller the tail on the original "conservative" RI, the smaller is the sedimentation rate of the dissociated nascent strand. For fractions 23, 27 and 31, the fraction of the radioactivity in the fast peak was 68, 49 and 51%, respectively.

These experiments strongly support the concept that either the viral strand or the nascent strand can be displaced from the RI during replication with equal probability. Both types of RI are eluted together from the BNC column, which explains the 50% resistance to RNase of the pulse label, but can be distinguished by centrifugation under partially denaturing conditions.

(c) *RNase sensitivity of the viral strand*

In the previous two sections, we have investigated the properties of the nascent strand, identified by a pulse-labeling procedure. An alternative approach, described in the following sections, is to label the viral strand of the RI. If cells are infected with [^{32}P]MS2 phage and the RI isolated, only the viral strand of the RI will be labeled. By experiments analogous to those described above, it is possible to determine whether or not the viral strand is displaced during replications. If the viral strand is displaced, the radioactive material in the viral strand should change from being fully resistant to being fully sensitive to RNase as the length of the nascent strand increases. Conversely, if the nascent strand is displaced, the radioactive material in the viral strand should remain very largely or fully resistant to RNase, independent of the length of the nascent strand.

An experiment has been described (Fig. 3, Kelly & Sinsheimer, 1967*b*) in which ^{32}P-parental-labeled RI is prepared, fractionated on a BNC column and the density of individual RI fractions measured in an ethidium bromide–cesium chloride density-

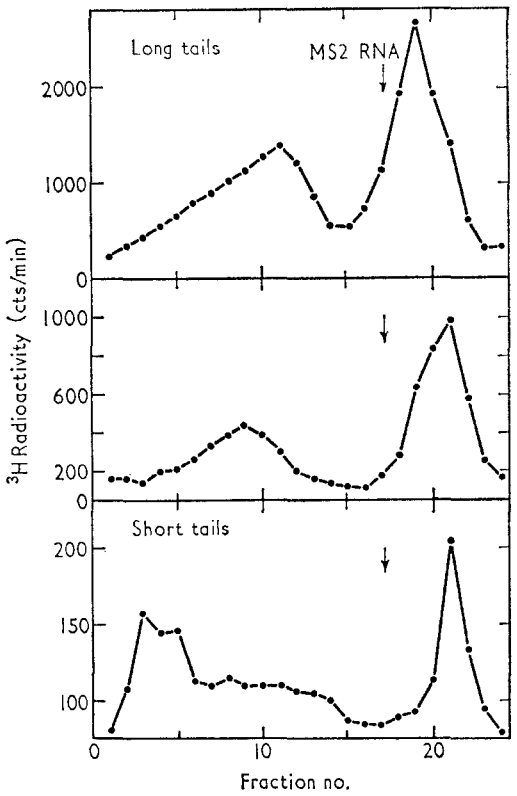

FIG. 5. Sedimentation properties of RI fractions with increasing tail length under partially denaturing conditions.

Fractions 23 (short tails), 27 and 31 (long tails) from a BNC column (Fig. 2) were centrifuged in 60% dimethyl sulfoxide using the conditions described in Fig. 3(a). The position of [^{32}P]MS2 RNA is indicated.

gradient. If the density of the RI fractions was used as a criterion for tail length, then, as the tail length increased, the viral strand of the RI became more sensitive to RNase.

This experiment thus implies that the viral strand of the parental RI can be displaced by the nascent strand, a result consistent with the observations of Lodish & Zinder (1966). However, in these experiments, as with the pulse label, no RI fraction has been isolated in which the sensitivity of the parental strand to RNase is greater than 50%. Thus this experiment is also consistent with the hypothesis that the replication of the parental RI can be semi-conservative or conservative with approximately equal probability.

(d) *Density measurements on RNase-treated [$^{13}C^{15}N^{32}P$] replicative intermediate*

The experiments just described used RI labeled with ^{32}P in the viral strand, and conclusions were drawn concerning the structure of the RI based upon the fraction of the ^{32}P label which remains in the core after RNase digestion. As another approach, the viral strand of the RI can be labeled with both radioactive and dense isotopes (^{32}P, ^{13}C and ^{15}N). After the tail has been removed with RNase, the density of the

core will be independent of tail length if the nascent strand is displaced during replication; whereas, if the viral strand is displaced, the density of the core must decrease as more of the viral strand becomes accessible to RNase. (Since only RNase-resistant material is studied in such an experiment, the possible artifact of small pieces of contaminating single-stranded RNA is avoided.)

To prepare RI density – labeled in the viral strand, cells were grown in broth to a concentration of 2×10^8 per ml. and infected at a multiplicity of one with [$^{13}C^{15}N^{32}P$]MS2. After eight minutes, the RNA was extracted from the cells and sedimented in a sucrose density-gradient (two hours at 65,000 rev./min, 5°C) to obtain RI fractions with different lengths of tail. The nucleic acids in those fractions which sedimented at 14 to 16 s, 17 to 20 s and 21 to 28 s were precipitated with alcohol. After resuspending each fraction and digesting with RNase to remove the tails, the resultant RNase-resistant duplexes (all 14 to 15 s) were isolated by a second sucrose gradient sedimentation. These fractions have been designated for convenience "15", "20" and "25 s", respectively, to indicate the *S*-value of the initial RI. ^3H-labeled fully light RNA duplexes were added as density marker to these ^{32}P-labeled cores from each of the fractions, and the samples were then centrifuged to equilibrium in a cesium chloride density-gradient at 65°C.

The distribution of ^{32}P and ^3H radioactivity in the fractions from these gradients is shown in Fig. 6. The density differences between the ^{32}P-labeled material and the marker were found to be $0{\cdot}016 \pm 0{\cdot}001$, $0{\cdot}015 \pm 0{\cdot}001$ and $0{\cdot}014 \pm 0{\cdot}001$ g cm^{-3} for the 15, 20 and 25 s fractions, respectively. This may be compared to the density difference of $0{\cdot}031$ g cm^{-3} observed between fully heavy and fully light duplexes (Kelly & Sinsheimer, 1967*b*). There is a skewing of the ^{32}P-labeled peaks toward the lighter density, especially in the 25 s fraction.

To determine if this result simply reflected a poor fractionation of the RI by sucrose gradient sedimentation, the same nucleic acid preparation was chromatographed on a BNC column and RI molecules of different tail lengths isolated. The cores derived from these RI molecules gave, on equilibrium density-gradient sedimentation, a result identical to that in Fig. 6, i.e. the radioactivity peak of the cores of all RI fractions is at the same density, which corresponds to that of a duplex in which one strand is "heavy", the other "light". Since we have shown that BNC columns fractionate RI according to tail length (Kelly & Sinsheimer, 1967*b*), we can conclude that the density of the major labeled component of the core is independent of the length of the tail.

A possible objection to this conclusion derives from the observation that scissions occur in single-stranded RNA centrifuged in a cesium chloride density-gradient for 48 hours at 65°C (Kelly & Sinsheimer, 1967*a*). If, during centrifugation, a break occurred in a hybrid-density duplex in the vicinity of the replication point, two double-stranded fragments would be produced, one fully light (unlabeled) and one half-heavy (^{32}P-labeled). Thus a duplex of constant density would be observed even if replication was semi-conservative. However, since it has been determined that 48 hours at 65°C in a cesium chloride density-gradient does not appreciably alter the sedimentation coefficient of core material, it is reasonable to conclude that such cleavage does not, in fact, occur.

The results of the experiment recorded in Fig. 6 are consistent with the observations described earlier (Kelly & Sinsheimer, 1967*a*) on unfractionated, RNase-treated RI, in which no density difference was observed between duplexes in which the heavy

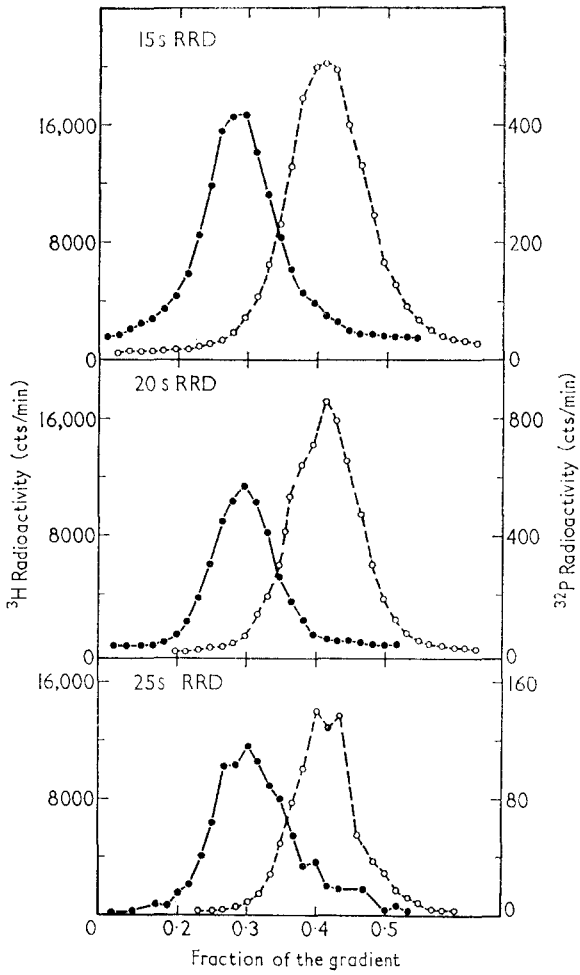

FIG. 6. Density of core material from RI labeled in the viral strand with ^{32}P, ^{13}C and ^{15}N. Cells were infected with [^{13}C^{15}N^{32}P]MS2 phage, and, after 8 min, chilled and the nucleic acids extracted. The RI was fractionated on a sucrose density-gradient into 3 fractions of approximately 15, 20 and 25 s. These were digested with RNase (0·1 μg/mc, 10 min at 37°C) and centrifuged on a sucrose gradient to obtain the 14 s core material. The latter was mixed with ^{3}H-labeled, fully light double-stranded RNA and then sedimented to equilibrium in a CsCl density-gradient (40 hr at 37,000 rev./min, 65°C). Fractions from these gradients were again digested with RNase (25 μg/ml., 30 min at 37°C) and trichloroacetic acid-precipitable radioactive material determined: —●—●—, ^{32}P cts/min; --○--○--, ^{3}H cts/min; RRD, ribonuclease-resistant duplex.

isotopes were in either the viral or the non-viral part of the RI. However, the present experiment could not be explained by the possible presence of a large pool of completed duplexes. Therefore it is again concluded that a fraction of parental RI molecules is involved in conservative replication. It should be emphasized, however, that these experiments are weighted in favor of finding conservative RI, since only the dense portion of the core is labeled with ^{32}P. Consequently, a duplex of lesser density than a half-heavy duplex will also have less radioactivity. If 50% of the RI molecules containing parental RNA were replicating semi-conservatively, only 33% of the total ^{32}P-labeled core material would be in duplexes of lesser density and would only be

detectable as a skewing of the ^{32}P peak toward the fully light density. While there is evidence for such skewing, the accuracy of the data is not sufficient to estimate to what fraction of semi-conservative RI molecules this asymmetry might correspond.

4. Discussion

Before discussion of the interpretation of these experiments, the assumptions on which they are based must be clarified. It has been assumed that the complex RNA structure under examination is, in fact, a replicative intermediate. This is justified by the experiments, which indicate that the RI is a precursor to single-stranded RNA *in vivo* (Fenwick *et al.*, 1964) and *in vitro* (Weissmann & Feix, 1966; Mills *et al.*, 1966); that the duplex is not conserved during replication (Kelly & Sinsheimer, 1967*a*); and that the RI molecule contains an infective viral strand (Ammann *et al.*, 1964). Implicit also in these experiments is the assumption of a model for the RI consisting of a double-stranded core to which only one single-stranded tail is attached (Kelly & Sinsheimer, 1967*b*). Although this model is supported by the observation that, on denaturing the RI, the nascent strand sediments as a single peak (Fig. 3(b)), evidence has been presented that a small number of RI molecules have indeed more than one tail (Granboulan & Franklin, 1966). The presence of a small number of molecules with more than one tail would complicate the discussion of these experiments but would, however, leave the conclusions unaltered.

Essential also to the experiments described here is the use of RNase digestion as a tool to distinguish single-stranded RNA from double-stranded RNA. If, in fact, the specificity of RNase action is more subtle than this, interpretation of these data is impossible. Finally, it has been supposed that it is a valid experimental approach to the study of the mechanism of replication to investigate the structure of the replicative intermediate. While this is not an unreasonable assumption, it ignores changes in structure which might take place upon purification.

When short pulses of radioactivity were used to label the nascent strand, and the RI was fractionated on a BNC column, it was apparent that the nascent strand had been displaced from the duplex (and thus become RNase-sensitive), but only in a fraction of the molecules. The converse experiment, in which the viral strand was labeled, gave the same result; this leads to the conclusion that either the viral *or* the nascent strand can be displaced during replication. When the viral strand was labeled with dense and radioactive isotopes, the density of the core material after the tail had been removed was in qualitative agreement with a model in which the nascent strand can be displaced from the duplex in a large fraction of the RI molecules. Because of the nature of the experiment, the presence of "conservative" RI molecules would effectively conceal the presence of "semi-conservative" RI molecules if both were present in approximately equal amounts. The results of these experiments, then, fit a model in which either the viral or the nascent strand is displaced with approximately equal frequency.

In these experiments, it has been assumed that a small amount of radioactivity is incorporated into complementary strands, during a pulse-label, relative to that incorporated into viral strands. In experiments similar to the above, Billeter, Libonati, Vinuela & Weissmann (1966) have shown by use of the specific dilution assay that under pulse-labeling conditions, 50% of the radioactivity of the plus type is sensitive to RNase. They also found that 10% of the radioactivity in RI was in minus or

complementary strands, and that 40% of this was sensitive to RNase. This may imply that transcription is not absolutely asymmetric.

If pulse-labeled RI is sedimented under partially denaturing conditions, the radioactivity peak with the sedimentation rate of RI is fully resistant to RNase, and the RNase-sensitive portion of the label now sediments as a single-strand RNA fragment. The best interpretation of this observation is that two classes of RI are fractionated by this procedure: the semi-conservative RI molecules, with a displaced viral strand, are not denatured; the conservative RI molecules, with a displaced nascent strand, partially denature, releasing the nascent strand from the duplex. If only a small number of hydrogen bonds holds a nascent strand to the duplex in a conservative RI (Fig. 1), the basis for this fractionation is easily explained.

From the areas of the peaks in Figs 3(a) and 4, it is obvious that both classes of RI are present in approximately equal amounts, except in fractions with short tails. In these, the fast (semi-conservative) component contains the majority of the pulse label. When such a fraction is sedimented under fully denaturing conditions, a peak corresponding to viral RNA is observed as well as a slower peak corresponding to the nascent strand. The preponderance of the fast component in these fractions therefore probably reflects the presence of duplexes which have completed a transcription and initiated another during the labeling period.

It is interesting to note that a purely conservative mechanism requires that the radioactivity is released from the duplex when transcription is complete, and so labeled complete duplexes would not appear (except, of course, through replication of the duplex). However, using a uniquely semi-conservative mode of replication, it would be impossible to explain the appearance of labeled single-strands of MS2 RNA (Fig. 2) after labeling times shorter than the transcription time.

The above analysis of the structure of the RI clearly leads to the conclusion that the duplex is transcribed both conservatively and semi-conservatively with approximately equal frequency. Since no duplexes are conserved during replication (Lodish & Zinder, 1966; Kelly & Sinsheimer, 1967*a*), it cannot be argued that one class of molecule always replicates conservatively, and the other semi-conservatively; all molecules must use both mechanisms. This could occur either if there is some mechanism for alternating between the two modes of replication, or simply if the two modes are used randomly with equal probability. It might be possible to distinguish between the two possibilities on kinetic evidence, but the data of Lodish & Zinder (1966) and Kelly & Sinsheimer (1967*a*) are not of sufficient accuracy.

Finally, there is some evidence *in vitro* that removing the MS2 synthetase enzyme from the RI makes the nascent strand less vulnerable to RNase digestion (Borst & Weissmann, 1965). It is therefore possible that the structure of the purified RI molecules is only indirectly related to the structure *in vivo*. This question can only be settled through examination of the structure of the replicative intermediate with the enzyme still attached.

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