Sindbis Virus RNA Replication. II. Strand Composition and Metabolic Fate of the Multi-stranded RNA Species

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SUMMARY

Double-stranded RNA from SB virus-infected cells was denatured and analysed on agarose-methylmercuric hydroxide gels. Equimolar amounts of three single-stranded species with mol. wt. of $4 \times 10^6$, $2.5 \times 10^6$ and $1.8 \times 10^6$ were found. Pulse and chase experiments in infected cells established a precursor-product relationship between the multi-stranded and single-stranded virus RNA species. The present results support the model in which the 49S and 26S species of virus RNA are synthesized in infected cells from two distinct replicating structures.

INTRODUCTION

The replication of Sindbis virus (SBV) RNA involves intermediates containing both positive and negative strands. These structures, termed replicative intermediates (RI), contain single-stranded (ss) non-hydrogen-bonded regions associated with a double-stranded (ds) core composed of complementary strands (Friedman, 1968; Simmons & Strauss, 1972; Segal & Sreevalsan, 1974). Digestion of RI with RNase yields the more extensively hydrogen-bonded cores which are referred to as replicative forms (RF; Simmons & Strauss, 1972; Eaton & Faulkner, 1973; Segal & Sreevalsan, 1974). Additionally, infected cells contain completely ds molecules, also termed RF (Levin & Friedman, 1971).

The role of the RI and RF in the replication scheme of togaviruses is not clearly understood. Simmons & Strauss (1972) reported that RI is the first species to be labelled during brief exposure of infected cells to radioactive nucleoside precursors. Additionally, they observed that the rate of ssRNA synthesis is reflected in the rate at which label is incorporated into the multi-stranded structures. These findings imply that RI serves as the nascent structure involved in virus RNA synthesis.

Treatment of the multi-stranded RNA species with RNase generates three species of RNase-resistant cores with mol. wt. of $8.5 \times 10^6$, $5.6 \times 10^6$ and $3.1 \times 10^6$, respectively (Simmons & Strauss, 1972; Martin & Burke, 1974). Based on the kinetics of labelling of these structures at the peak of RNA synthesis in infected cells, Simmons & Strauss (1972) postulated the existence of two different types of RI, both containing a complementary (negative) 49S RNA. They observed that the molar ratios of these structures varied depending on the length of the pulse with radioactive precursors. According to their model, the RI serving as a precursor to the $4 \times 10^6$ mol. wt. ss species (virion RNA) when treated with RNase generates RFI (mol. wt. $8.5 \times 10^6$). The other species of RI, the

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precursor to the 26S species (mol. wt. \(1.8 \times 10^6\)), has a gap in a specific region of the positive strand. The ss region in the negative strand across from this gap is susceptible to RNase and, consequently, two species of dsRNA, RFII and RFIII are produced after digestion with the enzyme. Both RFIII and RFII are thought to consist of duplexes of complementary ssRNAs with mol. wt. of \(1.8 \times 10^6\) and \(2.8 \times 10^6\), respectively. Although the 26S species of ssRNA found in infected cells corresponds to RFIII, comparable ssRNA for RFII has not been detected (Frey & Strauss, 1978). The objective of our work was to determine the role of the multi-stranded RNAs in the synthesis of the ss virus RNAs. We report on the metabolic instability of these structures during a pulse and chase experiment. Furthermore, we examined the strand composition of the dsRNA obtained from infected cells. Our results are consistent with the notion that two distinct replicating structures are involved in the synthesis of the 49S and 26S species of SBV RNA.

**METHODS**

**Materials.** Actinomycin D (Merck, Sharp and Dohme, West Point, Penn., U.S.A.) was used at a concentration of \(1.0 \mu\text{g/ml}\) to inhibit the synthesis of cellular RNA. Sodium dodecyl sulphate (SDS) was purchased from B.D.H., Poole, Dorset, U.K. \(^3\text{H-uridine}\) (sp. act. > 50 mCi/mmol), \(^{14}\text{C-uridine}\) (sp. act. > 50 mCi/mmol) and Biofluor were obtained from New England Nuclear Corp., Boston, Mass., U.S.A. Glucosamine hydrochloride, cytidine and ribonuclease A (RNase) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Uridine and pancreatic deoxyribonuclease I (DNase, RNase-free) were obtained from Worthington Biochemicals, Freehold, N.J., U.S.A. Methylmercuric hydroxide was purchased from Alfa Products, Inc., Danvers, Mass., U.S.A., and Dimethylaminopropionitrile (DMAPN) from Eastman Kodak Co., Rochester, N.Y., U.S.A. Tris-EDTA-saline (TES), consisting of \(1 \text{mM-tris (hydroxymethyl) aminomethane (pH 7.4)}\), \(1 \text{mM-EDTA}\) and \(100 \text{mM-NaCl}\), was used for resuspending nucleic acids. Saline-tris-EDTA (STE) consisted of \(100 \text{mM-NaCl}, 1 \text{mM-EDTA}\) and \(20 \text{mM-tris (pH 7.4)}\) and was used for CF-11 cellulose chromatography.

**Virus and cells.** The HR strain of Sindbis virus was used. Primary cultures of chick embryo (CE) cells were prepared and grown in Eagle’s minimal essential medium (MEM; Sreevalsan & Lockart, 1966). The method used for preparation of virus stocks free of defective particles was identical to that described by us recently (Czarniecki & Sreevalsan, 1979). Virus from a single plaque (obtained after three successive cycles of cloning) was used to infect CE cell monolayers at an m.o.i. of \(100/1 \times 10^6\) cells. After 15 h of incubation the cultures were harvested and the resulting suspension was used as seed virus. The details of the methods for the preparation of stock virus, infection and labelling of cells were identical to those reported recently (Czarniecki & Sreevalsan, 1979).

**Pulse and chase of \(^3\text{H-uridine in infected cells.** The procedure used was a modification of those reported by Wertz (1975), Scholtissek et al. (1972) and Murphy & Attardi (1973). Monolayers of chicken CE cells pre-labelled with \(^{14}\text{C-uridine (0.1 \mu Ci/culture)}\) for 24 h were infected with SBV stock. After the 30 min adsorption period, the cultures were incubated at 37 °C with medium containing actinomycin D (1 \(\mu\text{g/ml})\). At 2 h p.i. growth medium was replaced with that containing glucosamine (20 mm) and incubation continued at 37 °C. At 4 h p.i., the cells were scraped from the plates, centrifuged at 500 \(g\) for 10 min at 25 °C and resuspended in medium containing glucosamine (20 mm) but lacking calcium and magnesium. The cell suspension (\(5 \times 10^6\) cells/ml) was incubated at 37 °C with gentle stirring. \(^3\text{H-uridine (500 \mu Ci/ml)}\) was added; 1 min later the suspension was poured into ice-cold PBS and this mixture was, in turn, poured into ‘chase medium’ (containing 20 mm-unlabelled glucosamine, 5 mm-cytidine and 100 \(\mu\text{g/ml uridine})\). The suspension was
centrifuged at 500g for 10 min at 4 °C. The resulting pellet was washed with the 'chase medium' twice. The cells were maintained at 4 °C throughout the above steps to ensure no further incorporation of ³H-U-uridine. The cell pellet was resuspended in 'chase medium' and re-incubated at 37 °C. A pulse sample was withdrawn from the suspension at this time. Chase samples were collected at the end of 30 min incubation at 37 °C.

Isolation of RNA from infected cells and differential precipitation of RNAs. The procedure used was previously described (Czarniecki & Sreevalsan, 1979). The extraction and analysis of RNA samples from the pulse and chase experiments employed a slightly modified technique. The pellet obtained from infected cells was de-proteinized with phenol as described previously (Czarniecki & Sreevalsan, 1979). The aqueous layer obtained was extracted with ethyl ether three times. The sample was adjusted to contain 35% ethanol and then fractionated using CF-11 cellulose columns at 25 °C (Segal & Sreevalsan, 1974) to separate the single-stranded and multi-stranded forms of SBV RNA. Samples of radioactivity eluting in STE buffer containing 15% ethanol (representing ssRNA) or STE buffer (representing multi-stranded species) were used directly for electrophoretic analysis.

RNase digestion of virus RNAs. RNase (1.0 µg/ml) was added to the samples and incubated for 30 min at 37 °C. The final concentration of NaCl in the incubation mixture was 0.25 M. Less than 2% of the ssRNAs remained acid precipitable after enzymic digestion under these conditions.

Purification of salt-soluble RNA for denaturation. Virus RNA was isolated from cells infected with SBV and incubated in the presence of actinomycin D (1 µg/ml) and ³H-U-uridine (25 µCi/plate) for 6 h. The phenol-extracted RNA was salt-precipitated. The salt-soluble fraction was concentrated by precipitation with 2 vol. of alcohol and subjected to two cycles of chromatographic separation on CF-1 cellulose (Segal & Sreevalsan, 1974). The radioactivity eluting in STE buffer representing the replicative form (RF) was recovered, precipitated with alcohol and subjected to centrifugation on sucrose density gradients as described (Segal & Sreevalsan, 1974). The profile of radioactivity obtained after the above step indicated a fairly homogeneous peak with a sedimentation rate of about 23S. Samples of fractions representing the peak of radioactivity were used for denaturation by methylmercuric hydroxide.

Electrophoretic analysis of ssRNA. Polyacrylamide gels containing 2·5% (w/w) acrylamide, 0·13% (w/w) bisacrylamide and 0·5% (w/v) agarose were prepared according to a modification of the method reported by Schincariol & Howatson (1972). Conditions have been described previously (Czarniecki & Sreevalsan, 1979).

Electrophoretic analysis of dsRNA under non-denaturing conditions. Polyacrylamide gels containing 1·9% (w/w) acrylamide, 0·1% (w/w) bisacrylamide and 0·005% (w/w) agarose were prepared as described by Peacock & Dingman (1968). RNA samples (10 to 15 µg/100 µl or less) containing sucrose, at a final concentration of 6 to 10% and bromophenol blue were applied to the gels and electrophoresis was carried out at 100 V for 6 h at room temperature.

Electrophoresis of virus RNA under denaturing conditions. Agarose gels (0·9%) containing methylmercuric hydroxide at a final concentration of 5 mM were prepared as described by Bailey & Davidson (1976). Samples were diluted 1:1 with electrophoresis buffer (0·05 M-boric acid, 5 mM-sodium borate, 0·01 M-sodium sulphate, 1 mM-EDTA) and 10 mM of methylmercuric hydroxide. The mixture was incubated at 37 °C for 15 min, mixed with sucrose (6 to 10% final concentration) and bromophenol blue and applied to the gel. Electrophoresis was carried out at 100 V for 2·5 h at room temperature.

All gels were fractionated and assayed for radioactivity as described (Czarniecki & Sreevalsan, 1979).
Fig. 1. Sedimentation profile of salt-soluble SBV RNA on a sucrose density gradient. Infected cells were pulse labelled with $^3$H-uridine for 1 min at 4 h p.i. The RNA was isolated and fractionated using 2 M-LiCl and sedimented through a 15 to 30% sucrose density gradient as described in Methods. The arrows indicate the positions at which CE cell ribosomal RNAs sediment in an identical gradient.

Fig. 2. Acrylamide gel electrophoresis of salt-soluble RNA fraction. Infected cell cultures were pulse-labelled with $^3$H-uridine for 1 min. The virus-specific RNA was isolated and subjected to salt-precipitation. The salt-soluble RNA was recovered and samples were analysed on polyacrylamide gels, with or without RNase digestion: (a) salt soluble RNA; (b) salt soluble RNA after RNase digestion. Migration in these and all subsequent electrophoretograms is from left to right.

RESULTS

Strand composition of RF

Based on resistance to RNase and solubility in high salt, an RF with a sedimentation rate of 23S can be isolated from cells infected with SB or Semliki Forest (SF) virus (Simmons & Strauss, 1972; Martin & Burke, 1974; Bruton & Kennedy, 1975). This RF which migrates on acrylamide gels as a homogeneous species is converted by RNase treatment into three electrophoretically distinct species (RFI, RFII and RFIII) with apparent mol. wt. of $8 \times 10^6$, $5.6 \times 10^6$ and $3.1 \times 10^6$ (Martin & Burke, 1974). One of our aims was to determine the strand composition of dsRNA appearing in cells infected with non-defective SBV. Since cells infected with DI (defective interfering) forms of SB or SF viruses contain additional RFs corresponding to the size of the DI RNAs involved
Sindbis virus multi-stranded RNAs

Fig. 3. Electrophoretic analysis of the salt-soluble RNA species under denaturing or non-denaturing conditions. Virus-specific RNA was obtained from cells that had been labelled with \(^{3}H\)-uridine from 3 to 6 h p.i. After salt-fractionation, the salt-soluble RNA was purified as described in Methods; samples were mixed with \(^{14}C\)-uridine labelled 49S SB virion RNA and subjected to electrophoresis on agarose gels (a) without or (b) with methylmercuric hydroxide. ——, \(^{3}H\)-uridine labelled salt-soluble RNA; ○—○, \(^{14}C\)-uridine labelled 49S virion RNA. The apparent mol. wt. of the virus RNA species were estimated from the relative mobilities of 49S virion RNA and 18S and 28S ribosomal RNAs under denaturing conditions (▲—▲).

(Kennedy et al. 1976), virus stocks were prepared and used under conditions favouring minimal production of defective particles (see Methods).

Total RNA isolated from infected cells was fractionated by lithium chloride (Erikson & Franklin, 1966). The sucrose density sedimentation pattern and the electrophoretic mobility on polyacrylamide gels of the salt-soluble fraction representing dsRNA is shown in Fig. 1 and 2(a), respectively. In both instances only one species of RNA was detected. The absence of RFs smaller than this species argues for the presence of only non-defective virions in the stocks used. However, treatment with RNase, although resulting in no loss of radioactivity, yielded three distinct species corresponding to RFI, RFII and RFIII (Fig. 2b). These results are in agreement with those reported earlier (Martin & Burke, 1974; Segal & Sreevalsan, 1974).

The salt-soluble RNA was further purified by CF-11 cellulose chromatography (see Methods) to remove any contaminating single- or multi-stranded RNAs. The resultant preparation contained less than 0.001% RNase-sensitive material. The RNA samples were further purified by sucrose density gradient centrifugation and the peak fraction was then denatured by methylmercuric hydroxide (Bailey & Davidson, 1976) and analysed on agarose gels. The undenatured RNA migrated slower than the 49S ssRNA (Fig. 3a), a result consistent with the former species being double-stranded (Bailey & Davidson, 1976). Unlike the homogeneous peak observed in Fig. 3(a) the \(^{3}H\)-uridine specific radioactivity
Table 1. Distribution of radioactivity appearing in ribonuclease-resistant RNA species during various pulse periods

<table>
<thead>
<tr>
<th>Pulse period (min)</th>
<th>Molar proportion</th>
<th>Ratio</th>
<th>RFIII/RFII</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>2.9</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>1.1</td>
<td>2.8</td>
</tr>
<tr>
<td>30</td>
<td>1</td>
<td>1.3</td>
<td>2.4</td>
</tr>
<tr>
<td>180</td>
<td>1</td>
<td>2.3</td>
<td>2.4</td>
</tr>
</tbody>
</table>

The radioactivity found in the various species after electrophoresis was ascertained. The molar ratio was calculated by assuming mol. wt. of RF-I, RF-II, RF-III to be 8.0 x 10^6, 5.6 x 10^6 and 3.1 x 10^6 respectively (Simmons & Strauss, 1972: Martin & Burke, 1974).

The radioactivity associated with the dsRNA in the presence of methylmercuric hydroxide was distributed in three species with mol. wt. of 4.0 x 10^6, 2.5 x 10^6 and 1.8 x 10^6, respectively (Fig. 3b). Additionally, the distribution of radioactivity among the three species was equimolar. Approximately 95% of the input radioactivity was accounted for after denaturation, suggesting little or no incident degradation. Incubation of the dsRNA with methylmercuric hydroxide rendered them completely ribonuclease-sensitive indicating denaturation (results not presented). The salt-soluble RNA contained no structures corresponding to RF-II or RF-III (Fig. 1 and 2a). However, denaturation of the salt soluble RNA yielded not only the 4.0 x 10^6 mol. wt. species but also small species with mol. wt. of 2.5 x 10^6 and 1.8 x 10^6. These results best fit the model proposed by Simmons & Strauss (1972), i.e. that two different types of replicating structures exist, one consisting of a duplex of intact 49S RNAs; the other containing an intact 49S negative strand hydrogen-bonded to positive strands with mol. wt. of 2.5 x 10^6 and 1.8 x 10^6.

Kinetics of appearance of the three replicative forms

Based primarily on the kinetics of labelling of the three RFs at the peak of RNA synthesis, Simmons & Strauss (1972) observed that the molar ratios of these structures varied, depending on the length of the pulse with radioactive precursors. In contrast, Martin & Burke (1974) observed no change in the molar ratios using short or long-term pulses in SFV-infected cells. As pointed out by the latter authors the presence of defective particles in the virus stocks used may contribute to the apparently differing results. Therefore we re-examined the above issue using virus stocks prepared under conditions which favoured minimal generation of defective particles. Infected cells were exposed to 3H-uridine at 4 h p.i. for various lengths of time. The total RNA obtained from these samples was incubated with RNase and subjected to polyacrylamide gel electrophoresis to separate the different RFs. The radioactivity present in the fractions corresponding to each RF was computed and their molar ratios were obtained for each pulse period (Table 1). When cells were pulsed for 1 min, the ratio was 1:0.5:2.9, indicating radioactivity to be maximal in RF-III and minimal in RF-II. The proportion of RF-II:III approached unity only after 3 h of labelling time. These results are in agreement with those of Simmons & Strauss (1972) and reiterate their conclusion that RNA synthesis is occurring more rapidly in RF-III than in RF-II. Regardless of the time p.i. at which the pulse was carried out, the molar ratios remained constant (results not presented).

Fate of the multi-stranded RNA structures during a pulse and chase

We examined the precursor-product relationship between the multi-stranded structures and ss virus RNAs by a pulse and chase experiment. Newly synthesized virus RNAs in
infected cells are located in RIs and after RNase digestion, much of this material remains associated with the ds cores or RFs (Simmons & Strauss, 1972). Therefore, the metabolic stability of the RFs during a pulse and chase should reflect that of the multi-stranded structures (RI).

Preliminary experiments indicated that when infected cells were pulse-labelled with 3H-uridine for 1 min (at 4 h p.i.), only 10% of the radioactivity was salt-soluble. Similar results have been reported by Martin & Burke (1974) using SFV-infected cells. The salt-precipitable radioactivity was comprised of 60 to 70% RI-type molecules and the remainder represented single strands. However, the majority of the RI-type molecules remain at the origin of the gel during electrophoretic analysis. Multi-stranded RNA structures have been shown to be unstable during isolation procedures or storage (Bishop et al. 1969) and possessed a pronounced tendency to aggregate (Thach et al. 1974). This may, in part, explain the inability of these structures to enter polyacrylamide gels. Therefore, the amount of radioactivity associated with the various species of virus RNA during a pulse and chase experiment could not be quantified. As an alternative to salt-precipitation, multi-stranded structures present in the total RNA were separated by chromatography on CF-11 cellulose. When the material present in the STE buffer fraction was analysed by electrophoresis, 98% of the input radioactivity could be accounted for in the gel fractions.

We therefore employed the above method for the analysis of the various species of virus RNAs during a pulse and chase (see Methods). CE cells were exposed to 14C-uridine for 12 h, washed several times to remove unincorporated isotope and then re-incubated with medium for another 24 h before treatment with actinomycin D and infection with SBV.
Fig. 5. Single-stranded RNA species present during a pulse and chase. Samples representing the 15% ethanol-buffer fraction from the pulse and chase experiment of Fig. 4 were analysed by polyacrylamide gel electrophoresis: (a) pulse sample; (b) 30 min chase sample. •—•, ^3^H-uridine labelled virus-specific RNA; ○—○, ^14^C-uridine labelled 28S ribosomal RNA.

The cells were pre-labelled to correct for any variation in loss of cells or recovery of RNA from sample to sample. At 4.5 h p.i. the cells were pulse-labelled with ^3^H-uridine for 2 min and chased for 30 min (as described in Methods). The total nucleic acid extracted from the pulse and chase samples was subjected to chromatography on CF-11 cellulose. Samples of the STE buffer fraction representing multi-stranded RNA were subjected to electrophoresis before and after RNase digestion (Fig. 4).

Multi-stranded structures are eluted only in the STE buffer fraction during CF-11 cellulose chromatography (Franklin, 1966; Segal & Sreevalsan, 1974). Additionally, as mentioned in the previous section during a short pulse, only 10% of the total radioactivity represents completely dsRNA, as determined by salt-solubility. Therefore, the predominant peak of radioactivity in Fig. 4(a, b) probably represents the multi-stranded structures. During the chase period, there was a decrease in radioactivity in this species. Also, it should be noted that approx. 50% of the radioactivity appearing in the STE buffer fraction was associated with ssRNAs, a finding consistent with the observation of Engelhardt (1972) concerning the unusual behaviour of SBV RNAs on CF-11 cellulose columns due to secondary structure. The electrophoretic profiles of the RNase-resistant material present in the STE buffer fraction are shown in Fig. 4(c, d). Radioactivity present in all of the three RFs decreased during the chase period. The significance of an additional peak in fraction 60 (Fig. 4d) during the chase period is not understood at present.

The profiles in Fig. 5 show the distribution of radioactivity present in the ssRNA species. All four species of ssRNAs (49S, 38S, 33S and 26S) were present in both the pulse and chase samples and, as expected, the radioactivity corresponding to these species increased during the chase period, with the major increase occurring in the 49S species.
Table 2. Flow of radioactivity during a pulse and chase

<table>
<thead>
<tr>
<th></th>
<th>Amount of radioactivity* (ct/min x 10^-4)</th>
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<tbody>
<tr>
<td></td>
<td>RFI</td>
</tr>
<tr>
<td>Pulse (1 min)</td>
<td>6.0</td>
</tr>
<tr>
<td>Chase (30 min)</td>
<td>2.2</td>
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</table>

* Radioactivity found in the various RNA species, subsequent to electrophoretic analysis was calculated and normalized for differences in counting efficiency as well as for the volume used in the electrophoretic analyses.

Table 2 summarizes the data on the amount of radioactivity associated with the various species of virus RNAs during the above pulse and chase experiment as computed from the electrophoretic profiles (Fig. 4 and 5). Since the 38S and 33S species represent conformational forms of the 49S and 26S species (Kennedy, 1976; Czarniecki & Sreevalsan, 1979), they have not been treated as separate entities in the above calculations. Additionally, the amount of radioactivity representing ssRNA in Table 2 corresponds to the sum of that eluting in the STE buffer fraction and the 15% ethanol-buffer fraction (Fig. 4 and 5). In general, the amount of radioactivity in the structures generating all three RFs decreased while that found in the ss species increased. It is interesting to note that the amount of radioactivity disappearing from RFI (3.8 x 10^4 ct/min) is equivalent to the increase appearing as the 49S species during the chase (3.2 x 10^4 ct/min). Similarly, the radioactivity lost from RFIII (4.4 x 10^4 ct/min) approximates the net increase in the 26S species (4.1 x 10^4 ct/min). There is no analogous increase in a ss species to correlate with the loss in radioactivity of RFII.

**DISCUSSION**

Three species of RFs were isolated from cells infected with SB or SF virus (Simmons & Strauss, 1972; Martin & Burke, 1974; Segal & Sreevalsan, 1974). It has also been reported that RFII and RFIII could originate from the replication of DI particles (Martin & Burke, 1974). When the salt-soluble RNA from infected cells (representing RF), was analysed (Fig. 1 and 2), only one species was detected. RFII and RFIII were observed only after treatment of the salt-soluble RNA with RNase. Therefore, it is unlikely that the RFII and RFIII described in the present study originated as a consequence of the replication of DI RNAs.

Using competition hybridization, Bruton & Kennedy (1975) reported that RFI consists of a duplex of two 49S molecules, one of positive and one of negative polarity. Additionally, they reported the rate of negative strand synthesis reached a maximum at 2.5 h p.i. and then rapidly decreased. Due to poor labelling of RFII and RFIII, they were unable to determine the strand composition of those molecules. In the present study, we analysed the denaturation products of Sindbis RFs and ascertained their mol. wt. (Fig. 3b). The RF preparation used in this experiment was obtained from cells labelled at 4.5 h p.i., a time at which synthesis of negative strands is minimal as shown by Bruton & Kennedy (1975). Although we did not directly determine the polarity of the denaturation products, we infer that the majority of the radioactivity in Fig. 3(b) is associated with positive strands. The three species of ssRNA (mol. wt. 4.0 x 10^6, 2.5 x 10^6 and 1.8 x 10^6) obtained after denaturation were found in equimolar proportions. Additionally, the sum of the mol. wt. of the two smaller species (4.3 x 10^6) agrees with that of the intact 49S RNA. These results lend strong support to the notion that RFII and RFIII originate from a common structure, as originally proposed by Simmons & Strauss (1972).
Using short pulse labelling conditions, the rate at which radioactivity is incorporated into the multi-stranded RNAs should be a direct measure of the rate of synthesis of the single strands generated from them. Results shown in Table I indicated that the majority of the radioactivity was found in RFIII while small amounts were associated with RFII, suggesting that proportionately more ss molecules are synthesized from RFIII templates than RFII templates. It also appears that the rate of ssRNA synthesis from RFIII structures occurs two to three times faster than that from the corresponding RFI-type molecules.

The results of the pulse and chase experiment also support the model of two distinct replicating structures. The data provide additional evidence that the replicative intermediate corresponding to RFI serves as the precursor for the 49S species (mol. wt. $4.0 \times 10^6$) while that corresponding to RFII and RFIII generates the 26S species (mol. wt. $1.8 \times 10^6$). The results showing the loss of radioactivity associated with RFII during the chase period suggest the generation of a ss species with a mol. wt. of $2.5 \times 10^6$. The results from these experiments as well as those reported by Simmons and Strauss (1974) indicate that no free ss species with this mol. wt. is found in infected cells. A possible explanation is that this RNA species is degraded soon after its release from the replicative intermediate. The reported absence of poly(A) tracts in RFII molecules (Frey & Strauss, 1978) supports this proposed instability of the corresponding ssRNA.

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