A Kinetic Analysis of the Synthesis in BHK 21 Cells of RNAs Specific for Semliki Forest Virus

By L. KAARIAIENEN* AND P. J. GOMATOS
The Sloan-Kettering Institute, New York, New York, U.S.A.

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SUMMARY
A kinetic analysis of formation of Semliki Forest virus-specific RNAs revealed that 22 S RNA was synthesized first and accumulated within or on a structure not disrupted by homogenization, but disrupted by detergent treatment, presumably a lipid-containing membrane or vesicle. When sampled at the end of the latent period of virus growth the 22 S RNA consisted of a mixture of two RNAs, double- and single-stranded. The amount of double-stranded 22 S RNA remained constant thereafter with no complementary RNA synthesized after the latent period of virus growth.

Appearing during the latter portion of the latent period were 26 S RNAs and 42 S virus RNA. The rate at which 26 S RNA accumulated remained constant throughout infection thereafter, whereas that of 42 S virus RNA was maximal at the time of maximal virus accumulation.

INTRODUCTION
Semliki Forest virus, an arbovirus, contains RNA and has a lipid coat. The replication of this virus and other members of group A of arboviruses has been well studied in a primary cell line (Friedman, 1968; Friedman & Berezovsky, 1967; Friedman, Levy & Carter, 1966; Sonnabend, Martin & Mecs, 1967; Sreevalsan & Lockart, 1966; Burge & Pfefferkorn, 1967; Skehel & Burke, 1968; Ben-Ishai, Goldblum & Becker, 1968). At least three new species of RNA have been identified within the infected cell: 42 S RNA; 26 S RNA; and a heterogeneous RNA sedimenting at 16 S to 29 S and detectable within the RNA profile in sucrose gradients by resistance to RNase. The heterogeneous RNA, which possesses some properties of a replicative intermediate (Franklin, 1966), is the first virus-specific RNA synthesized and may be a precursor of the remaining virus-specific RNA species (Friedman, 1968). The 42 S RNA is infectious and is undoubtedly that which is in virus. The significance of the 26 S RNA in virus replication is unknown. RNA sedimenting at 26 S was produced by exposure of the 42 S RNA to high temperature or dialysis against water (Sreevalsan et al. 1968). That this RNA is, in fact, identical with the 26 S RNA found in the infected cell remains to be determined.

In the work we report here, the replicative intermediate was isolated free from the 42 S and 26 S RNAs and its kinetics of synthesis and structural properties were studied. RNAs were extracted from cells without phenol to avoid any change in their

* Present address: Department of Virology, University of Helsinki, Haartmaninkatu 3, Helsinki 25, Finland.
secondary structure (Geiduschek, Moohr & Weiss, 1962; Borst & Weissmann, 1965; Weissmann & Feix, 1966). Disruption of swollen cells by homogenization in hypotonic buffer, followed, after centrifugation, by a mild detergent treatment of the pelleted material resulted in a simple fractionation of the 42 S and 26 S RNAs from the RNAs in the replicative complex.

METHODS

Cells

BHK 21 cells, clone 13, a continuous cell line derived from baby hamster kidney (Stoker & Abel, 1962) were grown in Eagle’s minimal essential medium (MEM) (Eagle, 1959) supplemented with 5% foetal bovine serum. For virus production and preparation of cytoplasmic extracts, cells were grown at 37° as monolayers on the surface of 2 l. cylindrical bottles placed on rollers revolving at a rate of 30 rev./hr. Complete monolayers in plastic Petri dishes 60 × 15 mm. were used for plaque assay.

Virus

Semliki Forest virus, prototype strain, was obtained from Dr J. R. Henderson, Yale University School of Medicine. Virus stocks were prepared by infecting BHK 21 cells at a multiplicity of infection of 0.1 or 0.01. After a 1 hr adsorption period at 37°, 200 ml. of MEM supplemented with 0.2% bovine plasma albumin, fraction V, Armour (BSA), was added (virus growth medium). After 16 to 20 hr incubation at 37°, the medium containing released virus was centrifuged at 900 g and the pelleted material discarded. The resulting supernatant fluid was centrifuged at 78,000 g for 3 hr in a Spinco L 2 Ultracentrifuge. The pelleted material containing virus was resuspended in phosphate-buffered saline (PBS) (Dulbecco & Vogt, 1954), containing 0.5% BSA, and was stored at -90° (stock virus).

For purification of virus, either unlabelled or labelled with 32P, the culture fluid containing released virus was centrifuged at 10,000 g for 30 min. and the resulting supernatant fluid was centrifuged for 3 hr at 78,000 g; the pellet containing virus was resuspended in PBS, layered on a potassium tartrate gradient, 5% to 50% in PBS, and centrifuged at 4° for 2 hr at 24,000 rev./min. in the SW 25.1 rotor. Material identified as virus by haemagglutinating activity, at a density of 1.19 g./ml., was diluted with RSB-PVS (0.01 M-tris, pH 7.4, 0.01 M-KCl, and 1.5 mM-MgCl2 containing 20 μg./ml. of potassium polyvinylsulphate), and collected as a pellet after centrifugation at 4° for 90 min. at 40,000 rev./min. in the SW 50 rotor. The supernatant fluid was removed and the pellet containing virus was resuspended in RSB-PVS containing sodium dodecyl sulphate in a final concentration of 2%. RNA was extracted at 20° or 60° with phenol (Gierer & Schramm, 1956).

Titration

Plaque assay. Complete monolayers of BHK 21 cells in Petri dishes were inoculated with 0.25 ml. of virus diluted in PBS + 0.5% BSA and incubated at 37° in 5% CO2 in humidified air. After 1 hr adsorption the virus inoculum was aspirated and to each plate was added 8 ml. of overlay medium consisting of equal volumes of 1.8% Noble agar and twice concentrated reinforced Eagle’s MEM (Bablanian, Eggars & Tamm, 1965) containing 10% foetal bovine serum. After incubation for 2 days, 4 ml. of a
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second overlay containing 0.005% neutral red was added to each culture. The plaques were counted the next day.

Haemagglutinin assay. For haemagglutination of goose erythrocytes by Semliki Forest virus, the best pH is 5.8. Titrations were performed as described by Clarke & Casals (1958).

Preparation of cytoplasmic extracts

BHK 21 cells in the logarithmic stage of growth were washed once with MEM and infected at a multiplicity of infection of 20 to 40 with virus in a final volume of 25 ml. of MEM containing 0.2% BSA and actinomycin D, 0.15 μg/ml. At the end of the 1 hr adsorption period the cells were washed twice with 100 ml. of MEM, and 25 to 100 ml. of virus growth medium containing actinomycin D, 0.15 μg/ml., was added. Virus was added to cells at zero time in all experiments.

At various times during the virus growth cycle, [3H]uridine or carrier-free [32P]orthophosphate in respective final concentrations of 0.25 to 4 μc/ml. and 50 to 120 μc/ml. was added for a chosen period at 37°C. After the period of labelling with radioactivity, the medium was removed, and the cells were washed at 0°C with 50 ml. of PBS and 50 ml. of RSB-PVS sequentially. The cells were scraped into a small volume of RSB-PVS, allowed to swell for 10 min. at 0°C, and ruptured with 20 to 30 strokes in a Dounce homogenizer of small clearance. After 8 min. centrifugation at 250 g, the supernatant fluid, cytoplasmic extract 1 (CE-1), was collected. The pelleted material was treated with 2 to 4 ml. of RSB-PVS containing 0.44% sodium deoxycholate and 0.87% Tween 40 (Penman, 1966) and the mixture centrifuged for 8 min. at 250 g. The supernatant fluid from this centrifugation was cytoplasmic extract 2 (CE-2).

Analytical procedures

Cytoplasmic extracts 1 and 2 were treated at room temperature with sodium dodecyl sulphate, BRIJ 58, and EDTA at final concentrations of 1.95%, 0.5%, and 0.5 mm respectively. The treated sample was layered on a linear 15% to 30% sucrose gradient in RSB and centrifuged at 20°C in an SW 25.2 rotor, 12 hr at 23,000 rev./min. for CE-1 or 20 hr at 24,000 rev./min. for CE-2. Fractions were collected from below and absorbency of each fraction determined in the Zeiss PMQ II spectrophotometer. Acid-insoluble material labelled with radioactivity was obtained by precipitating the fractions with 5% trichloracetic acid after the addition of 50 μg. of BSA as carrier and collecting the resulting precipitate on membrane filters, Millipore, type-HA, 0.45 μ pore diameter. The amount of radioactivity on the dried filters placed in a solution containing 4 g. of 2,5 diphenyloxazole (PPO) and 0.05 g. of 1,4-bis-2 (5-phenyloxazolyl) benzene (POPOP) per litre of toluene was determined in a Tri-Carb scintillation spectrometer.

Unless otherwise stated, samples to be treated with pancreatic ribonuclease (RNase) were incubated at 37°C for 30 min. in 1.7× RSB with 3 μg./ml., and acid-insoluble material labelled with radioactivity was determined as described above. Sedimentation coefficients were estimated by the method of Martin & Ames, (1961) using BHK 21 ribosomal RNAs, 29 S and 19 S, as reference.
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Analysis of virus-specific RNAs

To pooled fractions from sucrose gradients containing the virus-specific RNAs were added sodium acetate and EDTA in final concentrations of 0.3 M and 0.1 mM, respectively. Ethanol (2 vol.) was added and the suspensions kept at -20° for 10 to 12 hr. The RNAs were collected as pellets by centrifugation for 20 min. at 38,000 g, dissolved in 0.1 M-NaCl, 0.01 M-sodium phosphate, pH 6.7, or in 1/10×KKC (1×KKC, 0.15 M-potassium chloride, 0.015 M-potassium citrate).

Chromatography on B-D cellulose

Benzoylated diethylaminoethyl cellulose (B-D cellulose) was purchased from Schwarz Bioresearch. The 22 S RNA in CE-2 in 0.1 M-NaCl, 0.05 M-sodium phosphate, pH 6.7, was applied on a 72×0.9 cm. column and eluted under constant pressure with an exponential gradient of NaCl from 0.1 M to 2 M in 0.05 M sodium phosphate, pH 6.7. Fractions of 4.5 ml. were collected. The salt of every eighth fraction was determined by relating its conductivity to those of standard solutions. Acid-insoluble material labelled with radioactivity was determined as described above.

Determination of base compositions

The RNA was hydrolysed with 0.3 M-KOH for 18 hr at 37°, potassium ions were removed by chromatography on carboxymethyl cellulose paper and base compositions were determined as described previously (Gomatos, 1967).

RNA annealing

Annealing conditions were as described by Gomatos (1968) for method (A), or Baltimore (1966) for method (B).

Labelled precursors and detergents

[3H]Uridine, 18 c/m-mole, was obtained from the Nuclear Chicago Corporation. Carrier-free [32P]orthophosphate was purchased from New England Nuclear Corporation. Sodium dodecyl sulphate (Matheson, Coleman and Bell) was recrystallized according to Mandel (1964). Sodium deoxycholate, enzyme grade, was obtained from the Mann Research Laboratories; Tween 40, polyoxyethylene sorbitan monopalmitate, from the Amend Drug Co.; BRIJ 58, polyoxyethylene (20) cetyl ether from Atlas Chemical Industries, Inc.

RESULTS

Growth curve

Incomplete monolayers of BHK 21 cells in 60 mm. plastic Petri dishes were inoculated with Semliki Forest virus at a multiplicity of 20. After a 1 hr adsorption period at 37° the cells were washed 3 times with MEM, 5 ml. of growth medium containing actinomycin D, 1 μg./ml., was added, and incubation was continued at 37°. At intervals, the medium was collected and centrifuged at 800 g for 10 min., and the supernatant fluid assayed for virus.

The latent period during growth of Semliki Forest virus was 180 min. (Fig. 1). Virus increased exponentially from 210 to 240 min. and greatest yields were reached
between 360 and 420 min. The final yields of virus produced in BHK 21 cells varied from 100 to 1000 p.f.u. per cell. The presence of actinomycin D, 0.15 to 1 µg./ml., in virus medium did not affect the growth cycle or virus yields.

**RNA synthesis in BHK 21 cells infected with Semliki Forest virus**

Cells were exposed to [3H]uridine from 90 to 225 min. after virus inoculation. After disruption of the preswollen cells by homogenization the homogenate was centrifuged at low speed to yield cytoplasmic extract 1 (CE-1). To identify new RNAs within or on structures sedimenting with nuclei and cellular debris without disrupting nuclei, the pelleted material was treated with Tween 40 sodium deoxycholate as described in Methods. After centrifugation at low speed, the supernatant fluid, CE-2, was collected. Both CE-1 and CE-2, after treatment with sodium dodecyl sulphate, and BRIJ 58 were analysed by sucrose density gradient centrifugation.

There were present in CE-1 two major species of RNA labelled with radioactivity (Fig. 2). These RNAs sedimented at 42 S and 26 S. Treatment with RNase in the presence of 1.7 × RSB rendered acid-soluble all the radioactivity in both RNAs. A small amount of material sedimenting at approximately 22 S was partially RNase-resistant.

Material in CE-2 was not released by homogenization, but was released by detergent treatment. It was thus separated from the contents in CE-1 by a detergent-sensitive structure, most likely a lipid membrane. The distribution of the RNAs labelled with radioactivity in CE-2 revealed a different pattern from that in CE-1. Most of the RNA in CE-2 sedimented between 22 S and 26 S with only a small amount of material sedimenting more rapidly than 29 S (Fig. 2). The RNAs in CE-2 were sensitive to
hydrolysis by RNase except for some of the RNA sedimenting at 22 S. The percentage of total 22 S RNA that was RNase-resistant varied, depending on the time of sampling, from 16 to 60% (see later).

**Kinetic analysis of synthesis of the virus-specific RNAs**

The clean separation of the virus 42 S RNA from the presumed replicative intermediate of Semliki Forest virus by preparation of CE-1 and CE-2 allowed both kinetic and chemical studies of the virus-specific RNAs. When $^{32}$P orthophosphate was the labelled precursor and was present from 0 to 120 min. after the addition of

![Graph](image_url)

**Fig. 2.** Distribution of radioactivity after sucrose density-gradient centrifugation of nucleic acid from BHK 21 cells infected with Semliki Forest virus: CE-1 and CE-2. Cells were exposed to $^3$H(uridine, 4 $\mu$C/ml. from 90 to 225 min. after infection. Actinomycin D, 0.15 $\mu$g/ml., was present during virus adsorption and growth. A, Cytoplasmic extract 1; B, cytoplasmic extract 2. ●–●, Acid-insoluble radioactivity in the fraction; ○–○, acid-insoluble radioactivity after RNase treatment.

virus, the only labelled virus-specific RNA detectable in CE-2 was the 22 S RNA (Fig. 3b, Table 1). During the latter portion of the latent period, from 120 to 180 min., the amount of radioactivity in the 22 S RNA increased 48-fold (Fig. 4, Table 1); during exponential growth and period of greatest accumulation of virus, the amount of label in the 22 S RNA increased less than twofold from that present at the end of the latent period.

The amount of material in the 22 S RNA resistant to hydrolysis by RNase decreased from 59% of total at 180 min. to 29% of total at 240 min., and to 24% at 345 min. When labelling with $^{32}$P was from 0 to 180 min., or from 120 to 240 or 345 min., RNase-resistant 22 S RNA contained $450 \times 10^3$, $302 \times 10^3$, and $302 \times 10^3$ counts/min.
respectively (Fig. 4). Thus, despite the increase in total 22 S RNA during exponential growth or period of greatest accumulation of virus, the amount of RNase-resistant 22 S RNA labelled with radioactivity decreased during those periods from that present at the end of the latent period.

There was a second species of RNA in CE-2 sedimenting at 26 S (Fig. 3a), first detectable at 180 min. and increasing thereafter. This species of RNA in 1 × KKC and 1 × RSB was hydrolysed completely to acid-soluble fragments by treatment for 30 min. at 37° with RNase, 3 μg./ml. The amounts of 26 S RNAs in CE-1 and CE-2 synthesized during each 60 min. period, from 120 min. onwards, remained relatively constant. The rate of accumulation of 26 S RNAs was greater than that of 42 S RNA early in infection, whereas the opposite occurred late in infection (Fig. 4, Table 1). The period
Table 1. Distribution of radioactivity among RNAs specific for Semliki Forest virus at various times after infection

Data in this table are derived from Fig. 3 by integrating the radioactivity within a particular RNA synthesized during a time period and subtracting from it the amount within that RNA synthesized in the immediately preceding time period. As can be seen from the experimental design in Fig. 3, the shortest period of labelling was 120 min.

<table>
<thead>
<tr>
<th>Period during virus growth (min.)</th>
<th>RNAs in CE-1</th>
<th>RNAs in CE-2</th>
<th>Incorporation of (^{32}P) into RNAs, fold increase during time period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early latent 0-120</td>
<td>42 S ND*</td>
<td>26 S ND</td>
<td>—</td>
</tr>
<tr>
<td>Late latent 120-180</td>
<td>415 887</td>
<td>26 S 357 763</td>
<td>x 152.0</td>
</tr>
<tr>
<td>Exponential 180-240</td>
<td>1025 853</td>
<td>22 S 248 280</td>
<td>x 1.0</td>
</tr>
<tr>
<td>Maximal accumulation of virus</td>
<td>5947 1693</td>
<td>26 S 959 216</td>
<td>x 1.8</td>
</tr>
</tbody>
</table>

* Not detectable. † Counts per min. \(\times 10^{-3}\).

Fig. 4. Data in this figure were derived from Fig. 3 by integrating the radioactivity within the particular RNA having accumulated by a certain time. The radioactivity within the 26 S RNAs in CE-1 and CE-2 were added and plotted together. □—□, 42 S RNA; ●—●, 22 S RNA; ○—○, 22 S RNA (RNase-resistant); ◇—◇, 26 S RNA.

of greatest labelling of the 42 S virus RNA coincided with that of greatest accumulation of virus, from 240 to 345 min., when 73 % of total infectious virus was synthesized.

The results are consistent with, but do not prove, the hypothesis that the 22 S RNA may be the replicative intermediate and serves as precursor to the 26 S RNA in CE-2 and that the 26 S RNAs in CE-2 and CE-1 are precursors of the 42 S virus RNA. This
can be answered unequivocally only by a pulse-chase experiment. A precursor–product relationship in RNA synthesis cannot with certainty be determined in animal cells because of the slowness in equilibration of RNA precursors and their chase from pools within animal cells (Canellakis, 1962). The experimental design shown in Fig. 3 and Table 1 was such that the precursor [3H]orthophosphate was present in each experiment for at least 2 hr before analyses of samples. This ensured that the virus RNAs were synthesized from pools of nucleotidyl precursors at a constant specific activity (Jeanteur, Amaldi & Attardi, 1968).

Fig. 5. Sucrose density-gradient analyses of [32P]-22 S RNA in CE-2. A, Untreated; B, RNase-treated. The 22 S RNA labelled during 0 to 180 min. after infection (depicted in Fig. 3a) was collected from the sucrose gradients by pooling fractions 11 through 14, precipitated with ethanol, and resuspended in 1 × RSB and 1 × KKC, in the presence or absence of RNase, 30 μg/ml. After incubation at 37°C for 30 min. the samples were analysed by sucrose gradient centrifugation as described in Fig. 2 except that centrifugation was for 18 hr rather than 20 hr.

Fig. 6. Chromatographic analysis on B-D cellulose of 32P-labelled 22 S RNA in CE-2. The 22 S RNA labelled during 0 to 180 min. after infection (depicted in Fig. 3a) was precipitated with ethanol after pooling contents of tubes 11 through 14, and the pellet was resuspended in 0.1 M-NaCl, 0.05 M sodium phosphate, pH 6.7. Chromatography was performed as described in Methods.
Further analyses of RNAs in CE-2

The 22 S and 26 S RNAs retained their original S values on recentrifugation (shown for the 22 S RNA in Fig. 5a). Double-stranded RNA with sedimentation value of slightly less than 22 S was not recovered from within the 22 S RNA by the use of RNase. The 22 S RNA labelled from 0 to 180 min. after infection (Fig. 3a) was 59% resistant to hydrolysis by RNase. After pretreatment of the 22 S RNA for 30 min.

Table 2. Analysis of base composition of 22 S RNA from BHK 21 cells infected with Semliki Forest virus and labelled with 32P from 0 to 180 min.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CMP</th>
<th>AMP</th>
<th>GMP</th>
<th>UMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.253</td>
<td>0.270</td>
<td>0.258</td>
<td>0.219</td>
</tr>
<tr>
<td>After recentrifugation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Sedimenting at 22 S</td>
<td>0.258</td>
<td>0.267</td>
<td>0.250</td>
<td>0.225</td>
</tr>
<tr>
<td>(b) Sedimenting at less than 22 S</td>
<td>0.245</td>
<td>0.302</td>
<td>0.239</td>
<td>0.214</td>
</tr>
<tr>
<td>After RNase and recentrifugation</td>
<td>0.258</td>
<td>0.261</td>
<td>0.251</td>
<td>0.231</td>
</tr>
<tr>
<td>After B-D cellulose chromatography</td>
<td>0.253</td>
<td>0.253</td>
<td>0.260</td>
<td>0.234</td>
</tr>
<tr>
<td>Expected for double-stranded virus RNA</td>
<td>0.257</td>
<td>0.243</td>
<td>0.257</td>
<td>0.243</td>
</tr>
<tr>
<td>Expected for triple-stranded virus RNA (2+ and 1− strands)</td>
<td>0.258</td>
<td>0.259</td>
<td>0.256</td>
<td>0.227</td>
</tr>
</tbody>
</table>

Sample with no treatment was the 22 S RNA shown in Fig. 3a and labelled from 0 to 180 min. The 22 S RNA after recentrifugation or RNase and recentrifugation was that shown in Fig. 5a and b, respectively. After recentrifugation, the contents in tubes 18 to 24 were pooled for analyses of base composition of 22 S RNA and those in tubes 25 to 34 were pooled for analysis of RNA released from 22 S RNA. The 22 S RNA eluted from B-D cellulose was depicted in Fig. 6. Samples in tubes 46 to 53 were pooled. The samples were processed for analyses of base compositions as described in Methods. Each base composition presented is the average of three determinations made on the same initial 22 S RNA but treated separately.

Table 3. Base composition of 32P-labelled RNA from Semliki Forest virus and BHK 21 cells infected with Semliki Forest virus

<table>
<thead>
<tr>
<th>Source of RNA</th>
<th>CMP</th>
<th>AMP</th>
<th>GMP</th>
<th>UMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified virus</td>
<td>0.259 ± 0.004</td>
<td>0.291 ± 0.003</td>
<td>0.255 ± 0.003</td>
<td>0.195 ± 0.003</td>
</tr>
<tr>
<td>From CE-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42 S</td>
<td>0.253 ± 0.002</td>
<td>0.283 ± 0.004</td>
<td>0.269 ± 0.006</td>
<td>0.195 ± 0.001</td>
</tr>
<tr>
<td>26 S</td>
<td>0.249 ± 0.001</td>
<td>0.294 ± 0.003</td>
<td>0.256 ± 0.004</td>
<td>0.201 ± 0.001</td>
</tr>
<tr>
<td>From CE-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26 S</td>
<td>0.248 ± 0.003</td>
<td>0.284 ± 0.004</td>
<td>0.262 ± 0.004</td>
<td>0.206 ± 0.004</td>
</tr>
<tr>
<td>22 S</td>
<td>0.252 ± 0.001</td>
<td>0.277 ± 0.004</td>
<td>0.268 ± 0.003</td>
<td>0.203 ± 0.003</td>
</tr>
<tr>
<td>Expected for double-stranded virus RNA</td>
<td>0.257</td>
<td>0.243</td>
<td>0.257</td>
<td>0.243</td>
</tr>
</tbody>
</table>

32P-labelled virus-specific RNAs were obtained from cytoplasmic extracts of actinomycin D-treated BHK 21 cells infected with Semliki Forest virus and labelled from 120 to 240 min. or 120 to 345 min. after infection as for Fig. 2. The RNAs were collected and base compositions were determined as described in Methods. Each base composition presented is the average of at least six determinations. The base compositions for the two time periods studied were the same.
with RNase and subsequent analysis by gradient centrifugation, 56% of the total initial 22 S RNA was recovered as acid-insoluble RNA (Fig. 5b). Only RNA sedimenting at less than 13 S was recovered.

The 22 S RNA labelled from 0 to 180 min. and isolated from the original centrifugation had a base composition which was not complementary, with AMP and UMP contents specifically not equal (Table 2). This base composition tended toward that of virus RNA (Table 3). Recentrifugation or RNase treatment followed by recentrifugation did effect a slight change in the base composition with a decrease in AMP and an increase in UMP content (Table 2). Released from the 22 S RNA by recentrifugation were single-stranded RNAs with sedimentation values of less than 22 S (Fig. 5a) and with a base composition similar to that of virus RNA ‘plus’ strands (Table 2).

To study the RNase-resistant RNA in the 22 S RNA in its native configuration without the use of RNase, the same 22 S RNA used for Fig. 5a was chromatographed
on B-D cellulose as described in Methods. RNA labelled with radioactivity eluted sharply between 0-5 and 0-6 m-NaCl (Fig. 6), the salt concentration at which DNA and the replicative form of RNA are eluted (Sedat, Kelly & Sinsheimer, 1967; Kelly & Sinsheimer, 1967). Of the material labelled with radioactivity, 16% was recovered in the main peak. The remaining RNA had a configuration such that it could not be eluted in quantity from the B-D cellulose even at 2 m-NaCl, nor with further elution with 2 % ATP in 2 m-NaCl.

The RNA eluting from B-D cellulose between 0-5 m and 0-6 m-NaCl (Fig. 6) had a sedimentation value of 22 S on recentrifugation and 92% of total was resistant to hydrolysis by RNase. Its base composition was close to, but not identical to that expected for double-stranded RNA of Semliki Forest virus replicative form (Table 2). Of the total 22 S RNA labelled during the latent period of virus infection, 16% was thus double-stranded RNA as judged by the criterion of B-D cellulose chromatography, RNase-resistance, and base composition. The 22 S RNA labelled after the latent period and the 26 S and 42 S RNAs synthesized during infection had the base composition of virus RNA 'plus' strands.

Single-stranded RNA could be separated and recovered from within the 22 S RNA labelled after the latent period by treatment with 1 m-NaCl or 2 m-LiCl as described by Franklin (1966) and Baltimore (1966). Both species of RNA, soluble or insoluble at the high salt concentration, sedimented in the main at 22 S (Fig. 7a, b). There was one important difference, however: in 1 x RSB the RNA soluble at the high salt concentration had an increased resistance to hydrolysis by RNase over that present in the original 22 S RNA; the RNA precipitated by high salt was completely sensitive to hydrolysis by RNase.

<table>
<thead>
<tr>
<th>Time after infection of labelling of 22 SRNA with 3H (min.)</th>
<th>RNase resistant (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method A</td>
<td>Method B</td>
</tr>
<tr>
<td>Annealing conditions</td>
<td>Quenched in ice after heating</td>
</tr>
<tr>
<td>90–210</td>
<td>5-6</td>
</tr>
<tr>
<td>150–210</td>
<td>2-5</td>
</tr>
</tbody>
</table>

The 22 S RNA was prepared from actinomycin D-treated BHK 21 cells infected with Semliki Forest virus by pulse labelling with [3H]uridine from 90 to 210 min. after infection, or from 150 to 210 min. Unlabelled RNA from Semliki Forest virus was prepared and the annealing conditions were as described in Methods. The amount of Semliki Forest virus RNA present in each annealing mixture was 2-3 μg.

Annealing experiments

To ascertain if there was present in CE-2 after the latent period any RNA complementary to virus RNA 'plus' strands and not in double-stranded RNA, the RNAs in CE-2 were tested in hybridization experiments. When the 22 S RNA was heated in 3 x KKC and exposed to annealing conditions or cooled quickly in ice, there was no reformation of the configuration in which the RNA was 16% to 60% resistant to hydrolysis by RNase: after annealing, only 5% of the 22 S RNA remained resistant. Addition of an excess of parental virus RNA 'plus' strands increased consistently the
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amount of 22 S RNA which was RNase-resistant after annealing (Table 4). At the times analysed, at most 5% of the RNAs in the 22 S RNA was complementary RNA.

The possibility existed that at the times studied there was already asymmetric labelling of the strands in the double-helical RNA with preferential labelling with radioactivity of the virus RNA ‘plus’ strands. After annealing conditions, 3% of 32P-labelled Semliki Forest virus 42 S RNA remained resistant to hydrolysis by RNase. This 3% was consistently increased to 8% by the addition of an excess of 3H-labelled 22 S RNA to the 32P-labelled 42 S virus RNA during annealing. Thus as a maximum at later times, no more than 10% of the RNA strands in the 22 S RNA are complementary to virus RNA. No complementary RNA was found in the two RNAs in CE-1 and the 26 S RNA in CE-2.

DISCUSSION

In BHK 21 cells infected with Semliki Forest virus there appear at least four new species of virus-specific RNAs, three of which have been identified before. As shown in this paper, the replicating RNA complex, the 22 S RNA, is attached to a membrane or located within a structure lined by membranes sensitive to disruption by detergents. On or in this membranous structure, which may be identical to the large round vacuoles lined on their inner surfaces with small vesicles (Acheson & Tamm, 1967; Friedman & Berezesky, 1967), there is no 42 S virus RNA, and thus the replicating complex is physically separated from the end-product of RNA synthesis, virus RNA.

The 22 S RNA, the first of the virus-specific RNAs to be synthesized, accumulated in large part during the latter portion of the latent period. Within the 22 S RNA there were two distinct species of RNA: one with complementary base ratios and double-stranded; the other, single-stranded with a base composition similar to that of virus RNA ‘plus’ strands. Heterogeneity was not seen in sedimentation values or in the elution pattern from B-D cellulose of RNAs in the replicating complex resulting from variation in the lengths of nascent strands attached to the parental duplex (Kelly & Sinsheimer, 1967). Instead, the replicative form and the single-stranded RNA derived from it had the same sedimentation coefficient. On recentrifugation, 25% of total 22 S RNA sedimented at values less than 22 S. These RNAs had a total base composition similar to that of virus RNA ‘plus’ strands and could represent nascent virus RNA ‘plus’ strands in the process of synthesis. There were few or no complementary RNA ‘minus’ strands newly synthesized during the exponential growth or greatest accumulation of virus. The amounts of ‘minus’ RNA strands and double-stranded RNA remained relatively constant or decreased after the end of the latent period. Labelling of 22 S RNA with radioactivity during the periods of exponential growth or maximal accumulation of Semliki Forest virus was totally asymmetric with label detectable only in virus RNA ‘plus’ strands.

During the period of maximal synthesis of 22 S RNAs, the latter portion of the latent period, the other virus-specific RNAs were first detectable. Within the same confines as the 22 S RNAs was 26 S RNA. In addition, 26 S RNA was present free in the cytoplasm. These RNAs had the same sedimentation value, were synthesized simultaneously and with similar kinetics, and had at all times of sampling the same base composition, that of virus RNA ‘plus’ strands. They were distinguishable only by their differential solubilities in 2 M-LiCl or 1 M-NaCl. Of the 26 S RNA in CE-2, 15% to 57% was soluble in the presence of high salt, whereas at most 9% of the
26 S RNA in CE-1 was soluble. Secondary structure of the particular RNA has been thought to determine solubilities of RNAs in high salt (Baltimore, 1966; Franklin, 1966). Because of the many similarities noted above, the 26 S RNAs in CE-1 and CE-2 could be one and the same RNA but in different configurational arrangement. Maximal synthesis of 42 S virus RNA occurred coincident with the period of maximal accumulation of virus, from 240 to 345 min. after infection.

The results obtained are consistent with the following replication scheme: (a) synthesis of complementary RNA ‘minus’ strands on the incoming parental virus RNA with the appearance on a membrane, or within a membrane-bounded vesicle, of double-helical 22 S RNA, maximal synthesis occurring during the latter part of the latent period; (b) synthesis on the double-helical RNA of 22 S single-stranded RNA with a base composition that of virus RNA ‘plus’ strands; (c) a postulated subsequent series of configurational changes occurring first within or on the detergent-sensitive structure with the 22 S single-stranded RNA acquiring a configuration such that it sedimented at 26 S even after it had emerged from its membranous attachment and 42 S after its association with protein to form nucleocapsid; and (d) acquisition of a lipoprotein coat before release of virus from the cell.

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REFERENCES


Synthesis of SFV-specific RNAs


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